

Modern Clinical Molecular Techniques

Peter C. Hu
Madhuri R. Hegde
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Editors

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 Springer

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Preface

Clinical Molecular Diagnostics is an ever-expanding field which has come to the forefront of clinical testing with the increasing demand for molecular pathology and the ideologies of personalized medicine. It is apparent that with any rapid changes to a field, there is a learning curve. Our hope is to assist those who are either familiar or unfamiliar with Molecular Diagnostics in the areas of Infectious Disease, Oncology, Pre-/postnatal, and Human Identity Testing.

Modern Clinical Molecular Techniques is divided into six parts. Part I focuses on current clinical molecular laboratory practices including the proper handling of specimens, DNA/RNA isolation, test validation, current general molecular techniques, summary reporting, and data storage. In Part II, we begin to take a more in-depth view on some of the more advanced protocols in clinical molecular infectious disease testing ranging from specimen identification to viral load testing using current diagnostic molecular techniques. Part III is focused on clinical molecular oncology testing. In this part, current clinical molecular diagnostic techniques are showcased in areas from detecting gene rearrangements, mutation analysis, cancer microarray analysis, and minimal residual disease monitoring. Part IV shifts the focus to a different molecular discipline – the area of molecular pre-/postnatal testing. In this part, current diagnostic molecular protocols will be described in detail for a deeper understanding of current test methodologies and their uses. Part V describes current human identity testing such as parentage testing and an overview of microsatellite testing. Part VI illuminates issues surrounding the current shortage of clinical professionals in the area of diagnostic molecular testing throughout the USA. This part focuses on the clinical training aspects of both bench technologists – those from accredited programs and those with on-the-job training – and medical and laboratory directors (M.D. and Ph.D levels). The chapters conclude with a look at another fast growing trend in point-of-care testing training.

Currently, there are many books in press that focus on various aspects of the information covered in this text. However, we feel that by narrowing the focus to current diagnostic molecular testing protocols and clinical training, the reader will find it easier to not only begin creating their own diagnostic molecular testing protocols, but also to implement clinical training when issues arise. Upon reading this text, keep in mind that although many protocols on molecular testing are included, the specifics of testing change as new technologies emerge in this ever-growing, ever-changing field and will require additional updates and continual education.

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Part I
Current Molecular Laboratory Methods

Chapter 1

Specimen Collection, Handling, and Processing

Lindsay Hengesbach and John A. Gerlach

Keywords Collection • Contamination • Identification • Inhibiting substance • Nucleic acid • Pre-analytic • Safety • Sample • Sample integrity • Sample shipping

1.1 Introduction

Specimen collection handling and processing are key to the pre-analytic phase of clinical laboratory testing. In computer jargon there is an acronym “GIGO” that stands for garbage in equals garbage out. It is also applicable to clinical laboratory testing. If the proper sample is not collected from the individual to be tested and if the sample is not handled and transported to the laboratory in an efficient and efficacious manner, there will not be usable diagnostic information provided. This chapter deals with the pre-analytic process of sample collection, handling and processing for diagnostic molecular testing.

1.2 The Collection and Processing of Specimens in the Clinical Molecular Laboratory

1.2.1 Introduction to the Pre-analytical Phase of Testing

Pre-analytical testing in a clinical laboratory encompasses all of the aspects involved in procuring and preparing the specimen before analytical testing takes place. This includes maintaining the integrity and confidentiality of the patient’s specimen; the proper collection of numerous types of specimens, all with different requirements; the transport of the specimen to and from the laboratory; the storage of these specimens while waiting for testing; and the processing of these specimens so they are ready for testing protocols. All of this must be accomplished, as well as maintaining a safe work environment by following Biosafety Level 2 precautions throughout the entire process. Often the complexity of the pre-analytical phase of testing is underrated. The pre-analytical phase of testing is one of the most critical because the majority of laboratory errors leading to misdiagnosis or mistreatment of a patient occur in the pre-analytical phase of testing [5, 12, 22].

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1.2.2 Specimen Identification

- It is the responsibility of the laboratory to “establish and follow” set guidelines and written protocols for maintaining the identity of the patient’s specimen throughout the entire testing process including the pre-analytical phase of testing [12].
- It is the laboratory’s responsibility to maintain the security of a patient’s identification by following the Human Insurance Portability and Accountability ACT (HIPAA) guidelines throughout the entire testing process [13].
- At a minimum, a patient’s sample must have a label containing a specimen identification number, the date of collection, the time of collection, and the specimen source. It is highly recommended that the name of the individual collecting the specimen is present as well [6, 7].

1.2.3 Laboratory Safety/Standard Precautions

Working in a biomedical laboratory poses an inherent risk of exposure to unknown pathogens. Each laboratory is responsible for issuing and enforcing operational procedures that follow recommendations put forth in the OSHA Bloodborne Pathogen Standard to prevent the occupational transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens [6, 27]. The appropriate safety precautions can significantly reduce the risk of exposure of mucous membranes and non-intact skin to potentially infectious agents. It is common to follow Biosafety Level 2 practices in a molecular diagnostics laboratory because the majority of specimens submitted for testing are human derived and therefore potentially infectious. Potentially infectious materials must be kept in a durable, leakproof container during collection handling, storage, and transport to the laboratory. Personal protective equipment including gloves, gowns, and face or eye protection must be utilized when in contact with human-derived specimens. This equipment should be removed before leaving designated areas. Molecular labs typically designate equipment to specific areas of the lab to prevent the contamination of specimens with spurious or amplified nucleic acids (DNA/RNA) [5]. Engineering controls such as a biological safety cabinet should be utilized to minimize exposure to aerosols and as a method for limiting exposure of the sample to contaminating DNA from the environment/other specimens [18].

1.2.4 Sample Acceptability

- Source: When an assay is validated by a laboratory, one of the criteria of the validation process is to use the range of samples that are expected to be received. When a laboratory receives a sample that is outside of those used in validation, it must either revalidate or reject the sample; therefore, the source of the sample is an important criterion.
- Volume: The sample volume must be sufficient to allow recovery of enough nucleic acid material to perform the assay. It is best to have excess, but it should be a minimal excess.
- Shipping: The sample should be shipped appropriately to the laboratory, arrive in a timely fashion without being overexposed to environmental influence, and should be protected from freezing and overheating.
- Storage: Once received, the specimen must be processed and stored properly in accordance with the laboratory’s validated protocol.

1.2.5 Specimen Collection

When collecting a specimen for molecular testing, it is critical to protect the specimen from contamination from exfoliated skin cells as well as to protect the individual collecting the specimen from potentially infectious materials and infectious agents. Gloves should be worn throughout the entire collection process and gloves should be changed frequently. There are numerous specimens from which nucleic acid can be obtained.

- Whole blood, plasma, and serum are common sources of samples for nucleic acid testing. Whole blood often yields a very high quality and ample quantity of nucleic acid for testing. The impact of anticoagulants on downstream analysis must be considered before collecting whole blood. The most common anticoagulants are ethylenediaminetetraacetic acid (EDTA) or acid citrate dextrose (ACD) [1]. Both chelate calcium as their mode of action. ACD is useful when prolonged shipping may be involved as it will help maintain the integrity of the nucleated cells. The DNA in plasma or serum is most likely from dead cells. It is a good source but it may not be of high molecular size [10]. On another note though, the RNA that is collected from plasma or serum may actually be the material targeted as it may be of viral origin [28].
- Dried blood spots are commonplace in newborn screening scenarios as well as studies that require shipping across large distances or long-term storage. There are several manufactures that supply the filter papers used and the basic premise is to collect a sample, most often capillary blood from a lancet poke (finger or heel) that is allowed to saturate a filter paper. Often there is a circle printed on the filter to demark the appropriate volume of sample needed. Blood is allowed to seep onto/into the filter until the circle area is filled. The sample is allowed to dry and then it is shipped or archived. If archived, it is usually vacuum-sealed with a desiccant [19, 26, 30].
- Anatomic samples, either fresh, frozen, fixed, and/or archived, are very useful in molecular diagnostic testing. Isolation methods, as discussed later, will need to take into account if there have been fixatives used. Cytological samples would fall within this group also. These are samples collected from body sites by a lavage/washing/flushing process. Cells are dislodged or washed away by the process and collected into a container that has preservative or transport medium. How the sample is collected needs to ensure that there is not contamination from the environment. The container and any solutions or preservatives used must follow the guidelines of how the test was validated [2, 9].
- Body fluids can be a source of nucleic acid material for testing as well as the nucleated cells that are present. It may be important that the collection and transport maintains the integrity of the cells present as both the fluid and the cellular nucleic acid materials may need to be tested separately. Again the caution here is to follow the collection procedure as it was validated during assay development [16].
- Swabs are a common collection method for nucleic acid testing, and there are mainly two paradigms. The first is a cheek swab, and it is useful when the samples are not being collected at the institution but are collected off-site at clinics and possibly by the client. There are many types of fibers available, and some even have coarse bristles. The concept is to brush or swipe the inside of the cheek to remove cells containing nucleic acids. Care needs to be taken in how hard the cheek is “scrubbed” as bleeding may ensue at which point there will be erythrocytes that may lead to complication during the isolation process. It is also a good idea to have the client rinse prior to the process to remove debris that may contribute to sample mass collected. A second approach is a swab of a lesion for submission, most often to identify a pathogen’s nucleic acid. Viral, bacterial, and fungal targets are commonly found using a swab from a lesion [11, 20, 23, 29].
- Saliva is another sample chosen when it is deemed best to avoid venipuncture or direct blood handling. A container is provided with a mark denoting the volume necessary to collect and the client is directed to “expectorate into the container.” It is the cells that are present in the saliva, the same that you would collect from the cheek swab, which will be source of the nucleic acids isolated [7, 14].

- Other sources: These may be pathogen cultures or possibly parasites that have been harvested. The forensics laboratories can fill volumes on what they receive to isolate nucleic acids for analysis. The clinical laboratory often pales in vast diversity of the forensics laboratory but there can be surprises. The caution here is to note that the assay being requested was validated for a particular range of samples and anything outside of that list needs to be thoroughly documented throughout the assay process and any variation needs to be included in the interpretation of the assay. Just because you can isolate nucleic acids from the material (most likely it is probable) does not mean the material will be of sufficient quantity and/or quality to provide a meaningful result. Caution needs to be taken when unusual circumstances are presented to the laboratory.

Special circumstances and/or materials and supplies need to be considered when the goal is to isolate RNA. There are special tubes and timeframes that need to be adhered to with viral load testing [8, 15]. Equipment and supplies need to be RNase-free. These should be addressed in the assay protocol, and these stipulations in the protocol must be adhered to for the sample collected to deliver a meaningful diagnostic value [31].

The integrity of a sample may suffer because inhibiting substances are often co-collected with the patient specimen [3, 4]. Materials such as the anticoagulant utilized (heparin), the presence of hemolysis (free hemoglobin), high levels of lipids or carbohydrates, and even the pharmaceutical agents (remember the samples are being collected from physically ill individuals that are being medicated) present at the time of collection may be co-isolated with the nucleic acids and impact the usefulness of the sample [4, 21]. When possible and there is a priori knowledge, avoid these circumstances. Often inhibiting substances are not detectable and can lead to false negative results. A listing of inhibiting substances and conditions is often a useful document to help guide specimen collection. These lists may be generic for all assays or specific for a particular assay. All individuals involved in sample collection need to be armed with this information to help preserve the integrity of the pre-analytic portion of laboratory testing.

1.2.6 Sample Transport

Once the sample is collected in accordance with assay protocol, it needs to make its way to the laboratory for testing. This requires transport. It may be as simple as moving within an institution by hand or as complex as preparing a sample for international shipping. At all times, there are two main concerns: maintaining the integrity of the sample in terms of keeping it from being contaminated as well as the physical extremes it may encounter. Once again the protocol for the assay needs to be followed, and the assay needs to be validated with samples that have endured the “usual” transportation methods used. The prevention of freezing and overheating during shipping as well as breakage are the most obvious perils.

The second major concern with shipping is protecting the “transporter” and the environment from the sample. At the very least, the samples being discussed are biological in nature and therefore, the practice of universal precautions should be applied as the process is designed. It is also probable that the material itself may be infectious, warranting different, much more stringent regimen of containment and labeling. Below is a minimal set of guidelines. It would be imprudent to go into extreme detail on shipping and transport regulations as the guidelines and regulations change quite regularly and the best practice is to continue to monitor shipping vendor sites and federal sites for updates. The federal sources would be the Centers for Disease Control and Prevention, the Department of Transportation, the Federal Aviation Administration, as well as Homeland Security [25].

- Samples should be in a double container (even if it is only double bagging), so that if the integrity of the first container is compromised, the second will still provide the protection intended.

- Liquid sample should have an absorbent material sufficient to soak up the material if the holding container leaks or is broken.
- The Centers for Disease Control and Prevention provides a list of highly infectious agents as well as a list of select agents. These need special permits and handling.
- Material transfer agreements may also be necessary by the shipping or receiving institution and may often be on a case-by-case basis.

1.3 Sample Accessioning or Processing

This is one of the crucial steps in the pre-analytic phase of a laboratory operation. It is here, where the sample is received, the test has been ordered and the integrity and the appropriateness of the sample is reviewed and logged. This process is where the decision on whether to proceed, reject, or document variation of conditions outside of those prescribed by the procedure or laboratory policies is made. Policies and guidelines, as stated throughout this section, are a necessity.

- Sample receipt involves the un-packaging of the sample and an assessment on whether to proceed with the test ordered or to notify the source that the sample does not meet criteria for acceptance. Regardless of whether accepted or not, the sample and test ordered need to be logged into the system for subsequent tracking.
- Decisions on sample integrity and appropriateness may result in sample rejection. This may be because the sample is
 - From an inappropriate source
 - Of insufficient quantity
 - Collected in the wrong anticoagulant
 - Displaying evidence of hemolysis
 - Showing evidence of extreme environmental exposure during shipping or storage
 - Misidentified or has no identification
- If a sample is rejected, it needs to be logged into the normal system and the rejection noted. There also needs to be documentation that the source is notified of the rejection and the reason, as well as how the rejected sample is discarded.
- Aliquots of the original sample may need to be prepared. This requires a written protocol for staff to follow. It is important here, as well as during collection and shipping, that the integrity of the sample be maintained to prevent contamination, and there needs to be safeguards for the individual preparing the aliquots. An easy way to achieve both goals is to perform this function in a biosafety cabinet using sterile supplies. It is important to note that labeling the aliquots should be the primary focus to help maintain the sample identity throughout the testing and storage process.
- Specimen storage will again be determined by the assay requirements as validated during the assay development. As a general set of guidelines:
 - DNA can be stored short term at 4°C and long term at –20°C or colder.
 - RNA is best stored –20°C or colder.
 - Samples pre-isolation – this will be variable and the best reference will be the protocol for the particular assay.

With storage of samples used in molecular diagnostic testing, a concern for ethical or legal issues arises that are not quite as evident with other sample biobanking. Genetic testing falls under continued public and legal scrutiny because of the ability to stratify people based on inherited traits and the potential to use that information for discriminatory purposes. This is a concern and can be more an emotional issue than a logical concern. It does put an additional onus on a molecular diagnostic

laboratory to thoroughly define the need to maintain a sample after an assay is completed and also to have clinically justifiable reasons for the retention and subsequent use of banked samples. It would be judicious for a laboratory involved in biobanking and one that uses banked samples to seek approval of an Institutional Review Board of the guidelines and circumstances [24].

The process of sample collection really encompasses more than a needlestick, skin scrape, or placing a piece of tissue into a container. Sample collection is at the heart of the pre-analytic phase of molecular diagnostic testing, and it is here where adherence to the protocols and policies of the laboratory ensures the provision of useful diagnostic information.

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Chapter 2

DNA/RNA Isolation and Quantitation

Jing Wang

Keywords DNA • RNA • miRNA • Extraction • Quantification

2.1 Introduction

2.1.1 DNA Extraction

Nucleic acids are very large biological molecules essential for life. DNA (deoxyribonucleic acid) is found mainly in the nucleus of the cell, and RNA (ribonucleic acid) is found mainly in the cytoplasm of the cell although it is usually synthesized in the nucleus. DNA contains the genetic codes to make RNA, and the RNA in turn then contains the codes for the primary sequence of amino acids to make proteins. Nucleic acid preparation is the important initial step for molecular diagnosis. There are varieties of methods available for nucleic acid preparation [1, 2]. Strategies for DNA extraction and DNA purification depend on the sample volume, the major sample type, and the downstream applications.

Extracted DNA has been widely used for clinical analysis such as genotyping, diagnosis of infectious diseases, transplant engraftment assessment, and other tests such as forensic and ancestry testing. Different extraction methodologies have been implemented and evaluated. The basic steps for DNA extraction include (1) cell or tissue lysis using detergents, proteinase K, and/or RNase; (2) protein removal, avoiding RNA and chemical contamination; and (3) DNA purification. The conventional manual DNA extraction procedures involve phenol-chloroform purification and precipitation using ethanol or isopropanol. Although the manual DNA extraction procedures can easily handle large sample volume, for example, 5–10 ml of blood, or different types of specimen, and yield high quality and quantity of DNA with low cost. However, the sample processing procedure is very time consuming. The automated DNA extraction provides the high throughput of sample handling and shorter processing time [3, 4]. It typically utilizes magnetic beads technology and has the advantage of standardized sample treatment and avoidance of error during sample handling and contamination due to intermediate

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process. However, the quality and quantity of DNA extracted by automated methods may not be as good as by manual methods [5, 6] and, therefore, may not be optimal for some molecular tests such as Southern blotting and microarray testing.

The DNA extraction protocol presented here is adapted from Gentra Puregene DNA extraction molecular weight nuclear and mitochondrial DNA from variety of sample sources, including whole blood, frozen solid tissue, cultured cell, and paraffin blocks.

2.1.1.1 Reagents and Equipments

Reagents and Consumables Required

Supplied by Gentra Puregene DNA extraction kit (QIAGEN Inc., Valencia, CA):

- Red cell lysis solution (155 mM NH_4Cl , 10 mM KHCO_3 , 1 mM EDTA, pH 8.0)
- Cell lysis solution
- RNase A solution (4 mg/ml)
- DNA hydration solution or 1× TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA)
- Protein precipitation solution
- Glycogen solution (20 mg/ml)
- Proteinase K (20 mg/ml)
- 100% Isopropanol
- 70% Ethanol

For cultured cells:

- 1× PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH 7.4)
- Phenol-chloroform
- Chloroform

For paraffin block:

- Xylene or Hemo-De (Nontoxic alternative/Scientific Safety Solvents)
- 100% Ethanol
- Microcentrifuge tube pestle

Consumables:

- 1-ml Microcentrifuge tubes
- 15-ml Centrifuge tubes
- Pipetman (2 μl , 100 μl , 1 ml) and tips

Equipment:

- Microcentrifuge (Eppendorf Centrifuge 5415D)
- Bench Top Centrifuge (Beckman Coulter Allegra™ X-22 or equivalent)
- Vortex mixer

Note:

- Wear gloves and a laboratory coat throughout the procedure.
- Be careful and avoid cross-contamination of samples and reagents.
- Make sure reagent tubs, tubes, and plates are clearly labeled and clean.
Dispose of all used materials and media as biohazard waste.

2.1.1.2 Protocol

Cell Lysis

DNA Extraction from Whole Blood

Note: This protocol is designed for extraction of 3 ml whole blood with expected yield of 50–150 µg of DNA. For best results, blood must not exceed 6 days before processing.

Control: A blank control is extracted along with each batch's blood samples. 3 ml of sterile water is replaced with the 3 ml of blood. After the extraction process, the blank is quantified and amplified in a PCR reaction to check for any contaminations in the extraction reagents:

1. Wear latex gloves when working under the biological safety hood and mix the tube of whole blood by inverting the tube six times.
2. Pour 3 ml of blood into a labeled 15-ml conical centrifuge tube.
3. Add 9 ml red blood cell lysis solution to the 3 ml of blood. Invert ten times to mix and incubate 5 min at room temperature; invert at least once during incubation.
4. Centrifuge for 5 min at 2,000× g (on the Beckman Coulter Allegra™ X-22). Remove supernatant, leaving the visible white cell pellet in the tube.
5. Resuspend the pellet in the residual supernatant by tapping tube; this greatly facilitates cell lysis in step 6 below.
6. Add 3 ml of cell lysis solution to the resuspended pellet. Incubate at 37°C for 2 h. Additional incubation at room temperature overnight can be added to ensure the solution is homogenous. This action can be further facilitated by placing the tube on a shaker. Samples are stable in cell lysis solution for at least 18 months at room temperature.

DNA Extraction from Solid Tissue

Note: This protocol is designed for 25–50 mg of solid tissue extraction; expect to yield 50–250 µg of DNA.

1. Dissect tissue sample quickly on dry ice. Work very quickly and keep tissue on dry ice at all times. Fresh tissue may also be used. Freeze the remaining tissue at –80°C or lower.
2. Coarsely ground 25–50 mg tissue with a surgical blade. Transfer the grounded tissue to a 15-ml centrifuge tube containing 3 ml cell lysis solution. Add 15 µl of proteinase K solution (20 mg/ml) to the lysate. Mix by inverting 25 times. Incubate at 55°C for 3 h or until tissue has completely lysed. The sample can be incubated at 37°C overnight for maximum yield.

DNA Extraction from Cultured Cells

Note: This protocol is designed for extraction of DNA from 1–2 × 10⁷ monolayer cultured cells; expect to yield 50–100 µg of DNA.

1. Pipette out the culture medium. Wash cells with 5 ml PBS at room temperature, then remove all PBS solution.
2. Detach cells by trypsinization or use a cell scraper. Transfer the cell to a 15-ml centrifuge tube.
3. Add 5 ml PBS to the harvested cells. Invert the tube two times.
4. Centrifuge 500× g for 3 min to pellet the cells.
5. Carefully discard the supernatant, leaving approximately 200 µl liquid.
6. Vortex the tube vigorously to suspend the cells. Add 3 ml cell lysis solution to the suspended cells. Vortex on high speed for 10 s.

7. Incubate at 37°C for 2 h. Additional incubation at room temperature overnight can be added to ensure the solution is homogeneous. This action can be further facilitated by placing the tube on a shaker. Samples are stable in cell lysis solution for at least 18 months at room temperature.

DNA Extraction from Paraffin Block

Sample Deparaffinization

1. Place 5–10 mg of finely minced tissue in a 1.5-ml tube. Add 300 µl Xylene or Hemo-De and incubate 5 min with constant mixing at room temperature.
2. Centrifuge at 13,000–16,000× g (Eppendorf Centrifuge 5415D) for 1–3 min to pellet the tissue. Discard the Xylene or Hemo-De.
3. Repeat steps 1 and 2 twice (for a total of three washes).
4. Add 300 µl of 100% ethanol to the tube and incubate 5 min with constant mixing at room temperature.
5. Centrifuge at 13,000–16,000× g for 3 min to pellet the tissue. Discard the ethanol.
6. Repeat steps 4 and 5 (for a total of two ethanol washes).

Cell Lysis

1. Add 300 µl cell lysis solution and mix by using 30–50 strokes with a microcentrifuge tube pestle.
2. Incubate lysate at 65°C for 15–60 min.
3. If maximum yield is required, 1.5 µl proteinase K solution (20 mg/ml) may be added to the lysate. Mix by inverting 25 times and incubate at 55°C until tissue particulates have dissolved (3 h to overnight). If possible, invert tube periodically during the incubation.

RNase Treatment (Optional)

4. Add 1.5 µl RNase A solution (4 mg/ml) to the cell lysate.
5. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15–60 min.

Protein Precipitation:

1. After cell lysis steps described above for different types, cool sample to room temperature.
2. Add 1 ml of protein precipitation solution to the cell lysate. For paraffin block DNA extraction, add 100 µl protein precipitation solution to the cell lysate.
3. Vortex vigorously at high speed for 20 s to mix the protein precipitation solution uniformly with the cell lysate.
4. Centrifuge at 13,000–16,000× g for 5 min. The precipitated proteins will form a tight pellet. If the protein pellet is not visible, repeat step 3 followed by incubation on ice for 5 min, then repeat step 4.

DNA Precipitation:

1. Pour the supernatant from protein precipitation protocol step 4 containing the DNA into a labeled 15-ml conical centrifuge tube containing 3 ml of isopropanol. If DNA yield is expected to be below 0.1 µg, add 3.0 µl glycogen solution (20 mg/ml) to the isopropanol.

Extra steps for paraffin block and cultured cell extraction:

- (a) Pour the supernatant containing the DNA into a clean 15-ml tube and add equal volume of phenol-chloroform. Mix by rocking gently back and forth for 15 s.
- (b) Centrifuge at 2,000× g for 5 min using the centrifuge.
- (c) Carefully transfer the upper phase of supernatant containing the DNA into a clean 15-ml tube and add equal volume of chloroform. Mix by rocking gently back and forth for 15 s. Save everything until DNA is recovered.

- (d) Centrifuge at $2,000\times g$ for 5 min.
 - (e) Carefully transfer the supernatant containing the DNA (upper phase) into a labeled 15-ml tube containing 6 ml of isopropanol.
2. Mix the tube by inverting gently 50 times.
 3. Centrifuge at $2,000\times g$ for 5 min. The DNA will be visible as a small white pellet.
 4. Pour off supernatant and transfer the DNA pellet and remaining liquid to a 1.5-ml centrifuge tube.
 5. Add 500 μ l 70% ethanol and invert tube several times to wash the DNA pellet.
 6. Centrifuge at $13,000\text{--}16,000\times g$ for 1 min. Carefully pour off the ethanol. Pellet may be loose, so pour slowly and watch out for the pellet.
 7. Invert and drain the tube on clean absorbent paper and allow to air-dry for 10–15 min.
 8. Estimate the size of the pellet and add 200–400 μ l DNA hydration solution.
 9. Allow DNA to rehydrate overnight with gentle rocking at 37°C or 30 min at 65°C .
 10. Measure the DNA concentration by using the NanoDrop spectrophotometer. Refrigerate DNA at 4°C .

2.1.2 RNA Extraction

There are two main methods for RNA extraction: phenol-based extraction and silica matrix or glass fiber filter (GFF)-based binding. Phenol-based reagents, such as TRIzol, contain a combination of denaturants and RNase inhibitors for cell and tissue disruption and subsequent separation of RNA from contaminants. Phenol-based isolation procedures can recover RNA species in the 10–200 nucleotide range, including miRNAs, 5S rRNA, 5.8S rRNA, and U1 snRNA. If a sample of “total” RNA was purified by the silica matrix column or GFF procedure, it will be significantly depleted in small RNAs. Extraction procedures using TRIzol Reagent are the recommended methods for isolating total RNA from biological samples that will contain miRNAs/siRNAs. TRIzol Reagent is a monophasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lysis, TRIzol Reagent maintains the integrity of the RNA while disrupting cells and dissolving cell components [7].

This protocol utilizes the powerful TRIzol Reagent extraction method which allows the rapid isolation of RNA. High yields of pure, undegraded total RNA can be recovered from even small quantities of tissue or cells.

2.1.2.1 Reagents and Equipments

Reagents and Consumables:

- RNAlater (Ambion Inc., Austin, TX)
- RNaseZAP (Ambion Inc., Austin, TX)
- TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA)
- RNase Away surface decontaminant (Fisher Scientific, Hanover Park, IL)
- Red cell lysis solution (155 mM NH_4Cl , 10 mM KHCO_3 , 1 mM EDTA, pH 8.0)
- Ultrapure DNase/RNase-Free Distilled Water
- Chloroform
- 100% Isopropanol
- 70% Ethanol (keep cold at 4°C)
- High salt buffer (0.8 M NaCitrate/1.2 M NaCl)
- 1 \times PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM)

Accessories Required:

- Refrigerated centrifuge
- Microcentrifuge
- Micropipettors
- Vortex mixer
- Glass-Teflon or power homogenizer

2.1.2.2 Protocol

Note: Always wear lab coat, gloves, and eye protection. Avoid contact with skin or clothing. Operate in a biochemical safety hood. Avoid breathing vapor. Use RNaseZAP to clean all tools, pipettes, bench, and scale area before starting.

Cell Lysis**RNA extraction from whole blood:**

Note: This protocol is designed to extract whole blood samples of 5–8 ml and expect to yield approximately 30 µg of RNA.

1. Transfer 5–8 ml of whole blood into a 50-ml polypropylene conical centrifuge tube. Bring volume to 50 ml with red cell lysis solution.
2. Invert the tube ten times to mix. Let stand at room temperature for 5 min.
3. Centrifuge for 5 min at 2,000× g. Remove supernatant, leaving the visible white cell pellet in the tube.
4. Gently resuspend the pellet in 1 ml of red cell lysis solution and transfer to a 1.5-ml microcentrifuge tube. Let it stand for 2–5 min.
5. Pellet cells by centrifuging at 13,000× g for 2 min at room temperature. Carefully aspirate the supernatant.
6. If the resulting pellet is still red, repeat the RBC lysis (steps 4–5) as needed.
7. Resuspend the pellet in 1 ml of sterile PBS.
8. Pellet cells by centrifuging at 13,000× g for 2 min.
9. Carefully aspirate the supernatant.
10. Add 1 ml of TRIzol solution to each tube and resuspend the cells.

RNA extraction from solid tissues:

1. Zero the balance with about 2 ml RNAlater in a weigh boat.
2. Place 50–100 mg sample in the weigh boat with RNAlater and record the amount of tissue on the extraction log.
3. Store excess tissue at –80°C.
4. Homogenize tissue samples in 1 ml of TRIzol Reagent per 50–100 mg of tissue using a glass-Teflon or power homogenizer. The sample volume should not exceed 10% of the volume of TRIzol Reagent used for the homogenization.

RNA extraction from cells grown in monolayer:

1. Rinse cell monolayer with ice cold PBS once.
2. Lyse cells directly in a culture dish by adding 1 ml of TRIzol Reagent per 3.5-cm-diameter dish and scraping with cell scraper.
3. Pass the cell lysate several times through a pipette. Vortex thoroughly.

Note: The amount of TRIzol Reagent added is based on the area of the culture dish (1 ml per 10 cm²) and not on the number of cells present. An insufficient amount of TRIzol Reagent may result in DNA contamination of the isolated RNA.

RNA Precipitation:

1. Incubate the homogenized sample for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.
2. Centrifuge 13,000× g for 15 min at 4°C to remove cell debris. Transfer supernatant to a new 1.5-ml tube. Discard the pellet, which is cellular debris and DNA.

Note: If there is an oily/fat layer on top, remove fatty layer with a pipetman then pour supernatant into new tube. If the pellet moves, remove liquid with a pipette.

3. Add 0.2 ml chloroform per 1 ml TRIzol to each tube and close tubes. Vortex 15 s to mix.
4. Incubate samples at room temperature for 10 min. Centrifuge 13,000× g for 15 min at 4°C. Transfer *aqueous phase* (top layer) into a new tube.

Note: The RNA isolation can be stopped at this step. The collected *aqueous phase* can be stored at –80°C or immediately enriched for small RNAs (see Sect. 2.3).

5. Add 0.25 ml high salt buffer per 1 ml of TRIzol to each sample. Add 0.25 ml isopropanol per 1 ml TRIzol to each sample. Mix well by gently inverting tubes three to four times.
6. Incubate at room temperature for 20–30 min. Centrifuge 13,000 rpm for 15 min at 4°C.
7. Pour liquid (high salt/isopropanol) into a liquid waste container leaving the pellet behind. Tap tube on a paper towel to get as much liquid out as possible and place tube back on ice.
8. Add 1 ml cold 70% ethanol to each sample. Vortex pellet gently to remove residual high salt/isopropanol. Centrifuge 13,000× g for 1 min at 4°C.
9. Air-dry pellet for 10–30 min on ice with KimWipe on top of the open tubes. If pellets are thick, drying time may increase. Make sure the pellet is completely dry as residual ethanol can inhibit downstream applications.
10. Dissolve pellets using Ultrapure DNase/RNase-Free Distilled Water on ice. Amount of water depends on size of the pellet.

Note:

- Collect TRIzol and RNase Away into the phenol waste container in the hood.
- RNAlater waste can go down the sink while running the water for 10 min.
- Make sure bench paper in the hood is clean with no spilled TRIzol. If not, dispose of it immediately and replace with new bench paper.
- For microarray experiments, total RNA isolated by TRIzol method needs to be further cleaned using RNeasy cleanup protocol.

2.1.3 miRNA Enrichment

In the past 10 years, research interest in microRNAs (miRNAs) has rapidly expanded. miRNAs are small (~22 nucleotides) noncoding RNAs expressed by virtually all eukaryotes. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs). They have been shown to regulate target gene expression in various organisms.

Several unique physical attributes of miRNAs, such as small size and lack of poly-adenylated tails, have made them elusive and challenging to study.

The aqueous phase from step 4 of RNA precipitation contains total RNA. The miRNA enrichment procedure introduced here is conjunction with above total RNA extraction. Millipore Microcon filter units were used to separate large RNA species, mainly mRNA, and small-sized RNAs, including miRNA. The miRNA can be further enriched by using a second Microcon filter unit.

2.1.3.1 Reagents and Equipment

- 1 mM Tris, pH7.5
- Millipore Microcon YM-100 centrifuge filter unit (Millipore, Billerica, MA)
- Millipore Microcon YM-3 centrifuge filter unit (Millipore, Billerica, MA)
- Microcentrifuge

2.1.3.2 Procedures

The aqueous phase from “*RNA precipitation*” step 4 is to be used for enrichment of small RNAs, including miRNA:

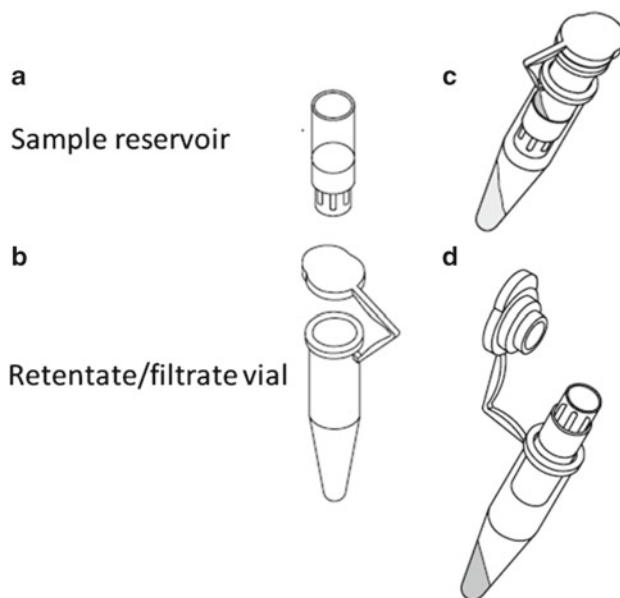
1. Insert a sample reservoir (see Fig. 2.1a) of Micocon YM-100 centrifuge filter unit into a vial provided with the unit (see Fig. 2.1b, c). Add aqueous phase described above to the reservoir. The maximum volume for each reservoir is 0.5 ml. Use multiple filter units when aqueous phase volume is greater than 0.5 ml.
2. Close the reservoir with attached cap (see Fig. 2.1c). Centrifuge at 13,000×g for 6 min.
3. Separate the vial from the sample reservoir.
4. The flow through in the vial contains small RNA species with size shorter than 300 nt, which include miRNA.

Note: The remaining in sample reservoir of YM-100 unit from step 3 contains large RNA species and can be collected and used for mRNA analysis.

- (a) Add 10 µl of 1 mM Tris, pH7.5 to the sample reservoir filter.
 - (b) Invert the reservoir and place in a new vial (see Fig. 2.1d).
 - (c) Centrifuge at 1,000×g for 3 min. Save the purified RNA (mainly mRNA) at –80°C for future use.
5. Assemble a sample reservoir from Microcon YM-3 filter unit into a vial provided with the unit (see Fig. 2.1c). Add the flow through from step 4 into the reservoir.
 6. Close the reservoir with attached cap (see Fig. 2.1c). Centrifuge at 14,000×g for 5 min.
 7. Discard the flow through. The remaining in sample reservoir of YM-3 unit contains small RNA species.
 8. Add 10 µl of 1 mM Tris, pH7.5 to the sample reservoir filter.
 9. Invert the reservoir and place in a new vial (see Fig. 2.1d).
 10. Centrifuge at 1,000×g for 3 min to transfer concentrated RNA to the collection vial, about 10–15 µl in total.
 11. The enriched miRNA from this step can be directly used for labeling or other procedures or saved at –80°C for future use.

Fig. 2.1 Microcon filter unit.

(a) Sample reservoir. (b) Two identical vials are supplied with each unit, one for filtrate and one for retentate. (c) Assembled device during concentration spin. (d) Retentate transfer for recovery spin.



2.2 Quantification of DNA and RNA Concentration by Nanodrop

2.2.1 Reagents and Equipments

- NanoDrop® ND-1,000 UV-Vis spectrophotometer
- Activated type XV calf thymus DNA standards

2.2.2 Standardization of the Instrument

1. Reconstitute 5 mg of activated type XV calf thymus DNA with 5 ml of water, to yield a final stock concentration of 1 mg/ml. Mix the sample well by vortexing for at least 1 min.
2. Prepare standards (low: 50 ng/μl, mid: 100 ng/μl, and high: 200 ng/μl) by diluting the 1 mg/ml activated type XV calf thymus stock DNA in DNA hydration solution. Aliquot into approximately 1-ml portions in 1.5-ml screw-cap tubes. Store at -0°C .
3. Use a spectrophotometer to determine the absorbance values of the controls. Ensure that baseline readings are stable for the controls.
4. Ensure that controls are equilibrated to room temperature prior to use on the spectrophotometer. The reading for the standards should be approximately $\pm 10\%$.

2.2.3 Quantification

2.2.3.1 Blank:

1. Turn on the software by clicking the icon on the screen. Choose appropriate user folder to store your data.

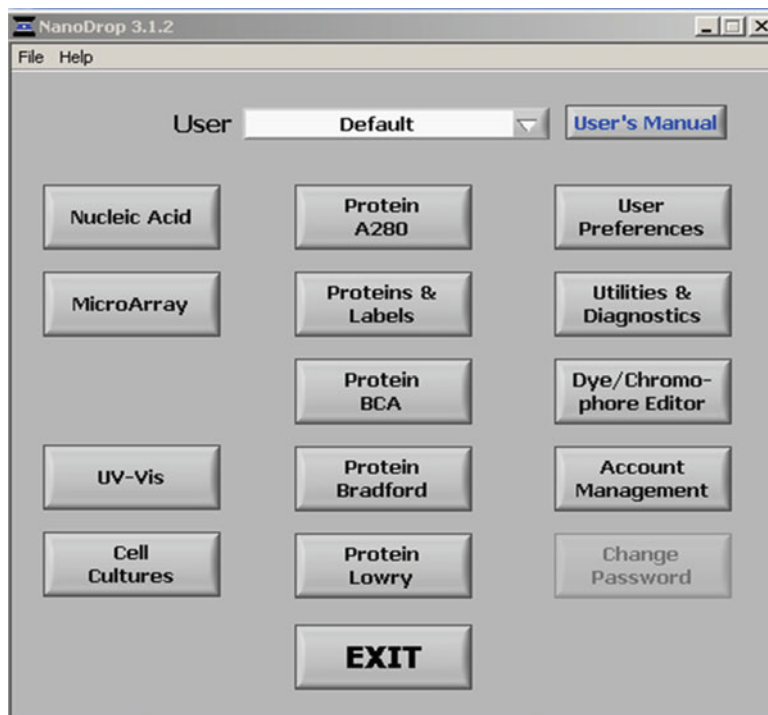


Fig. 2.2 Application modules. Select nucleic acid module. This module automatically calculates concentration and purity of nucleic acids

2. Select “Nucleic Acid” and then “DNA” or “RNA” analysis (see Fig. 2.2).
3. Load 2 μ l of 1X TE buffer (or carrier liquid used with your sample) onto the lower measurement pedestal and lower the sampling arm into the “down” position.
4. Click on the “Blank” button.
5. Wipe the blank from both measurement pedestal surfaces with a laboratory wipe. Measure another blank solution as though it were a sample by loading a fresh 2 μ l 1X TE buffer on the lower measurement pedestal and then click on the “Measure” button. The result spectrum should vary no more than 0.005 A from the zero baseline.
6. If the result spectrum varies more than 0.005 A from the zero baseline or has a negative value, repeat process in step 5, until the spectrum is within 0.005 A. If the baseline is still not within 0.005 A, repeat steps 3 and 4.

Measurement:

1. Start the report by selecting the “Start Report” button. This is very important, as the reading will not record unless this button is selected before performing the sample readings.
2. Start measurements by loading 2 μ l of the previously prepared low, mid, and high calf thymus standards. The readings for the standards should be less than or equal to 10% of expected values (50, 100, and 200 ng/ μ l).
3. Load 2 μ l sample and record result (see Fig. 2.3).

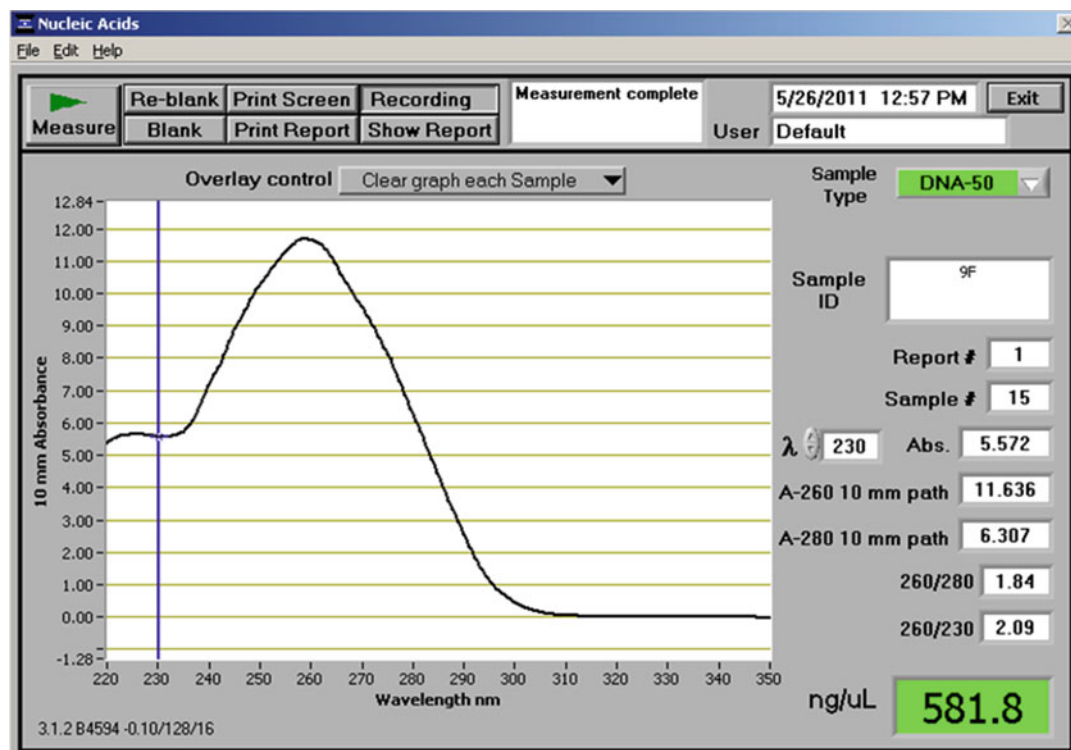


Fig. 2.3 Nucleic acid measurement. *Sample type*: “DNA-50” for dsDNA and “RNA-40” for RNA. The default is DNA-50. λ and *Abs*: the user selected wavelength and corresponding absorbance. *A260 10-mm path*: absorbance of the sample at 260 nm represented as if measured with a conventional 10-mm path. *A280 10-mm path*: sample absorbance at 280 nm represented as if measured with a conventional 10-mm path. *260/280*: ratio of sample absorbance at 260 and 280 nm. *260/230*: ratio of sample absorbance at 260 and 230 nm. *ng/μL*: sample concentration is based on absorbance at 260 nm and the selected analysis constant

Note:

- Both DNA and RNA are measured with a spectrophotometer. Measure the absorbance at 260 and 280 nm or scan absorbance from 220 to 320 nm (a scan will show if there are other factors affecting absorbance at 260 nm).
- Absorbance readings at 260 nm should lie between 0.1 and 1.0 to be accurate. Sample dilution should be adjusted accordingly.
- The A_{260}/A_{280} ratio should be 1.7–1.9 for a “pure” DNA and should be ~2.0 for a “pure” RNA. If the ratio is low in either DNA or RNA assessment, it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. If the ratio is above 2.0 in a DNA assessment, this may indicate the presence of RNA. RNA can be removed by increasing RNase incubation time from 30 to 60 min at 37°C.
- The A_{260}/A_{230} ratio should be 1.8–2.2. This is the secondary measurement of nucleic acid purity. If the ratio is low, this may indicate the presence of co-purified contaminants.

2.3 Nucleic Acid Storage

1. A working stock of DNA can be stored at 2–4°C for several months. For long-term storage, DNA should be stored at –20°C.

2. Storage of RNA

- (a) Split RNA into aliquots with no more than 300 µl per tube.
 - (b) If RNA will be used within 1 month, store in H₂O at −20°C.
 - (c) If RNA will not be used for 2 months or longer, precipitate it and store at −80°C.
3. Note that the solution in which the nucleic acid is eluted will affect its stability during storage. Pure water lacks buffering capacity and an acidic pH may lead to acid hydrolysis. Tris or Tris-EDTA buffer contains sufficient buffering capacity to prevent acid hydrolysis.

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Chapter 3

Test Validation

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Keywords Accuracy • Calibrators • Controls • Laboratory-developed test • Limit of detection • Precision • Reference range • Reportable range • Sensitivity • Specificity • Trueness • Validation • Verification

3.1 Introduction

3.1.1 Regulatory Considerations

3.1.1.1 Regulation of Clinical Laboratory Tests in the United States

All laboratory tests in the United States are considered medical devices and are regulated by the Food and Drug Administration (FDA) under the Code of Federal Regulations (CFR) Title 21 [1]. By contrast, clinical laboratory operations are regulated by the Clinical Laboratory Improvement Amendment (CLIA), which is administered by the Centers for Medicare & Medicaid Services (CMS), in conjunction with FDA and the Centers for Disease Control (CDC) (42 CFR Parts 405, 410, 416–418, 440, 482–485, 488, 491, 493, and 494) [2]. Every laboratory that examines “material derived from the human body for the purpose of providing information for the diagnosis, prevention, or treatment of any disease” is subject to CLIA. CLIA regulations were extensively modified in 2003 to emphasize quality for the entire testing process and to accommodate new technologies (42 CFR 493) [3]. So, while FDA regulations govern manufacturing, approval/clearance, and classification of clinical laboratory test kits or systems, CLIA regulations dictate minimum standards for demonstration of adequate test performance and continued assurance of quality testing within the clinical laboratory.

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Table 3.1 Performance characteristics to be evaluated according to type of test (42 CFR 493.1253) [2]

Type of test	Required parameters to establish
FDA-cleared or FDA-approved tests (unmodified)	<ul style="list-style-type: none">• Accuracy• Precision• Reportable range (and linearity, as applicable)• Reference intervals (normal values) for laboratory patient population
Modified FDA-cleared or FDA-approved tests or non-FDA-cleared tests (i.e., LDTs)	<ul style="list-style-type: none">• Accuracy• Precision• Reportable range (and linearity, as applicable)• Reference intervals (normal values) for laboratory patient population• Analytical sensitivity (lower limit of detection)• Analytical specificity (including interfering substances)• Any other characteristic required for test performance

Proof of adherence to minimum performance standards, as set forth by CLIA, is generally accomplished by laboratory registration, voluntary accreditation programs, and periodic on-site inspections performed by CLIA or various state agencies or private nonprofit accreditation organizations with deemed status from CMS, including the College of American Pathologists (CAP), American Society of Histocompatibility and Immunogenetics, and the Joint Commission (42 CFR 493.551) [2].

While one might expect perfect alignment of requirements between CLIA and the various accrediting agencies, this is not always the case. Therefore, it is important to understand the specific requirements set forth by the applicable accrediting agencies to meet CLIA regulations in regard to test verification, validation, reporting, quality control/assurance, proficiency testing, etc. It is also important to understand the FDA clearance designations for laboratory tests and reagents to determine the type of validation or verification that must be performed.

3.1.2 FDA-Cleared/Approved Products

In vitro diagnostic (IVD) products are reagents, instruments, and systems intended for use in the diagnosis of disease or other conditions in patients (21 CFR 809.3(a)) [1]. Laboratory tests are FDA-cleared/approved for IVD use through two main processes, premarket notification [510(k)] or premarket approval (PMA). Tests successfully submitted through the 510(k) process are labeled as *FDA-cleared*, while tests successfully submitted through the PMA process are labeled as *FDA-approved*.

Prior to revision of CLIA on April 24, 2003, the performance characteristics of an FDA-cleared/approved test established by the manufacturer could be accepted by the laboratory without further in-house validation (42 CFR 493.1253(a)) [2]. Following the 2003 final rule, laboratories must verify certain performance specifications of the manufacturer, including accuracy, precision (trueness), reportable range and linearity, as applicable, and reference interval (normal values) relevant to the test population for that laboratory (42 CFR 493.1253(b)(1)) [2]. Verification of analytical sensitivity and analytical specificity is not required, although limit of detection should be verified for quantitative assays. Also, these test characteristics may need to be confirmed as part of the evaluation of reportable range for qualitative and semiquantitative assays (Table 3.1).

Many clinical laboratories modify FDA-cleared/approved tests. Common modifications include changes in sample collection devices, altering specimen storage length or conditions, testing of alternate

sample types, dilution of samples, performance of an alternate nucleic acid extraction method, or use of a different manufacturer's instrumentation. Such off-label use of FDA-cleared/approved tests necessitates validation of the product in a manner similar to that prescribed for laboratory-developed tests (LDTs) below (42 CFR 493.1253(b)(2)) [2].

3.1.3 *Laboratory-Developed Tests (LDTs)*

Sometimes referred to as “home brew” tests, LDTs are non-FDA-cleared/approved assays that are developed, validated, and performed by clinical laboratories. Generally, LDTs include a combination of individual reagents either prepared or purchased by the laboratory. In 1997, the FDA developed guidelines for manufacturers to provide reagents known as analyte-specific reagents (ASRs) that would be of high quality and manufactured in compliance with good manufacturing practices (GMPs) for use in FDA-cleared/approved tests or LDTs (21 CFR 864.4020) [1]. ASRs may only be sold to manufacturers of diagnostic tests, CLIA-certified laboratories, and certain other nonclinical laboratories. ASRs in the context of molecular testing are defined as “...nucleic acid sequences and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens” and cannot be marketed as stand-alone diagnostic tests (21 CFR 809.10(e), 809.30, and 864.4020) [1]. When feasible, it is recommended that components labeled as ASRs are purchased for use in these assays.

LDTs require more extensive studies of the performance characteristics of the assay than is required for nonmodified FDA-cleared/approved tests, including accuracy, precision, reportable range, reference interval, analytical sensitivity, and analytical specificity (Table 3.1). However, laboratories are not required to verify or establish performance specifications for any test system used by the laboratory before April 24, 2003 (42 CFR 493.1253(b)(2)) [2]. Laboratories are required to include a disclaimer on all patient reports using LDTs, such as: “This test was developed and its performance characteristics determined by [laboratory name]. It has not been cleared or approved by the U.S. Food and Drug Administration” (21 CFR 809.30(e)) [1].

Because of the increasing growth and complexity of LDT use in US clinical laboratories, FDA officials have recently focused on the issue of regulatory oversight of these tests. Proposals have been made for a more comprehensive regulatory system, including FDA review, mandatory registries, and/or changes to CLIA. While discussion of the most optimum regulatory framework for LDTs will continue to evolve, it is reasonable to believe that more strict oversight of such testing will be enforced in the near future with near certain impact on test validation processes.

3.1.4 *RUOs and IUOs*

The FDA deems Research Use Only (RUO) reagents and kits as “in laboratory research phase of development and not represented as an effective in vitro diagnostic product” [4]. As such, they are not to be used for diagnostic purposes. RUO products are not registered with the FDA and are essentially unregulated, although manufacturers may eventually seek clearance or approval for their clinical diagnostic use. Unlike ASRs, manufacturers may sell these products freely to anyone but are encouraged to obtain attestation from researchers that they will not be used for diagnostic purposes. Use of RUO assays is not reimbursable by CMS and most nongovernmental payers [5]. Investigational Use Only (IUO) products may only be used in well-controlled clinical trials to establish clinical performance characteristics in support of 510(k) submission or PMA application to the FDA [4].

Unless exempt, all such evaluations must be issued an investigational device exemption (IDE) by the FDA and IRB-approved before initiation of the study [6].

3.2 Verification Versus Validation

Verification is commonly interpreted as the process of confirming the analytical performance characteristics established by the manufacturer of an FDA-cleared/approved test when it is used according to the package insert. In the simplest of terms, “Does this test work?” This one-time process should confirm that certain performance specifications of the test, including accuracy, precision, reportable range, and reference intervals, when applied to the laboratory’s patient population, are comparable to those established by the manufacturer (42 CFR 493.1253(b)(1)) [2, 7, 8].

The term *validation* is commonly used for the process of establishing performance characteristics of a modified FDA-cleared/approved assay or LDT or test system in which performance specifications are not provided by the manufacturer. In the simplest of terms, “Is this the correct test?” [8] In addition, it can encompass the continued evaluation of test performance over time, through periodic monitoring of control parameters. So, the term may also include “Does this test *still* work?” For these tests, CLIA requires that the laboratory independently establish performance characteristics for accuracy, precision, reportable range, reference intervals appropriate for the laboratory’s patient population, analytical sensitivity, analytical specificity (to include interfering substances), and any other characteristics, as applicable (42 CFR 493.1253(b)(2)) [2]. Unfortunately, these two terms are used inconsistently and interchangeably by various accreditation agencies, despite the fact that they describe different processes.

3.3 Development Phase

3.3.1 Why Bring in a New Test?

The test evaluation process should begin by defining the problem. What exactly is the reason for considering the development of a new test or adoption of a new testing platform? Generally, this is driven by new information, new technology, and a clinical need for a better test. A new test or instrument may offer better analytical performance than an existing method, capabilities to detect a wider spectrum of organisms or genotypes, faster turnaround, or opportunities of automation. It may be driven by test volume issues; perhaps an old test is not able to handle the increasing test volume or is labor intensive. Or, one of cost; cost savings may come from switching to a cheaper test, from better reimbursement, from bringing a test in-house as opposed to referring it out, from consolidating multiple tests on one platform, or from automating processes. Alternatively, a new test may offer the possibility of safer, less-invasive specimen collection for the patient population. Determining the clinician’s readiness to use the test, how it would alter patient care, and expected turnaround times are critical to assessing the clinical utility of the assay. Technical standards and evidence-based guidelines for testing and clinical management of genetic disorders (e.g., American College of Medical Genetics), neoplasms (e.g., National Comprehensive Cancer Network), or microorganisms (e.g., CDC; Association of the Infectious Diseases Society of America) should be consulted to establish the clinical utility and indications for the test. Defining these factors up-front and enlisting the input from all vested parties, including clinicians and laboratorians, is critical to initiating the evaluation process.

Table 3.2 Factors to consider in initial paper assessment of new test/testing platform

<i>Analytical performance</i>	Sensitivity, linear range, accuracy, complexity of the target (single or multiple), equivocal results, interferences, controls and calibrators, quality control
<i>Specimen</i>	Type, anticipated test volume, stability, storage, preparation
<i>Instrument</i>	Ease of use, throughput, footprint, required facilities, test menu, technical support, interfacing, reporting, random access, ancillary instrumentation
<i>Cost</i>	Purchase/lease/reagent rental, reagent costs, reagent stability, consumables, instrument cost, hands-on time, throughput, number of controls per run, anticipated repeat testing, royalties
<i>Logistics</i>	Available resources, compatibility with existing tests, tests competing for same instrument, laboratory workflow, dedicated workspace, personnel training and expertise, patent issues, consenting

3.3.2 The “Paper” Comparison [9]

This phase of the evaluation process involves defining the analytes to be tested and the appropriate methodology. Careful selection of a testing platform is important; an improper method choice can be costly to the laboratory not only in terms of financial and labor expenditures during the evaluation process, but also in terms of potentially damaged relationships with clients [9]. It can be exceptionally frustrating to have committed to a test platform, only to discover some major problem during validation. Therefore, intensive research of available testing options, prior to committing to any one test method or platform, is key to having a successful outcome. A good starting point is to confer with colleagues who have already been through the same decision-making process. Posting an inquiry on a society’s Listserv can often secure valuable feedback. Other valuable resources include the technical literature available online, in package inserts, from vendors, or published in professional journals.

This initial review of available tests/platforms and their performance characteristics, considered within budgetary or physical constraints of the laboratory, should help in narrowing the field of potential candidates. Once this has been accomplished, a more comprehensive evaluation of the remaining contending methods can be undertaken. This should include a more in-depth examination of many of the factors presented in Table 3.2. Valuable insight can be gained by observing the operation of the test/instrument in another laboratory or having a vendor arrange a demonstration on-site.

When available, FDA-cleared/approved tests and adjunct instrumentation should be chosen over LDTs, although the laboratory director certainly has discretion when the FDA-cleared/approved test is found to be inferior to the LDT in documented studies [7]. A list of FDA-cleared/approved molecular diagnostics tests is maintained by the Association for Molecular Pathology (AMP; www.amp.org under “Resources” tab; accessed 27 August, 2011). While significantly more expensive than LDTs, these tests have undergone rigorous laboratory and clinical investigation and evaluation by experts in the relevant scientific field. Moreover, comprehensive assessments of clinical sensitivity and specificity, which are usually beyond the financial or logistical capabilities of most laboratories, have been verified. The use of FDA-cleared/approved tests also avoids any potential intellectual property (IP) rights that may be associated with a test. In the absence of an FDA-cleared/approved test, the laboratory may choose to develop a test method. When feasible, LDTs should employ the use of high-quality ASRs. When implementing an LDT, it is important to search for potential IP claims related to the test being considered [10].

3.3.3 Initial Experiments

Once a specific test or testing platform has been identified that appears to meet the specifications for the laboratory, there will be a run-in phase before formal evaluation of the assay begins [7–12]. For LDTs, this will involve assay design and optimization, including identification of target analytes,

design and selection of primers and probes, refinement of assay conditions, selection of appropriate controls and calibrators, evaluation of extraction and detection processes, specimen types and collection methods, and general workflow issues. For all tests, this will involve pilot testing to gain familiarization and experience with the method/test platform and to identify any critical factors that may affect performance. Such testing may explore the linear range and limit of detection (LoD) of the assay but since assay conditions are often being adjusted during this feasibility phase, generated data are generally not used for validation/verification purposes [7, 9]. The extent of this prevalidation/verification process will depend on the complexity of the assay, experience of personnel with the technology, and whether it is an FDA-cleared/approved test or an LDT. This initial assessment should provide a sense of how good the test will be clinically and whether it will be a good fit for the laboratory.

3.3.4 Validation/Verification Plan

The next step in the process is to write the validation/verification plan [7–12]. This should outline the main experiments to be performed to determine the performance characteristics of the test. The design will differ depending on whether the test is FDA-cleared/approved, a modified FDA-cleared/approved test, an LDT, whether single or multiple analytes are targeted, whether qualitative or quantitative, and whether the analyte is frequent or rare, among other factors (Table 3.3). Where possible, validation

Table 3.3 Performance characteristics to be validated/verified according to type and regulatory status of the new assay

Type of test	Regulatory status	CLIA-required parameters to establish	Comments
Qualitative	FDA-cleared/approved	Accuracy Precision Reportable range Reference intervals	Although confirmation of LoD is not required by CLIA, laboratories may want to confirm LoD as part of an evaluation of reportable range
	Modified FDA-cleared/approved or LDTs	Accuracy Precision/trueness Reportable range Reference intervals Analytical sensitivity (LoD) Analytical specificity (including interfering substances) Any other characteristic required for test performance	
Quantitative	FDA-cleared/approved	Accuracy Precision Reportable range (linearity) Reference intervals	Evaluation of reportable range includes confirmation of linearity of the assay. As part of this study, the LoD and LoQ should also be confirmed
	Modified FDA-cleared/approved or LDTs	Accuracy Precision Reportable range (linearity, LoQ) Reference intervals Analytical sensitivity (LoD) Analytical specificity (including interfering substances) Any other characteristic required for test performance	

(continued)

Table 3.3 (continued)

Type of test	Regulatory status	CLIA-required parameters to establish	Comments
Semi-quantitative	FDA-cleared/approved	Accuracy Precision Reportable range Reference intervals	Determining the reportable range may require confirmation of cutoff points of the assay including evaluation of LoD, although this is not specifically required by CLIA
	Modified FDA-cleared/ approved or LDTs	Accuracy Precision Reportable range Reference intervals Analytical sensitivity Analytical specificity (including interfering substances) Any other characteristic required for test performance	

LoD limit of detection, LoQ limits of quantification

experiments should be performed by an experienced analyst, which will avoid errors due to inexperience. When feasible, technologists should be blinded to the status of each sample used in these studies. To reduce systematic errors, sample testing order should be randomized from run to run. Establishing performance characteristics of a study can be an expensive proposition; therefore, judicious planning of experiments will ensure good use of specimens, reagents, and time. With careful planning, data for one experiment can also be used for another. No definitive protocols for statistical analysis of data generated during validation/verification studies have been established; however, several publications provide valuable guidance on this topic, which is beyond the scope of this chapter [7–9, 12–25]. Consultation with a statistician before validation/verification implementation may ensure a more robust plan. Also, a plan for obtaining suitable samples and controls and calibrators for the formal evaluation process should be prepared at this time.

3.4 Materials for Validation/Verification Studies

3.4.1 Specimen Sources

Ready access to a sizable number of appropriate specimens for assay validation/verification purposes can be a challenging proposition. Samples for validation/verification studies most often consist of archived patient samples, commercially available materials, mock samples prepared by the laboratory, or some combination of the three [7, 8, 10, 12]. When choosing archived patient samples for use in validation/verification studies, it is important to determine the stability of the analyte during storage since this could adversely affect results [26]. Frequently, a test that is currently being referred to a reference laboratory will be targeted for in-house validation, in which case, prior to referral, samples may be split, one part used in validation studies, and results correlated between laboratories. For archived samples acquired from an outside laboratory, differences in collection, storage, and transport should be considered since these could negatively impact results.

Standardized commercial or reference material is only available for a limited number of commonly tested analytes [7, 27]. As a result, there is often considerable variability in test results between assays and laboratories, especially in quantitative testing. Several groups, including the World Health

Organization International Standards (<http://www.nibsc.ac.uk>; accessed August 27, 2011) and the Joint Committee for Traceability in Laboratory Medicine (<http://www.bipm.org/en/committees/jc/jctlm>; accessed August 27, 2011), have been actively engaged in the establishment of internationally standardized working reagents for use in nucleic acid-based tests. Currently, such reference materials with assigned international standard (IS) units are available for HIV-1, HIV-2, hepatitis A, hepatitis C, human papillomavirus types 16 and 18, parvovirus B-19, *Plasmodium falciparum*, influenza, and *BCR-ABL1*, and others are planned. IS controls are also available for a variety of genetic disorders, including Prader-Willi and Angelman syndromes, factor V Leiden, prothrombin 20210GA mutation, fragile X syndrome, and hemophilia A, and multiple infectious agents for use in nonquantitative molecular assays. A list of other molecular diagnostic controls, reagents, and proficiency panels is maintained on the website for the AMP (www.amp.org; accessed 27 August, 2011).

When archived patient samples or reference materials are not available, it is possible to make laboratory-specific reference materials by spiking known target into pooled negative samples or other appropriate matrix. Materials used for spiking can include cell lines, microorganisms, plasmid constructs containing the appropriate target sequence, proficiency test material, and calibrators, among others. Spiked material should account for <10% of the final product to maintain integrity of the sample matrix. Human cell lines, DNA containing specific mutations, microorganisms, and isolated genomes of microbes are commercially available from organizations such as American Tissue Cell Culture (ATCC; Manassas, VA), Coriell Institute for Medical Research (Camden, NJ), and German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). Judicious care should be taken in selecting materials to be used in the construction of these standards/controls as copy numbers of genetic mutations can vary by cell line, which may not be representative of many patient samples. Ideally, reference materials should be constructed in such a way that they can be subjected to nucleic acid extraction in a similar manner as clinical samples. Spiking reference material into extracted DNA or RNA that is negative for the analyte just prior to PCR analysis is not equivalent to subjecting the same specimen to lysis and/or nucleic acid extraction [7].

3.4.2 Specimen Numbers

“How many samples are needed to validate a test?” While 20–30 is considered the “rule of thumb” for statistical purposes, there is no set number specifically required by guidelines. Numbers are often based on general conventions, but a number can be generated based on statistical methods [28]. Some statistical tools for calculating power and sample size are available at www.statpages.org/#Power (accessed 27th August, 2011). The number of samples chosen for individual experiments in the validation/verification process will vary by circumstance and may be influenced by a variety of factors, including the nature of the assay (qualitative, quantitative, etc.), regulatory status of the assay, clinical significance of assay results, cost of reagents, and sample availability [7, 8, 12, 16]. Finally, some specimen types, such as cerebrospinal fluid (CSF), may not be readily available for comprehensive analysis; nevertheless, such types of specimen may still be acceptable for routine clinical testing [29]. The CAP suggests inclusion of a disclaimer statement that the sample type has not been validated when testing these rare specimens [30].

3.4.3 Controls and Calibrators

In establishing any molecular assay, due consideration must be given to the choice, composition, and use of controls and calibrators. This will be dictated by multiple factors, i.e., whether infectious disease or genetic disease, qualitative or quantitative, single or multiple targets, amplification or

hybridization-based assay, availability of materials, etc. It is important to understand the controls/calibrators that will be required for continued performance of the assay and to include them in validation studies, so that they are well characterized by the time the new assay is put into use. Requirements regarding the type, quantity, and frequency of controls are dependent upon the nature of the assay (qualitative, semiquantitative, quantitative) as well as the assay's regulatory status (FDA-cleared/approved test, modified FDA-cleared/approved, or LDT) [7, 8, 12, 16]. When designing controls/calibrators, one should consider their function in assuring the quality performance of the assay. Functions of assay performance that may require monitoring by the use of controls/calibrators can include any or all of the following: specimen lysis, extraction, nucleic acid purification, reagent efficacy and purity, amplification conditions, detection, specimen inhibition, and quantitation.

Controls/calibrators may be prepared in-house or obtained commercially or from other sources. If a commercial test system provides some but not all control/calibrator materials, it is the laboratory's responsibility to define and validate additional control material beyond those recommended by the manufacturer to meet requirements of the relevant accrediting agency [7, 8, 12, 16]. Where feasible, controls/calibrators should have a matrix that simulates that of clinical specimens because components of the matrix may affect detection of the analyte. Since control/calibrator materials will be used repeatedly over a potentially long period of testing, preparation of a durable supply is recommended; however, due consideration should be given to storage. Potential deterioration of controls due to freeze-thaw or long-term storage should be explored as part of the validation/verification process. For positive controls and calibrators that generate a quantified value, expected ranges should be established and monitored using standard Westgard rules and Levey-Jennings plots, where appropriate.

3.4.4 Controls

Examples of some controls that may be used in molecular assays follow:

3.4.4.1 Reagent Contamination/Negative/No-Template Control

This control, which is variably referred to as the “no-template,” “negative,” or “blank” control, is common to all nucleic acid amplification assays and controls for the possibility of intersample contamination with isolated nucleic acids or previously amplified products. Generally, this control consists of water or buffer in place of any nucleic acid template, but ideally it should comprise a specimen that lacks the targeted sequence. The inclusion of more than one reagent contamination control, interspersed between patient samples, is strongly recommended whenever large numbers of patient samples are included. This control can also be used to assess background noise generated by reagents in the assay. Where possible, this control should be taken through the entire assay process.

3.4.4.2 Positive Control

At least one positive control is needed for all molecular assays. Positive controls may consist of matrix or negative samples spiked with target (cultured cells, microorganism, artificial constructs such as plasmids) or previous positive clinical samples. For genetic tests, heterozygous variant controls are often used since normal and variant alleles are represented. It is preferable to have positive controls for each of the analytes detected by the assay, but this may not be feasible in all instances, especially when running highly multiplexed assays. In lieu of multiple individual positive controls or a pooled analyte control, individual positive controls can sometimes be rotated between each run of the assay.

If a positive control is unavailable for a specific targeted sequence (i.e., rare genetic disorders), an alternative control procedure could include bidirectional sequencing [12].

It is generally recommended that the positive control contains a low concentration of target sequence that is still reliably detectable (close to the lower limit of detection (LoD)) to challenge the assay and minimize the risk of contamination of patient samples. For quantitative assays, two positive controls are mandated for every run (low and high concentrations of analyte), usually set at medically relevant decision points in the reportable range. As with the negative control, it is often necessary for positive controls to be taken through the entire assay process, including lysis and extraction to assure adequate function of the assay.

3.4.4.3 Inhibition (Internal) Control

This control is used to monitor individual patient specimens for substances that could interfere with amplification, potentially producing a false-negative result. This type of control should be included if reaction inhibition is a significant source of false-negative results. Use of inhibition controls may be relaxed or discontinued if it is determined that inhibition rates are medically acceptable; however, this should be proven experimentally and documented. Testing for inhibition in patient samples can be accomplished by multiple methods [7, 8]. A small amount of the same or similar (*homologous*) or different (*heterologous*) target sequences can be added to a second aliquot of the patient specimen and run in parallel with the original aliquot to test for inhibition (*extrinsic control*). Some assays also add the inhibition control target within the same reaction used for analyte testing. Alternately, target sequences inherent to the host genome, often referred to as “housekeeping genes” (*heterologous intrinsic control*), are coamplified with the target sequence. Some commonly targeted genes used as intrinsic controls for genetic and cancer testing include β -globin, β -actin, and glyceraldehyde-3-phosphate dehydrogenase. To avoid competition in amplification, concentration of internal controls should be kept to a minimal level and should produce products that are of a size that is in excess of that of the largest targeted analyte.

3.4.5 Calibrators

Calibrators are used to evaluate and adjust a test system’s quantitation of the analyte being detected relative to the actual concentration of analyte present. Calibration verification assures the operator that the assay has remained stable throughout the reportable range for the analyte when testing patient samples, and therefore patient results are set accurately. Calibrators are constructed with known concentrations of the analyte. These can consist of commercially available standards or reference materials, proficiency samples in the appropriate matrix, or previously tested patient specimens. The reader should consult regulations and guidelines for how calibrators should be used [16, 17, 25, 31].

3.5 Analytical Validation/Verification

3.5.1 Analytical Sensitivity

Analytical sensitivity indicates the ability of a test to give a positive result for specimens containing the analyte (e.g., mutation or infectious agent) being measured [7, 8, 16, 17, 32]. There are various contextual definitions of this term that the reader should be aware. Some authors use it strictly when

referring to qualitative and semiquantitative tests, whereby it is defined as the ability to obtain positive results in concordance with positive results from a reference method (a.k.a. positive agreement) [7, 8, 16, 17, 32]. Positive agreement is considered under the section on Accuracy below. However, most associate analytical sensitivity with the term *lower limit of detection* (LoD), which is the least concentration of analyte that can be reliably detected (often set at $\geq 95\%$ of samples with analyte at that concentration) in a given specimen type [7, 8, 16, 17, 32]. Obviously, LoD applies to quantitative and semiquantitative tests. For qualitative tests, LoD is often defined as the lowest concentration of analyte that gives an informative test, for instance, the concentration of DNA or RNA that will give an accurate result $\geq 95\%$ of the time. LoD must be determined for all anticipated specimen types and for all analytes represented in the assay. Ideally, LoD determinations should be performed including the DNA/RNA extraction process as part of the verification/validation experiments, as nucleic acid extraction is a source of variability in test performance.

There are numerous factors to be considered when designing analytical sensitivity experiments and multiple ways in which LoD can be determined. In infectious disease testing, LoD is generally easy to define, but it is important to consider that LoD may differ by organism genotype or subtype and that this difference may be clinically relevant [7, 8, 16, 17, 32]. In genetic and cancer testing, the LoD can be harder to define in a clinically relevant manner and can pose more of a challenge for experimental design. For genetic testing involving detection of constitutional mutations, which are basically present in every cell of the body, LoD is often not of special concern. One quick approach to defining the LoD for these mutations is to dilute samples with known mutations with normal samples [12]. Also, one can determine the minimum concentration of DNA needed to perform the assay [8]. However, the ability to detect some types of genetic mutations may demand a more comprehensive analysis. For instance, assays that produce variable sizes of PCR fragments depending on the type of mutation present (e.g., trinucleotide repeat expansion disorders) will likely have variable LoDs; large expansions of GC-rich sequences may be quickly outcompeted by normal-sized alleles in a PCR assay. Also, LoD determinations for mitochondrial DNA mutations may be influenced by the type of mutation, type of specimen, and degree of heteroplasmy. For molecular assays designed to detect a wide spectrum of mutations associated with a particular genetic disorder, it may not be possible to determine the LoD for all mutations, and ongoing evaluation of LoD following assay implementation may be unavoidable [12].

For cancer testing, the LoD is often defined as the minimum amount of tumor as compared to normal cells that must be present in a sample to yield a valid result [8]. The amount of tumor cells in clinical samples that could potentially be used in studies to define LoD can often be very difficult to determine, and often measurements rely on relatively inaccurate methods or subjective criteria. For instance, in a test to detect *KRAS* mutations in formalin-fixed, paraffin-embedded (FFPE) tissue sections of colorectal cancer, one could use a subjective visual assessment of the number of tumor cells relative to normal cells observed on an H&E-stained section; however, this assumes that each tumor cell has the mutation. Alternatively, one could use cell lines that have *KRAS* mutations to create serial dilutions; one could even simulate FFPE processing of these cells, but these cells will never have the complex matrix features of clinical tissues, nor will they be representative of the highly variable processing of specimens that occurs in most surgical pathology suites.

3.5.1.1 Study Approach

Many different specimen or surrogate sample types can be used for LoD testing. In infectious disease testing, it is often practical to make spiked surrogate samples of known concentrations to use in LoD testing. For cancer testing, “pure” tumor samples, tumor cells microdissected from tissue sections, or well-characterized cell lines can be used to create mock samples of varying concentrations by spiking into normal tissue samples. Plasmids or extracted nucleic acid can also be used for creating surrogate samples for LoD testing; however, these methods are generally less desirable.

Common methods employed to evaluate LoD include testing multiple replicates of serial dilutions to determine the concentration at which $\geq 95\%$ of samples are detected, as well as probit analysis [7, 16]. When testing replicates over a series of dilutions, a minimum of 20 replicates at each dilution should be tested. CLSI recommends 20 samples for *confirmation* of a manufacturer's claim of LoD but suggests a minimum of 60 data points (12 measurements each from 5 samples) to *establish* an LoD [24, 32]. Various authors recommend 20 data points at and on either side of the anticipated LoD, as determined by the initial analyte dilution series [7, 8, 12, 17]. In this scheme, at least 19 of 20 replicates must be positive to meet threshold criteria. Any concentrations with < 19 replicates positive are determined to be below the LoD of the assay. In probit analysis, replicates at various concentrations are also tested, and the LoD is determined by a statistical regression model. Commercial statistics software is available that will perform probit analysis. Ideally, these experiments should be performed over a series of runs over multiple days so that variations are appropriately incorporated. Finally, the LoD may be determined in conjunction with the limit of blank (LoB), the distribution of values obtained from measurement of multiple blank negative or normal samples [17, 32]. The LoB is most important to consider in methods in which a negative result generates a detectable signal in the assay. Obviously, the LoD should not fall within the range of possible results generated by a true negative sample. LoD and LoB determination methods are described in CLSI documents EP17-A and MM-06-A2 [24, 32].

Interfering substances should be explored in the context of LoD determination since their effects are often acutely apparent at lower concentrations of the analyte. Positive samples could be spiked with potential confounding substances or other analytes (see Sect. 3.5.6 below).

3.5.2 *Quantitative Tests and LoQ*

Quantitative molecular assays have a limited range over which the measured value of an analyte is acceptably accurate and precise without dilution or concentration or other pretreatment of the primary specimen. This range is commonly referred to as the linear range or analytical measurement range (AMR) [7, 8, 16, 24, 29, 32]. The extremes of this range are referred to as the upper and lower limits of quantification (LoQ). In many assays, the lower LoQ is not the same as the lower LoD because of a general loss of precision at the extremes of analyte concentration. Confirmation of an assay's linearity as well as determination of the AMR/upper and lower LoQ is required for all quantitative assays.

3.5.2.1 *Study Approach*

Linearity is determined by testing sequential known concentrations of an analyte and plotting them against the expected value [7, 8, 16, 24, 29, 32]. Optimally, these concentrations should be evenly spaced and include the anticipated minimum and maximum values that can be accurately measured. However, it is often useful to expand the concentration range in these experiments beyond the anticipated upper and lower concentration limits to demonstrate the limits of linearity of the assay. Serial tenfold dilutions are commonly used. Commercial calibration material is available for many analytes and is a good choice for these experiments. When possible, material calibrated to known internationally standardized working reagents should be used. Despite calibration to a known standard, variability can still exist between testing platforms and specimen processing, so it is important to know how the concentrations were established for the calibration standards to be used.

For FDA-cleared/approved assays, a minimum of three replicates of at least five different concentration levels over the expected linear range of the assay should be tested. Greater numbers (seven to nine concentrations tested in triplicate) should be used for LDTs [32]. Results should be plotted with

the measured log-transformed (log base 10) results on the y-axis and the expected results on the x-axis. Commonly, a simple linear regression analysis is used to evaluate the data. The r^2 value describes the fit of the regression line with values approaching 1.0 indicating a linear relationship between x and y . Values approaching 0.0 indicate that there is no linear relationship between x and y . Y-intercept values that are significantly different from zero indicate bias between the assay and expected value [7]. Additional experiments should be considered at the lower LoQ to confirm precision, if this value is different than the LoD.

In addition to establishing the AMR, the laboratory should also verify the AMR for each reported analyte at least every 6 months and after recalibration or major maintenance to instrumentation or major changes to the protocol [7, 8, 12, 31]. AMR verification performed after introduction of an assay may use commercial calibrators or standards, PT materials, or patient samples with known results and comprise at least three samples that span the measurement range. Criteria for acceptability of values should be established by the laboratory.

3.5.3 Reportable Range

The *reportable range* is the range of results for which a system has been proven to yield (numerically) accurate results [7, 8, 12, 16, 31]. For qualitative tests, the reportable range could simply be positive (detected) and negative (not detected), or the genotypes of the patient or organism detected by the assay. For quantitative tests, the reportable range includes the AMR and the *clinically reportable range* (CCR), which is the range that is clinically acceptable to report and might include dilution, concentration, or other pretreatment of specimens before analysis. So, the CCR may be wider than the AMR, although sometimes the CCR can be determined by clinically relevant limits, which might be narrower than the AMR. Establishing the CCR is a clinical decision to be made by the laboratory director. If a test result is less than or greater than the CCR, it is reported as less than the lowest value or greater than the highest values of the CCR (e.g., <50 copies/mL or >500,000 copies/mL). When a dilution or concentration is performed on a patient sample to achieve a value on the AMR, an appropriate comment should be added to the report.

3.5.3.1 Study Approach

Defining the reportable range of an assay may not require specific validation experiments. For qualitative tests, this will be simply “negative or positive” or a list of the genotypes that the assay detects. For quantitative tests, experiments demonstrating the AMR as well as experiments demonstrating the validity of diluting or concentrating samples will likely suffice to determine the reportable range.

3.5.4 Reference Range

The *reference range* is the range of values for a specific analyte found in a normal (healthy) population [7, 8, 12, 16]. The term reference range is preferred over “normal range” since the reference population is a defined population (e.g., pediatric, male, female) rather than an ill-defined concept of normal. Reference ranges allow the clinician to compare a patient’s results to a specific population or normal reference. Reference ranges are often more applicable to clinical chemistry type tests rather than molecular assays [33]. Nevertheless, the same information is also obtained to determine background levels of molecular assays as part of analytical sensitivity experiments.

The reference range must be established or verified for each reported analyte and specimen source (e.g., blood, urine, cerebrospinal fluid), when appropriate. In addition, it must be verified any time there is a major modification to the methodology or instrumentation or a change in the patient population.

3.5.4.1 Study Approach

An adequate number of normal (negative) patients should be tested to establish the measureable values associated with this population. The results obtained with abnormal patients should all reside outside the reference range. Alternatively, reference range(s) established by the manufacturer or cited in the literature may be appropriate.

3.5.5 Analytical Specificity

Analytical specificity indicates the ability of a test to give a normal or negative result for specimens that lack the analyte (e.g., mutation or infectious agent) being measured [7, 8, 12, 16]. Ambiguity also exists in defining this term. Some authors use it strictly when referring to qualitative and semiquantitative tests, whereby it is defined as the ability to obtain negative results in concordance with negative results obtained from a reference method (a.k.a. negative agreement). Negative agreement is considered under the section on Accuracy below. CLSI and the FDA define it as the ability of a test to correctly identify or quantify an analyte in the presence of interfering substances. Clinical specimens may contain various endogenous or exogenous (contamination) substances that may interfere with the performance of a test and potentially generate the wrong result.

3.5.5.1 Study Approach

Careful optimization of protocols must be undertaken to maximize target recognition and avert the potential for codetection of nonspecific targets. For LDTs using primers and probes, these should be carefully designed or selected. Searches of databases for homologies between primers/probes and targeted and nontargeted sequences are essential. This becomes particularly important when designing multiplexed assays to mixed source samples (e.g., multiple species of bacteria or viruses). Primers and probes should be checked for potential interfering polymorphisms that may underlie these sequences; this should also be performed periodically following implementation of the assay since updates to databases are made. These actions should be documented and included as part of the validation summary. Various technical publications [34] and guidelines (e.g., <http://www.ambion.com/techlib/tn/134/13.html>; accessed August 27, 2011) and publically available primer design and analysis tools (Table 3.4) may aid the laboratory in navigating this process. Alternatively, commercial services are available, whereby clients can simply provide a specified reference sequence to be targeted, and primers/probes are designed and synthesized by the vendor.

For infectious disease testing, specificity of the assay should be demonstrated using multiple microbial strains/isolates/variants of the intended target, genetically related microorganisms, microorganisms that can cause similar clinical conditions, and microorganisms that can be found in the same specimen types that will be used in the assay. For all assay types, false positives should be rerun and/or checked by an alternative method(s), where possible. If any cross-reactivity is noted that cannot be easily resolved, a plan should be developed to reflexively confirm all positive results from the screening assay (e.g., additional reflex probe-, restriction enzyme-, or sequence-based testing). Molecular

Table 3.4 Publicly available primer design and analysis tools

Online tool	Description
AutoPrime	Primer design for real-time PCR measurement of eukaryotic gene expression (http://www.autoprime.de/AutoPrimeWeb) ^a
CODEHOP	<i>C</i> onsensus- <i>D</i> Egenerate <i>H</i> ybrid <i>O</i> ligonucleotide <i>P</i> rimers designed from protein multiple sequence alignments (http://blocks.fhcrc.org/blocks/codehop.html) ^a
ExonPrimer	Design of intronic primers for PCR amplification of exons (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html) ^a
IDT SciTools	Variety of tools for primer/probe analysis and design (http://www.idtdna.com/scitools/scitools.aspx) ^a
NetPrimer	Variety of tools for primer/probe analysis and design (http://www.premierbiosoft.com/netprimer/) ^a
PathoGene™	Coding sequence finding and primer design tool for microbial, viral, or fungal genomes (http://pathogene.vbi.vt.edu/) ^a
Primer3	Primer selection tool for PCR amplification of DNA (http://frodo.wi.mit.edu/primer3/) ^a
Primer-BLAST	Finding primers specific to PCR template (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) ^a
PrimerDigital	Webtools for PCR, qPCR, in silico PCR, and oligonucleotides (http://primerdigital.com/tools/)
PrimerX	Automated design of mutagenic primers for site-directed mutagenesis (http://www.bioinformatics.org/primerx/) ^a
Primo Pro	PCR primer design (http://www.changbioscience.com/primo/primo.html) ^a
QuantPrime	Primer design for real-time qPCR on any eukaryote transcriptomes (http://www.quantprime.de/) ^a
SiteFind	Primer design for site-directed mutagenesis (http://www.utmb.edu/scccb/software/sitefind.html) ^a
SNPCheck	Check for primer binding and underlying polymorphisms (https://ngri.manchester.ac.uk/SNPCheckV2/docs.htm)
UCSC in silico PCR	Searches sequence database using given pair of PCR primers (http://genome.ucsc.edu/cgi-bin/hgPcr) ^a

^aAccessed August 27, 2011

amplification methods for microbe detection that are based on SYBR® Green or melt-curve analysis will typically require a secondary confirmatory test during assay development. Any limitations to test interpretation imposed by cross-reactivity should be clearly specified in the patient report.

In order to accurately assess the true specificity of a test, all attempts should be made to minimize potential sources of exogenous substances that could lead to false positive results. These can occur during the preanalytical or analytical phases of specimen processing. Cross contamination can occur during sample processing (e.g., contamination of fetal samples with maternal cells collected during amniocentesis). The surgical pathology suite is also a source for sample-to-sample contamination that can occur during grossing of specimens or cutting of sections; personnel should receive instructions on appropriate handling of specimens that will be used for molecular analysis. Likewise, the molecular laboratory should have physical and processing safeguards to prevent inadvertent contamination of specimens/reactions. Where feasible, only ASRs or molecular biology grade reagents that are certified free of contaminating nucleic acids or other potentially interfering substances should be used in LDTs.

3.5.6 Interfering Substances

Apart from cross-reactive substances showing some homology to targeted sequences that detrimentally impact the analytical *specificity* of a test, other (endogenous or exogenous) substances and collection/processing steps have the potential to adversely influence the *sensitivity* of an assay.

The laboratory should investigate potential common interferences, if these have not been previously identified by an instrument-reagent manufacturer or appear in the published scientific literature. Potential sources for interfering substances should be investigated for each step of specimen collection, storage, and test analysis. The wrong type of collection vial or media, the absence of specimen stabilizers, improper sampling, improper storage, and the presence of hemoglobin, fats, medications, or residual reagents from nucleic acid extraction or stabilization procedures, or contaminating copurified DNA or RNA can all change the performance characteristics of a test. Multiplex assays tend to be particularly susceptible to interferences because of their complexity. Any limitations to test interpretation imposed by interfering substances should be clearly specified in the patient report.

3.5.6.1 Study Approach

Testing of potential interfering substances should occur at the highest concentration expected to be found clinically (or higher). This may be done by spiking aliquots of clinical specimens (with analyte concentrations near medical decision points) with suspected interfering substances or by purposely mishandling specimens (e.g., to cause hemolysis or nucleic acid degradation). Studies should be performed for each specimen matrix type.

3.5.7 Accuracy and Trueness

Accuracy measures the closeness of the agreement between the results of a single measurement and the true value [7, 22]. Alternatively, the term *trueness* refers to the closeness of the agreement between the average result obtained from a large series of measurements and the true value. CLIA recommends that performance characteristics of any new assay be compared against a “gold standard.” This “gold standard” may be an existing (comparative) method, in which case, the accuracy/trueness of the results of the new method can be *verified* by comparison to the results of the comparative method. Alternatively and preferably, if standardized reference materials or a definitive method are available, the accuracy/trueness of the results of the new method can be *established* relative to the results of the reference materials/method (recovery method).

Method comparison studies are frequently used when deploying a new piece of testing equipment or test method to replace an existing testing platform. The intent of this exercise is to demonstrate that the two methods (old and new) are statistically similar, such that the two tests can essentially be used interchangeably or the new test can reasonably replace the old test. Generally, when comparing a new method to an established assay, the new method should demonstrate comparable performance to the “gold standard” method, without significant loss of diagnostic power. Perfect correlation (equivalence) of results between the two tests is not anticipated, but any differences should be clinically insignificant.

There can be certain challenges in designing an effective method comparison. Frequently, the impetus for adopting the newer technology is the superiority of the new test; newer molecular methods are often more sensitive than existing “gold standard” methods. Without comparative data from the “gold standard” method that spans the full detection range of the new test, additional well-characterized reference materials will be required to explore the LoD of the new test. Dideoxynucleotide sequencing is frequently viewed as a reasonable “gold standard” with which to compare other molecular methods that are designed to detect sequence variants; however, the analytic performance characteristics of Sanger sequencing may be vastly different from the method being compared. For instance, the LoD for Sanger sequencing is generally considered to be approximately 20%, which may be substantially higher than many other molecular methods.

Resolution of discrepant results between the two tests in a method comparison experiment may be problematic, potentially requiring testing by a third test method or by another independent laboratory. This becomes especially problematical for quantitative assays in the absence of IS reference materials since the performance characteristics of the same test at another laboratory may be quite different from that demonstrated locally. Discrepant results may also be resolved by comparing with clinical data. Additional challenges are encountered when evaluating multiplexed assays, where potentially multiple comparative methods have to be used to investigate the accuracy of each analyte. Statistical analyses of these data likewise become complex.

3.5.7.1 Study Approach

Method comparisons can be performed for assays that generate qualitative, semiquantitative, or quantitative types of data. Generally, ≥ 30 (CLSI suggests ≥ 40) samples should be analyzed in duplicate for both methods over at least 5 days along with routine quality controls. If close agreement between the new and comparative method is expected, then samples may be tested singly. Use of reference materials or other materials with known concentrations or activities is suggested in establishing or verifying accuracy.

For qualitative methods, test results are categorized into positive or negative with equal numbers of each category being tested. Percentage agreement, false-positive and false-negative rates, and sensitivity and specificity should be calculated for the new assay relative to the “gold standard” and displayed in a concordance table. If disproportionate numbers of positives and negatives are used, it may be more practical to separate overall test agreement into agreement values for positive and negative specimens. McNemar test for symmetry can be used to detect bias of one assay over the other, while Cohen’s kappa can be used to check for agreement between the two tests; generally, kappa values ≥ 0.8 are considered good agreement, while a value of 1.0 indicates perfect agreement.

For semiquantitative methods, results may be grouped into a number of different alphanumeric or numeric states, such as positive, negative, and equivocal; or homozygous normal, homozygous variant, and heterozygous; or with variable cutoffs for positive and negative. Generally, ≥ 30 samples should be tested with equal numbers of specimens in each category. A receiver-operator characteristic (ROC) curve may be helpful in delineating cutoff values [8].

For quantitative assays, samples should be chosen with results distributed over the reportable range of both assays. If the analytic ranges of the comparative and evaluation methods are different, additional reference materials may be required to assess the entire analytical range of the new test. Test results from both methods can be subjected to linear regression analysis (Deming, least square, or Passing-Bablok) with establishment of 95% confidence intervals (CI). A scatter plot allows for visual inspection of the type of error inherent in the evaluated method. Comparison of data generated by the two methods should be especially similar at existing positive–negative cutoffs and crucial medical decision points of quantitative assays. Outliers, nonlinearity, slope significantly different from one, and intercept significantly different from zero should be thoroughly investigated before establishing similarity between the two methods. In addition, a Bland-Altman or Tukey mean-difference plot with calculation of 95% CI can be made to analyze the agreement between the two sets of data. Data for this plot require prior log transformation. If data show bias between the two methods, then a larger study (typically 100) is needed.

In the absence of an independent comparative test to measure the accuracy of a new test, defining an appropriate “gold standard” can be problematic or impractical. However, one approach is to use the pathologic or clinical diagnosis as the comparator, which is ultimately defined by a variety of separate clinical tests and/or observations. In this scenario, the analysis is similar to assessment of a qualitative test; data are displayed in a concordance table and analyzed for bias and agreement, as described above.

3.5.8 Precision

Precision is described as the “closeness of agreement between independent test results obtained under stipulated conditions” and is used as a descriptor of random error associated with the overall operation of the assay [7, 8, 35]. In quantitative assays, this value is generally expressed as imprecision using standard deviation (SD) and/or coefficient of variation (CV). When defining precision for a specific test, it is important to consider all processes that might introduce variability into test performance. These can include differences between operators, instruments, or equipment, variation of performance between runs of the same day or between days, or variation in lots of reagents, among others. Repeatability is the term used to describe variability within a single run and will likely demonstrate the least difference in results between replicates. Reproducibility is the term used to describe when there is variability in one or more factors such as run, day, or operator [7, 8, 32].

3.5.8.1 Study Approach

Experiments to test precision can be performed using commercial standards, control material, or patient samples and, at a minimum, should include ten replicates of two to three sample concentrations [16]. A common experimental design used for assessing precision in chemistry assays evaluates replicates at multiple levels over 20 days [36]. However, this model may not be economically feasible for some molecular assays. It is important to use a single lot, batch, or source of control material for precision experiments to eliminate variability in sample as a contributor towards error. When testing precision of a quantitative assay, it is important to include known concentrations close to the LoD as well as concentrations that represent the AMR, including any potential values at medical decision points. Often times, assays are less precise at the extremes of concentration. For qualitative tests, it is important that one of the concentrations tested is close to the LoD since high positives will not adequately challenge the assay. The design and statistical analyses employed for precision experiments can be quite variable; therefore, it is recommended that additional references, preferably specific to the type of assay under development, are reviewed prior to undertaking this task [7, 32].

3.6 Clinical Validation/Verification

Often the clinical performance of a new test is something that gets forgotten in the whole validation exercise. Indeed, assessment of clinical performance specifications for new tests is not included as part of CLIA regulations, although laboratories are required to prove that all testing meets clinical expectations. *Clinical validity* (or performance) refers to the ability of a test to detect or predict a specific clinical disorder or phenotype, so it addresses how useful a test is clinically in making a diagnosis or prognosis. This can be a difficult element to assess in certain circumstances, often requiring long-term studies and gathering of data from other areas of the laboratory and clinical charts. Because establishing clinical validity often requires a much larger number of patient specimens and data than may be immediately accessible to the laboratory, CAP states that it “may require extended studies and monitoring that go beyond the purview or control of the individual laboratory. In such cases, it is acceptable to provide documentation in the form of peer-reviewed studies in the scientific literature.” However, it is important that laboratory directors use sound judgment in evaluating these studies since the patient population in many of these studies may be quite different from the local population. Cited studies should support the laboratory’s stated indications for testing. Any departures from clinical validity claims in the literature or changes in clinical validity values as the result of internal review of

data must be included in patient reports. It is important to select the patient population carefully when performing any clinical validation/verification exercise, but this becomes particularly poignant when making clinical validity calculations. Two elements need to be considered in assessing clinical validity: clinical sensitivity and clinical specificity.

3.6.1 Clinical Sensitivity

This is a measure of how well that positive test results correlate with patients having the underlying clinical condition that is being considered; the likelihood that a positive test result correctly determines the patient's condition. This can be assessed by determining the proportion of patients with positive test results and correlating these with the proportion of patients with the clinical disorder or phenotype being considered. This can be written as:

$$\text{Sensitivity} = \frac{\# \text{ true positives}}{\# \text{ condition positives}} = \frac{\# \text{ true positives}}{\# \text{ true positives} + \# \text{ false negatives}}$$

So, if 100 patients with a confirmed condition are tested and 57 test positive, the sensitivity of the assay is 57%.

3.6.2 Clinical Specificity

This is a measure of how well that negative test results correlate with patients lacking the underlying clinical condition that is being considered, i.e., the likelihood that a negative test result correctly determines the patient's condition. This can be written as:

$$\text{Specificity} = \frac{\# \text{ true negatives}}{\# \text{ condition negatives}} = \frac{\# \text{ true negatives}}{\# \text{ true negatives} + \# \text{ false positives}}$$

So, if 100 individuals with no disease are tested and 98 test negative, then the specificity is 98%. Note that calculations of sensitivity and specificity do not accommodate test results that are indeterminate. Indeterminate results are usually handled in one of two ways: they can be excluded from the analysis but the number of exclusions should always be stated when quoting sensitivity, or they can be added to the false negative category, which essentially gives a “worst-case” assessment of test sensitivity.

3.6.3 Predictive Values

Predictive values are used to assess the *probability* that a particular test result reflects the underlying condition being studied. Unlike sensitivity and specificity, predictive values are not a function of the test but rather depend on the prevalence of the disease in the population. The *positive predictive value* (PPV) is the probability that disease is present when the test is positive and can be written as:

$$\text{PPV} = \frac{\# \text{ true positives}}{\# \text{ testing positive}} = \frac{\# \text{ true positives}}{\# \text{ true positives} + \# \text{ false positives}}$$

Negative predictive value (NPV) is the probability that disease is absent when the test is negative and can be written as:

$$\text{NPV} = \frac{\# \text{ true negatives}}{\# \text{ testing negative}} = \frac{\# \text{ true negatives}}{\# \text{ true negatives} + \# \text{ false negatives}}$$

Generally, predictive values can only be accurately assessed in large cross-sectional studies and may require a much larger number of positive patient specimens than are immediately accessible to the laboratory. If the number of patients with a specific condition is low in the population used for test validation, then the predictive values of the test will be inaccurate. Predictive values should only be calculated when the ratio of patients in the disease group to patients in the healthy control group is equivalent to the prevalence of the disease in the population [7, 8, 12].

3.7 Other Experiments

The studies discussed in the foregoing text are by no means comprehensive. Other experiments may be designed to investigate other aspects of the performance of a new test, as deemed necessary by the laboratory and clients. Additional characteristics of a new test that may prove valuable include stability of specimens, controls, calibrators, and other reagents, potential sources of carryover or contamination (“checkerboard” experiments), etc.

3.8 Validation/Verification Data Summary

At the completion of the validation exercise, the laboratory director should compile a succinct summary of the validation process, evaluation findings, and conclusions. Any discrepant results should be highlighted and an explanation given as to how discrepancies were resolved. The validation summary document can take various formats, but the template in Table 3.5, which also includes a checklist of additional features not discussed in this chapter, such as communication with medical staff and IT, updating laboratory documents, and billing issues, may help in the construction of a comprehensive document. This should be readily available to inspectors who may inspect the laboratory. Documentation of all validation and verification procedures must be retained by the laboratory for the duration of the test and for at least 2 years after discontinuation of the test. Of note, each laboratory is responsible for the conduct of its own validation/verification studies relevant to its patient population. These studies are not directly transferrable to another clinical laboratory.

3.9 Quality Control/Assurance

Once the test has been validated/verified and implemented, additional performance data related to preanalytic, analytic, and postanalytic processes in the assay should continue to be collected over time by monitoring of various quality control/assurance measures. Documentation of longitudinal assessments should be made of the performance of controls, of rejected and equivocal specimens, of turnaround times, of proficiency testing, and following review of a percentage of patient reports with normal and abnormal results. Additional analytic monitors may include tracking of Ct values and melt temperatures of controls, signal ratios in FISH assays, background values, failed runs or reactions, and lot-to-lot checks. Such monitoring of parameters related to the test affirms the validation/verification

Table 3.5 Sample validation/verification plan and summary sheet

Test validation/verification summary

Assay: *Title* (Procedure # *XX*)

Methodology: (e.g., *FISH, PCR-RE, PCR sequencing, PCR-reverse blot, real-time PCR*):

Regulatory status:

- ☐ FDA-cleared/approved assay
- ☐ Modified FDA-cleared/approved assay
- ☐ Laboratory-developed test
 - ☐ LDT with ASRs
 - ☐ LDT with no ASRs

Specimen types tested: (e.g., *peripheral blood, bone marrow, CSF, urine, plasma, sputum, etc.*)

Type of specimen/matrix	# Specimens

Specimen types acceptable for clinical testing: (e.g., *peripheral blood, bone marrow*; note that testing matrices other than those listed in manufacturer's instructions constitutes a modification of an FDA-cleared/approved test):

Analytical characteristics

Analytical sensitivity: (i.e., *lower limit of detection*; e.g., *dilution series*)

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Analytical specificity: (i.e., *ability to identify/quantify analyte in presence of interfering substance*; data on interferences also may be obtained from manufacturer or published literature)

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Accuracy: (i.e., *agreement in results*; e.g., *parallel testing with reference method, exchange with another laboratory*)

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Precision: (i.e., *the ability of a test to produce the same result on repeated testing of the same target*; e.g., *replicate testing within and/or between runs*)

Within-run error (reliability), between-run, between-day, and between-operator error (reproducibility)

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Reference range: (i.e., *the range of results expected in the "normal" or "healthy" population*)

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Reportable range: (i.e., *results that can be reliably reported*; e.g., *homozygous wild type, heterozygous or homozygous mutant for a genotyping assay; or linear range of quantitative tests*)

Method, experimental design, and statistical analysis:

Samples (number and type):

(continued)

Table 3.5 (continued)

Test validation/verification summary

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Other characteristics:

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Clinical characteristics

Clinical sensitivity: (*proportion of patients with positive results with disorder; prevalence of the disease in a relevant population*)

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Clinical specificity: (*proportion of patients with negative results without disorder*)

Method, experimental design, and statistical analysis:

Samples (number and type):

Data to be used for other experiments:

Expected/acceptable performance:

Actual results:

Checklist

- ☐ Optimization of assay on an adequate number of each of the expected specimen types
- ☐ Results of new method compared with that of comparative/reference method
- ☐ Reference and/or reportable ranges established
- ☐ Written procedure prepared and approved by the laboratory director
- ☐ Laboratory work sheets developed and congruent with procedure in procedure manual
- ☐ Reporting criteria established and report template developed containing text for all expected findings (positive (quantitative/qualitative), negative, indeterminate, inconclusive, as appropriate)
- ☐ Turnaround time defined
- ☐ Test added to LIS
- ☐ QC procedures established
 - ☐ Schedule for instrument maintenance checks developed
 - ☐ Data tracking forms/files developed
 - ☐ Training checklist developed
 - ☐ Plan for continued supply of controls/calibrators
 - ☐ Plan for proficiency testing
- ☐ Staff trained in performing assay and competency documented
- ☐ CAP informed of change in testing activity, as appropriate
- ☐ Billing process completed and approved
- ☐ Test added to requisition
- ☐ Test added to website
- ☐ Hospitals/clinics/physicians informed of availability of new testing and provided with other relevant information
- ☐ Specimen collection/transport/shipping procedure developed and distributed to relevant parties
- ☐ Reagent inventory/safety manual/MSDS sheets revised, as appropriate

Approval:

I have reviewed this validation study, and the performance characteristics of the method are considered acceptable for patient testing.

Signature of laboratory director

Date

process and allows for early detection and correction of nonconformities. A full comprehension of the performance characteristics and clinical validity of any new test may take years. Failure to meet minimal performance expectations at any point in this long-term assessment may require the test to be modified following reiteration of the evaluation process.

3.10 Summary

Federal regulations define the main performance characteristics that must be investigated prior to the implementation of any clinical assay in order to assure quality testing. This chapter outlines the basic principles underlying validation and verification in the implementation of molecular tests in the clinical laboratory. The key components of the process are described and practical approaches to assessing each performance characteristic are provided. These are summarized in Table 3.6. Careful planning of validation/verification procedures is emphasized to assure efficient and economical use of limited laboratory resources in this process. A template validation/verification summary (Table 3.5) is provided as a guide to document the validation/verification process for the purposes of quality assurance and accreditation.

Table 3.6 Validation/verification experiments for performance characteristics^a

Accuracy

Type: patient or surrogate samples, controls, or reference materials

Method: ≥ 30 specimens (FDA-cleared/approved) or ≥ 50 specimens (modified or LDT) compared to “gold standard” or current method

Acceptable result: determine acceptable % agreement based on expected assay performance

Precision

Type: patient or surrogate samples, controls, or reference materials

Method: quantitative – 2–3 concentrations plus a negative in duplicate or triplicate, 1 run/day over multiple days with goal of 10–20 data points per concentration (FDA-cleared/approved) or 15–20 data points per concentration (modified or LDT); qualitative – 1–2 positive (close to LoD) and 1 negative in duplicate or triplicate, 1 run/day over multiple days with goal of 10–20 data points each (FDA-cleared/approved) or 15–20 data points each (modified or LDT)

Acceptable result: determine maximum % CV acceptable based on expected assay performance

Comment: determine variability within-run, between-run, within-day, between-day, and between-technologist depending upon expected utilization of assay

Reportable range

Type: controls or calibration materials in appropriate matrix (preferred) or well-characterized patient samples

Method: quantitative – 5–7 concentrations within stated AMR in triplicate (FDA-cleared/approved) or 7–11 concentrations below, within, and above expected AMR in triplicate (modified or LDT)

Acceptable result: results should be linear within stated AMR (FDA-cleared/approved); linearity needs to be established for determination of AMR (modified or LDT)

Comment: experiments should be designed to confirm or establish LoQ

Reference range (normal values)

Type: negative patient samples

Method: 10–20 (FDA-cleared/approved); ≥ 20 (modified or LDT)

Acceptable result: all samples are expected to be negative

Comment: any positives should be reanalyzed and/or evaluated by a reference method where available

Analytical sensitivity (LoD)

Type: controls or reference materials in appropriate matrix (preferred) or well-characterized patient samples

(continued)

Table 3.6 (continued)

Method: quantitative/qualitative – 1 concentration at the anticipated LoD, over multiple runs/days for a total of 20 data points (FDA-cleared/approved)* or 20 data points at and on either side of the anticipated LoD, over multiple runs/days (modified or LDT) or, for probit analysis, a minimum of 12 data points for each of 5 different concentrations is recommended

Acceptable result: $\geq 95\%$ expected positive at LoD concentration

Comment: *not required by CLIA for FDA-cleared/approved assays but may be necessary to confirm reportable range (see above) in quantitative or semiquantitative assays and may also be helpful in confirming acceptable performance of assay for clinical use

Analytical specificity

Type: patient and/or surrogate samples containing or spiked with potentially interfering substances

Method: evaluate related sequences *in silico*; test potential interfering endogenous and exogenous substances (drugs, specimen transport media, stabilizers, nucleic acids, microorganisms, etc.) and potential sources of contamination that could cause false positive results

Acceptable result: preferably no interferences should be observed, otherwise list in limitations of test

Comment: not required by CLIA for FDA-cleared/approved assays

^aStudies indicated in the table should only be used for general guidance. Actual design of experiments will vary due to assay target, method, regulatory status, and resources available. Ultimately, it is the responsibility of the laboratory director to establish the protocol necessary for adequate validation/verification of an assay

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Chapter 4

In Vitro Amplification Methods in Molecular Diagnostics

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Keywords In vitro amplification • Polymerase chain reaction • Genetic testing

4.1 Introduction

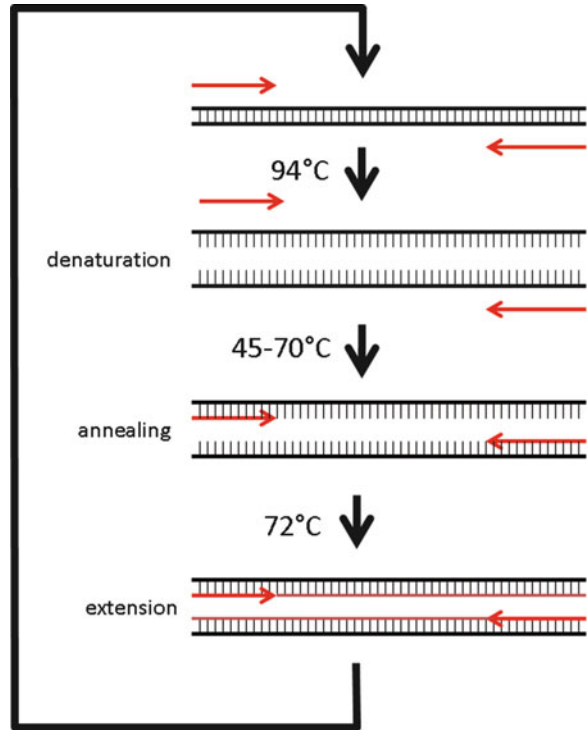
The polymerase chain reaction (PCR) was the first in vitro nucleic acid amplification method developed, and it revolutionized life sciences [1]. PCR has been instrumental in determining the etiology of many diseases, as well as the development of diagnostic testing for those diseases. During the past 25 years, additional methods of in vitro nucleic acid amplification have been developed based on the same principle. The aim of this chapter is to review some of the amplification methods that are being utilized in clinical testing. To this end, we will start with a review of the basic concepts underlying targeted PCR amplification and its use in clinical testing. As an example of a PCR-based diagnostic assay, we will provide a protocol for detection of trinucleotide repeat expansions in the *HTT* gene that causes Huntington disease. In addition, we will briefly review the principles and clinical applications of related methods such as multiplex PCR, real-time PCR, as well as massively parallel microdroplet PCR and digital PCR. In the last section, as opposed to targeted PCR amplification, we will discuss whole genome in vitro amplification methods including primer extension preamplification, degenerate oligonucleotide primed-polymerase chain reaction, OmniPlex, linker adaptor PCR, and multiple displacement amplification. The aim of this chapter is not to provide a complete review of all the different uses of PCR that have been developed over the past 25 years, indeed entire books have been written on that subject, but to provide a general introduction to the uses of PCR in clinical testing.

4.2 Polymerase Chain Reaction Background and Principles

During PCR, the targeted DNA sequence is amplified 100,000 to over one-million-fold, greatly enhancing the ability to detect low levels of target nucleic acid [2]. The technique utilizes a set of oligonucleotides called primers. The primers are generally 20–40 nucleotides long, and each primer is complementary to a sequence at the 5' and 3' ends of the target region of DNA. One primer is

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Fig. 4.1 Polymerase chain reaction amplification



designed to hybridize to the antisense strand, referred to as the forward primer, and the other primer is designed to hybridize to sense strand, referred to as the reverse primer. PCR utilizes repeated cycles of DNA denaturation, primer hybridization, and DNA polymerase extension to achieve amplification (Fig. 4.1). These activities are controlled by the temperature of the sample using a thermal cycler. Denaturation is accomplished through use of high temperatures (typically 94–95°C) that break the hydrogen bonds that hold the two strands of DNA together. After denaturation, the sample is cooled (to typically 45–70°C) to allow hybridization or annealing of the oligonucleotide primers to complementary sequences present at either end of the target sequence. Following hybridization, a DNA polymerase extends the primers using the target sequence as a template. The DNA polymerase used in PCR is a modified form of the enzyme isolated from a thermostable microorganism that is able to withstand the elevated temperatures required by PCR. The initial thermostable enzyme utilized in PCR was from the bacterium *Thermus aquaticus* and is therefore referred to as Taq DNA polymerase. Subsequent modifications to this polymerase, as well as polymerases from other thermostable organisms, are now routinely used in PCR. Typically, the optimum temperature for polymerase activity of these enzymes is approximately 72°C. Therefore, in many protocols, three temperature steps (94°C denaturation, 45–70°C hybridization, and 72°C extension) are used. However, in some PCR protocols, the hybridization and the extension are performed at a single temperature, thus only two temperature steps are used. After one cycle of denaturation, primer hybridization, and extension, the DNA polymerase creates two new strands of DNA (plus the two original strands), which are complementary to the target sequence. After two cycles, the four strands are converted to eight strands of target sequence. This exponential increase of the DNA continues for as many cycles as the reaction runs, resulting after ten cycles in about a thousandfold increase, after 20 cycles in a millionfold increase, and after 35 cycles in a billionfold increase (Fig. 4.2).

The key advantage of PCR is that it requires a minimal amount of DNA. In fact, amplification of a target sequence from a single DNA molecule can be performed. By increasing the copy number of the

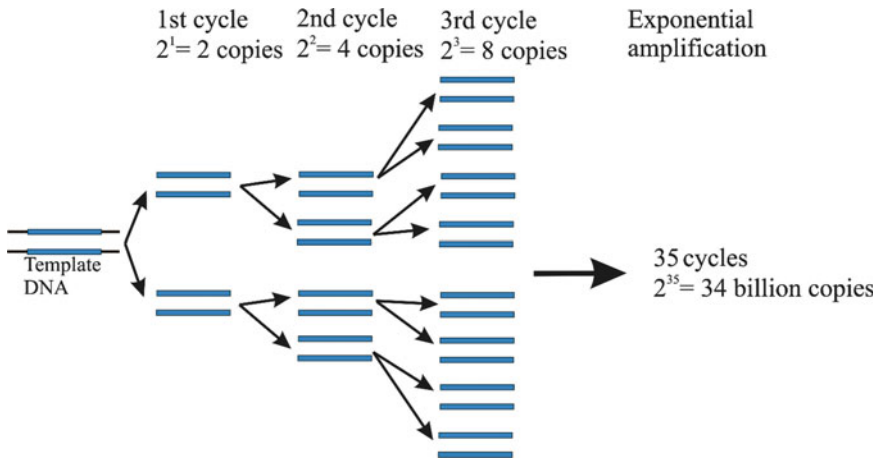


Fig. 4.2 Exponential increase in copy number after PCR

target sequence, PCR allows the use of detection methods, such as restriction enzyme digestion or DNA sequencing, as well as many other methods, to test for the presence or absence of a nucleotide change. In addition, PCR assays can also be designed to test directly for the presence or absence of a nucleotide change, either a single nucleotide substitution, a deletion, or an insertion, by a technique referred to as allele-specific PCR. In allele-specific PCR, the primers are designed so that a specific PCR product is produced only if the nucleotide change is present in the genomic DNA sample.

PCR is robust, and the purity of the target sequence generally is less critical because as long as a one or a few target sequences are able to bind the primers, the target can be amplified to a level where it ultimately can be detected. However, because of this sensitivity, there is the potential for false-positive and false-negative results due to the presence of contaminating DNA. The contaminating DNA may be genomic DNA or previously amplified PCR products. Amplified fragments of DNA may be aerosolized into the work environment when sample tubes are opened following amplification; therefore, special precautions must be observed because contamination of a sample by even one of these small fragments can cause an incorrect result [3]. Pipettes, gloves, clothing, equipment, and reagents can spread amplicons easily [4]. Physical separation of pre-PCR and post-PCR work areas is a basic requirement in clinical laboratories [4]. Use of hydrophobic filtered tips or positive displacement pipettes is another standard to prevent contamination of pipettes. Great care must be exercised to avoid contamination during PCR, especially in clinical laboratories where PCR is utilized daily on a large scale.

During PCR amplification of genomic DNA, two alleles are amplified simultaneously (except for cases of hemizygoty, such as genes on the X and Y chromosomes in males). Another potential issue of PCR is failure or amplification of one of the alleles, referred to as allele dropout, resulting in a false-positive or a false-negative result. Allele dropout is caused by a DNA sequence variation on one of the alleles that interferes with primer hybridization inhibiting amplification. Sequence variations located toward the 3' end of the primer binding site are more likely to inhibit binding and subsequent PCR than variations located toward the 5' end. PCR primers should be designed to avoid segments of the human genome where a sequence variant is known to be located. However, a comprehensive catalog of all human variation has yet to be generated. Thus, even though a nucleotide variant has not been documented for a site, there is always the potential that any given sample may have a variant in the primer binding site and allele dropout could occur.

4.2.1 *Practical Considerations of PCR*

By following a few simple rules, the vast majority of amplifications will work on the first attempt with little empirical optimization. However, there are some instances when a particular region of DNA proves difficult to amplify by PCR and extensive empirical work will need to be done to achieve efficient amplification. Factors such as primer sequence, product length, and primer annealing temperature will differ for each assay. Below are some of the factors that should be kept in mind when designing a PCR assay.

4.2.2 *Specificity of PCR Primer Pairs for the Target Sequence*

The selection of the correct pair of primer sequences for the PCR reaction may be the most critical parameter for successful PCR. The primer set must hybridize specifically to the target sequence with as little hybridization as possible to other sequences that are also present in the sample, including the other PCR primer, as well as primer self-hybridization (known as a primer dimer). Approximately 45% of the human genome is composed of interspersed repetitive sequences that are mainly transposon-based repeats such as LINE and SINE elements. In addition to interspersed repeats, there are simple repeats such as homopolymeric tracts, short tandem repeats (STRs), and satellite DNA that can interfere with amplification. These sequences should be avoided in the design of PCR primers since primers that anneal to these sequences will anneal to multiple sites in the genome. Identifying the location of these repeats within the sequence of interest is the first step in design of a PCR assay. Tools such as the RepeatMasker on the UCSC genome browser (<http://genome.ucsc.edu>) are useful in determining the location of these repeats. Once unique regions are identified, oligonucleotides between 20 and 40 bases can be designed to hybridize to these sites. Oligonucleotides less than 20 bases will have an increased likelihood of hybridizing to multiple regions in the genome by random chance.

In addition to high copy number repeats, many genes will have a homologous segment (or several homologous segments) in the genome known as pseudogenes. The pseudogene may consist of the entire sequence of the gene or only a portion of the gene. If a gene has a pseudogene, an alignment between the pseudogene sequence and the active gene sequence should be generated to identify regions unique to the active gene. BLAT searches using the UCSC genome browser, along with tools such as *in silico* PCR offered on the UCSC web site, can be used to help design primers that target the desired sequence and will not cross hybridize to a homologous sequences. Designing the primers that target and amplify the specific sequence of interest will ensure that the correct template is generated for downstream analyses.

4.2.3 *GC and AT Content*

Regions of the genome that gave a high GC content or a high AT content can be difficult to amplify. Elevated GC content can reduce the efficiency of denaturation resulting in reduced PCR product. Stretches of poly C or poly G should be avoided, as these can promote nonspecific annealing. Similarly, stretches of poly A or poly T should also be avoided, as these may open up stretches of the primer-template complex. Ideally, primer sequences should be designed to have a GC content between 45% and 55%.

4.2.4 *Primer Melting Temperature (T_m)*

In general, higher annealing temperatures result in increased specificity of the primer for its target. However, if the annealing temperature is too high for a primer, inefficient hybridization will occur resulting in inefficient amplification. The melting temperature or T_m is a direct function of primer length and GC content. Ideally the primers should closely match each other in their melting temperatures, or amplification efficiency will be reduced and may even lead to the failure of the PCR. Online T_m calculators can be used to determine theoretical melting temperature. As a starting point, the annealing temperature used in a PCR reaction is set 5°C below the estimated T_m for the primer pair.

4.2.5 *General Comments on Primer Design*

There are free web tools such as Primer3 (available at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) or commercially available programs such as Oligo 6 primer analysis software (Molecular Biology Insights Inc., Cascade, CO; <http://www.oligo.net>) to help design primers. An “ideal” primer will have a 50% GC content with a near random nucleotide composition and will be 20–40 bp long resulting in a T_m of 56–62°C. However, primers can only be designed from the available sequence, and sometimes these ideal conditions cannot be achieved. Compromise is the key, and it is very unusual to be unable to design a primer pair that will work after some optimization.

4.2.6 *Components of the PCR Reaction*

There are several basic components present in every PCR reaction. In addition to the primers and the template DNA, other reagents needed are: a buffer to control the pH of the reaction, such as Tris (HCl); monovalent ions, typically KCl; divalent cations, typically $MgCl_2$; deoxynucleoside triphosphates (dNTPs); and a thermostable enzyme such as Taq DNA polymerase. The typical PCR reaction volume typically ranges from 10 to 200 μ l.

Magnesium is required as a cofactor for DNA polymerases. In addition, magnesium cations can stabilize primer hybridization, and excess magnesium can lead to mispriming, where primers anneal to other sites in the genome besides their intended target sequence. Typically, the amount of $MgCl_2$ has to be determined empirically for optimum specificity and efficiency of the reaction, but in general the optimum final concentration of $MgCl_2$ is somewhere between 1 mM and 5 mM. A modified form of Taq DNA polymerase that is enzymatically inactive till it is heated to 95°C for several minutes (referred to as HotStart Taq) is also often used to increase specificity of the reaction. In addition, reagents such as DMSO and betaine, as well as nucleotide analogs such as 7-deaza-GTP, can be added to increase the efficiency and specificity of PCR. In summary, the concentrations of the various components of the PCR, specifically the $MgCl_2$, and the annealing temperature in the thermal cycling are used to optimize the efficiency and specificity of the PCR reaction.

4.2.7 *Analysis of PCR Products*

After thermal cycling, the efficiency and specificity of the reaction can most easily be assessed by agarose gel electrophoresis. After PCR, a single DNA fragment or amplicon should be evident. If a discrete band of the expected size is not visible, or if several bands are present, this indicates that further optimization is required. In addition to agarose electrophoresis, other electrophoretic methods such as polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis can be

used to assess the efficiency and specificity of the reaction. For analysis by capillary electrophoresis, one of the PCR primers must be labeled with a fluorescent dye for detection. It is important to note that Taq DNA polymerase has a tendency to add a single adenine overhang to the 3' end of the PCR product resulting in the production of a DNA strand that is 1 bp greater than the targeted sequence. Thus, two PCR products, 1 bp different in length, may be evident after analysis. Amplicons 1 bp different in size will not be resolved by agarose gel electrophoresis, but may be evident after PAGE or capillary electrophoresis.

4.3 An Example of the Use of PCR in Clinical Testing: Huntington Disease

To illustrate how PCR is used in a clinical test, we provide a protocol for testing for Huntington disease (HD), a progressive neurological disorder characterized by motor, cognitive, and psychiatric disturbances. HD is caused by expansion of a CAG trinucleotide repeat that encodes for glutamine in the *HTT* gene located on chromosome 4p16 [5]. Individuals with 10–35 CAG repeats are not affected with HD. Individuals with expansions of 40 CAG repeats or greater will develop disease with longer CAG repeat carriers developing disease earlier in life. Individuals who carry alleles with between 36 and 39 repeats may or may not develop HD [6]. These are referred to as partially penetrant alleles. Expansion of the CAG repeat introduces additional glutamines into the huntingtin protein, and it is believed that these additional glutamines alter the structural and biochemical properties of the protein leading to neuronal degeneration. Testing CAG repeat expansions is appropriate for individuals with clinical features indicative of HD and also for asymptomatic adults with a positive family history of Huntington disease. The test for HD consists of PCR amplification of the region containing the CAG repeats and calculating the number of CAG repeats by determining the size of the amplicons by capillary electrophoresis.

4.3.1 *Huntington Disease Protocol of PCR Amplification and Capillary Electrophoresis*

4.3.2 *Reagents for PCR*

dH₂O (HPLC purified) (Ultra Pure)
 10× PCR buffer (without MgCl₂) (Roche)
 DMSO (Fisher Scientific)
 dNTPs (Roche)

dATP (3.0 mM)
 dCTP (3.0 mM)
 dTTP (3.0 mM)
 dGTP (0.75 mM)
 7-deaza-GTP (5 mM) (Roche)

MgCl₂ (25 mM) (Qiagen)
 HotStar Taq (Qiagen)

Primers

HD for (10 pmol/μl) – 5'-ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC-3'
 HD_{CAG} revTet (10 pmol/μl) – 5'-GCG GTG GCG GCT GTT GCT GCT GCT G3'

(Note: 5' end is labeled with TET)

HD_{CCG} revFam (10 pmol/μl) – 5'-AAA CTC ACG GTC GGT GCA GCG GCT CCT C3'

(Note: 5' end is labeled with 6-FAM)

4.3.3 Controls

Accurate determination of the number of CAG repeats is critical for HD testing. A result that is off by one repeat can result in a change of classification for that allele from pathogenic to nonpathogenic or vice versa. Clinical laboratories utilize in-house validated controls to ensure accurate sizing. Our laboratory has used cloned fragments containing 35 CAG and 40 CAG repeats originally obtained from the laboratory of Dr. Michael R. Hayden in the Department of Medical Genetics, University of British Columbia. We use these controls because their repeat lengths are at the boundaries used for classification of pathogenic and nonpathogenic alleles. In addition, we use previously tested samples that have very large expansions (>80 CAG repeats) to verify that we are able to amplify these expansions on that day. Reference material from immortalized cell lines are also available and used by many clinical laboratories [7]. These standard controls, along with a negative control and a no template control, should be amplified in parallel every time the test is performed with a patient sample.

4.3.4 Procedure

1. Assemble the required reagents (see above). Thaw 10× PCR buffer, dNTP mix, and primers (either CAGrev or rev – see above).
2. Label 0.2-ml PCR tube with the patient's initial and number. Each run should have: a 35-repeat control, a 40-repeat control, a positive control, and a negative control, and a no template control.
3. To each tube add 3 μl of 15 ng/μl patient or control DNA.
4. Set up the following reactions for all patients and controls. *Taq* should be added to the reaction mix last, when all other tubes have been prepared. The mixtures can be scaled to generate enough PCR cocktail for each of the samples in the run.

4.3.5 PCR Cocktails

Reagent	Cocktail 1× (μl)
HTT CAG PCR Cocktail	
HPLC-dH ₂ O	9.0
DMSO	2.0
10× Buffer without MgCl ₂	2.0
MgCl ₂ (25 mM)	0.6
dNTP	1.3
7-deaza-dGTP (5 mM)	0.6
HD for (10 pmol/μl)	0.6
HD CAG RT (10 pmol/μl)	0.6
HotStar Taq (5 U/μl)	0.3
Final volume	17.0

(continued)

Reagent	Cocktail 1× (μl)
HTT CAG + CGG PCR Cocktail	
HPLC-dH ₂ O	9.0
DMSO	2.0
10× Buffer without MgCl ₂	2.0
MgCl ₂ (25 mM)	0.6
dNTP	1.3
7-deaza-dGTP (5 mM)	0.6
HD for (10 pmol/μl)	0.6
HD CCG R F (10 pmol/μl)	0.6
HotStar Taq (5 U/μl)	0.3
Final volume	17.0

- Place 0.2-ml tubes containing the patient and control DNAs on a −20°C block. Add 17 μl of reaction mix to each set of tubes.
- Place the tubes in a thermal cycler. The samples should be cycled with the following parameters:

95°C, 15 sec
 95°C, 1 min
 65°C, 2 min
 4°C, hold

} 27×

4.3.6 Fragment Analysis

The HD_{CAG} revTet amplification primer is labeled at the 5' end with the fluorescent dye TET which allows for visualization of the PCR product during capillary electrophoresis. The manual should be consulted for details of use of the capillary electrophoresis instrument. Below is the protocol followed by our laboratory for an Applied Biosystems 3730×1 DNA Analyzer.

- In a new 0.5-ml tube, mix formamide with the GeneScan 500 ROX size standard (Applied Biosystems) in the following proportions: 18 μl formamide + 0.5 μl 500 ROX size standard.
- Mix well by vortexing.
- Aliquot 18 μl formamide/marker cocktail into sample plate.
- Transfer 2 μl of PCR product into same plate.
- Place septa on sample plate.
- Mix well by vortexing.
- Quick spin samples in a centrifuge.
- Denature 3–4 min by heating at 95°C (use PCR machine or heat block).
- Load the sample plate for electrophoresis according to manufacturer's instructions.

4.3.7 Data Analysis and Interpretation

After electrophoresis, the PCR products are visualized and analyzed by the GeneMapper 4.0 (Applied Biosystems). During PCR amplification, there is slippage of the polymerase due to the presence of the CAG trinucleotide repeat. The slippage results in PCR products that appear as a stair-step pattern of peaks, with the peaks differing in size by 3 bp (Fig. 4.3). The largest and most abundant product,

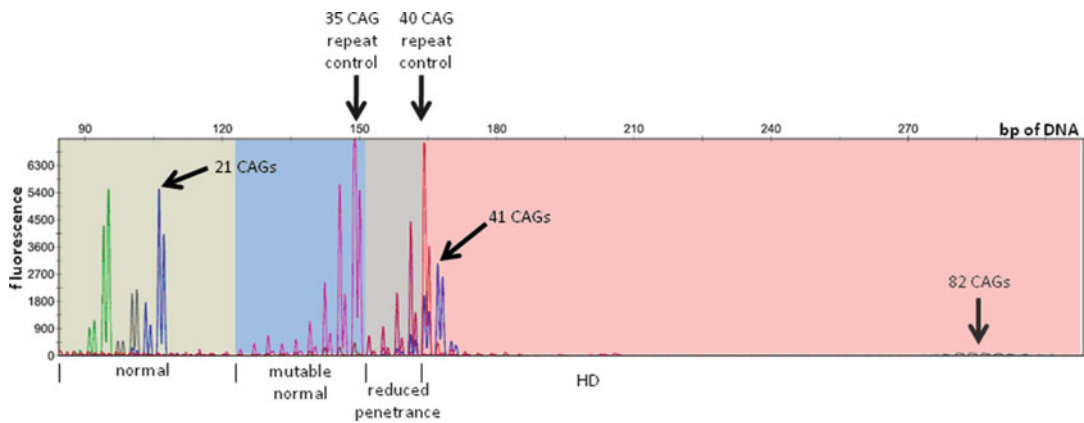


Fig. 4.3 Huntington disease CAG repeats testing

typically at the end of the stair-step, reflects the true size of the CAG repeat. Larger CAG repeats (>40 CAGs) produce a pattern that is more “mounded” in appearance with the tallest peak shifted to the middle. As with all trinucleotide repeat disorders, the amount of PCR product generated from the longer CAG repeat alleles is significantly less than the shorter alleles. The peaks have a “split” appearance due to the tendency of Taq DNA polymerase to add a nontemplate adenine to the end of the PCR product.

The tallest peak is used to calculate the CAG repeat length. The amount of flanking sequence in the amplicon containing the *HTT* CAG repeat is 46 bp. Therefore, the number of CAG repeats can simply be calculated by subtracting 46 bp from the size of the tallest peak in bp and dividing that number by 3 (number of CAG repeats=(bp of tallest peak –46)/3).

The American College of Medical Genetics Huntington Disease Working Group for the interpretation of HD gene mutation testing defines the *HTT* alleles as follows [8]:

CAG repeat range	Category description	Predicted phenotype
≤26	Normal allele	Unaffected
27–35	Mutable normal	Unaffected
36–39	HD allele with reduced penetrance	Unaffected/HD
≥40	HD allele	HD

Individuals who carry alleles with 35 or fewer CAG repeats on each chromosome will not be affected with HD. However, alleles at the higher end of this range, 27–35 CAG repeats, are less stable during meiosis than alleles with 26 or fewer CAG repeats. Therefore, these alleles may expand during transmission to the next generation and for this reason are referred to as mutable normal alleles. Individuals with alleles that have 36–39 CAG repeats may or may not develop HD, and these alleles are referred to as partially penetrant alleles. These alleles are also unstable and may change in size during transmission from generation to generation. Individuals that carry an *HTT* allele with 40 or greater CAG repeats will develop HD at some time during the course of their life. In these individuals, the length of the CAG repeat is inversely correlated with the onset of disease.

Due to the polymorphic nature of the repeat, most individuals will have two distinct CAG repeat patterns, one for each allele. However, a proportion of individuals in the population will be homozygous for the same-size CAG repeat on each allele and only one repeat will be detected by PCR. PCR amplification of very large CAG repeats (>90 CAGs) is not possible. Therefore, if a single CAG

repeat is detected it is important to determine if that individual is homozygous for that CAG repeat tract length or do they carry one normal size repeat on one chromosome and a large expansion that is not amplified by PCR on the other chromosome. To address this problem, a second PCR can be done that includes an adjacent polymorphic CGG repeat in the PCR product to distinguish between these two possibilities. For this reaction, the combined *HTT* CGG+CAG repeat can be calculated by subtracting the flanking sequence (171 bp) and dividing by 3 (number of CGG+CAG repeats = bp of tallest peak – 171/3). If one normal allele is detected by the *HTT* CAG PCR and two alleles are detected by the *HTT* CGG+CAG PCR, this indicates that the patient is homozygous for a normal size CAG. If only one normal allele is detected by the *HTT* CGG+CAG PCR, either the individual is homozygous for both the CAG and CGG repeats or they carry a normal allele on one chromosome and a large expansion of the other chromosome that is not able to be amplified by PCR. In these cases, Southern analysis can be used to test for the presence of large CAG expansions.

4.4 Other Targeted In Vitro Amplification Methods

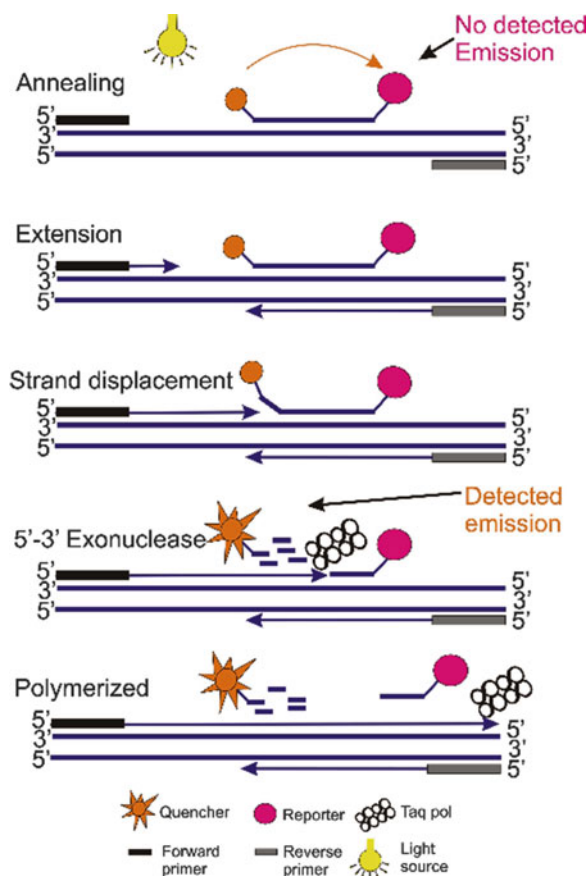
4.4.1 Multiplex PCR

There are many variations of traditional single-site-targeted PCR utilized in clinical testing. One variation is multiplex PCR where multiple primer pairs specific to several different targets are included in the same amplification reaction. The same criteria used for design of single-site PCR should be used for multiplex PCR. In addition, the design should take into account the possibility of interference from amplification of the other PCR products in the multiplex reaction. Coamplification of different targets in the same PCR can serve several purposes: (1) other segments of the genome can be used as an internal control for the quantity and quality of the genomic DNA; (2) for the hemizygous X chromosome and Y chromosome in males, failure of amplification of several contiguous loci in a single reaction is indicative of a large deletion that encompasses these loci; (3) several different segments of one gene or several segments of different genes can be amplified for subsequent analysis by downstream methods such as allele-specific hybridization and oligonucleotide ligation analysis; and (4) fragment sizing of several polymorphic short-tandem repeat tracts can be used in identity testing or as an indicator of integrity of the DNA mismatch repair pathway in DNA isolated from tumor tissue. Clinical disorders tested for using multiplex PCR include identification of *DMD* gene deletions in Duchenne and Becker muscular dystrophy [9], identification of Y chromosome deletions in males with infertility [10, 11], Ashkenazi Jewish mutation carrier screening using allele-specific PCR [12], cystic fibrosis mutation detection using oligonucleotide ligation analysis [13], identity testing for maternal cell contamination studies in prenatal testing [14], and microsatellite instability analysis for hereditary nonpolyposis colorectal cancer (HNPCC). These are only a few examples of the many different assays used in clinical laboratories that utilize multiplex PCR.

4.4.2 Real-Time PCR

Another variation of the basic PCR technique is real-time PCR, where the production of the PCR product is monitored during the course of the thermal cycling and the rate of amplicon production is used to estimate the relative amount of the target DNA in the sample. This monitoring is accomplished with the use of a camera capturing and quantifying the fluorescence at specific wavelengths during thermal cycling. Real-time PCR platforms utilize different detection formats. Fluorescent technologies employed are either nonspecific, using dyes such as SYBR Green I or SYBR Gold, which intercalate into the PCR product during the reaction, or specific, using probes to detect specific sequence

Fig. 4.4 DNA amplification by real-time PCR using TaqMan chemistry. The TaqMan probe anneals to the target, and the target-specific primers get extended. The TaqMan probe is displaced and digested by the exonuclease activity of the enzyme, thus, light emission is detected



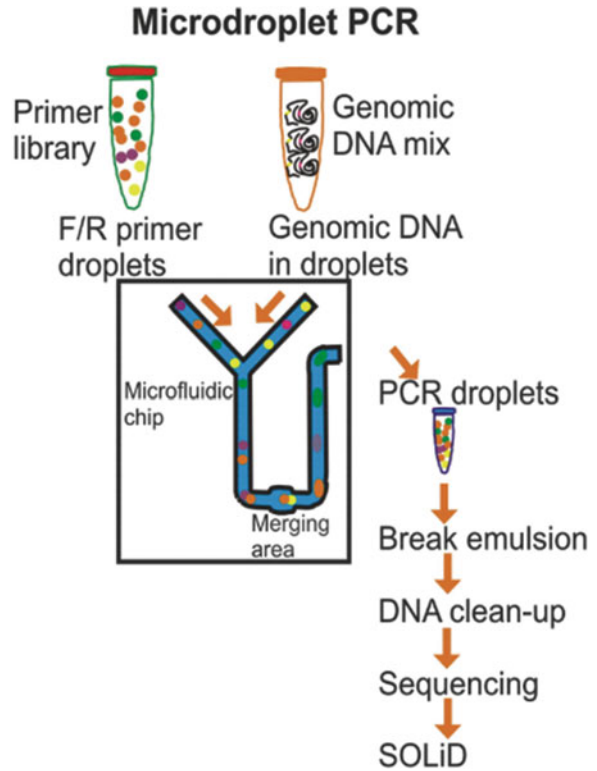
amplification in the PCR (Fig. 4.4). A number of different fluorescent probe chemistries have been employed in real-time PCR assays including TaqMan® (5' exonuclease) probes, HybProbes, molecular beacons, and Scorpion probes. While the mechanism of fluorescent signal generation is different for each of the probe chemistries, the fluorescent signal generated by the probes or minor groove-binding dyes is directly proportional to the amount of PCR product generated [15, 16]. Real-time PCR is quantitative, with a broader dynamic range than conventional PCR. Advantages of real-time PCR include that it is a rapid, accurate, and highly sensitive technique and can be utilized for both qualitative and quantitative analysis and for genotyping. These advantages have led to an explosion in the development of real-time PCR assays for diagnostic purposes driven by the need for faster and more accurate diagnostic tests.

4.5 Massively Parallel Targeted In Vitro Amplification Methods

4.5.1 Microdroplet PCR

An article by Frazer and colleagues in collaboration with RainDance Technologies reported the simultaneous amplification of 3,976 products using microdroplet PCR technology [17]. RainDance Technologies uses a microfluidics technology in which individual pairs of PCR primers for the genomic regions of interest are segregated in water in emulsion droplets and then pooled to create a

Fig. 4.5 Microdroplet PCR (RainDance): A microfluidic nozzle creates aqueous droplets (8 pl) of forward- and reverse-targeting primers in oil, which are dispensed into a channel in a microfluidic chip. Fragmented genomic DNA and associated PCR reagents are separately dispensed as aqueous microdroplets (14 pl) and paired together with the primer droplets at a 1/1 ratio. Paired droplets pass through a merging area for coalescence into PCR-competent microdroplets, which are collected into a single tube for thermal cycling



primer library (Fig. 4.5). Separately, emulsion droplets containing genomic DNA and PCR reagents are prepared. Two separate droplet streams are created, one with primer-library droplets and the other with droplets containing genomic DNA/PCR reagents. The two streams are merged, and primer-library droplets and genomic DNA/PCR reagent droplets are paired in a 1:1 ratio. As paired droplets proceed through the microfluidic channel, they pass an electrical impulse that causes them to physically coalesce. The coalesced droplets containing individual primer pairs and genomic DNA/PCR reagents are deposited in a single tube and amplified by the PCR on a standard PCR machine. After amplification, the emulsions are disrupted, and the amplicons are pooled and processed for next-generation sequencing. In a proof-of-principle study, the authors reported an 84% capture efficiency with 90% of the targeted bases showing uniform coverage of 435 exons of 47 genes using the microdroplet PCR method sequenced with either the Roche/454 or Illumina/Solexa platform [17]. This study demonstrated that the microdroplet technology is well suited for processing DNA for massively parallel enrichment of specific subsets of the human genome for targeted resequencing.

4.5.2 Digital PCR

Digital PCR is dependent upon microfluidic devices with the ability to perform highly parallel analyses in a single PCR step. The development of microfabricated valves and pumps by multilayer soft lithography (MSL) confers the ability to design integrated fluidic circuits (IFCs) and to manufacture microfluidic chips cost effectively [18]. A number of functional modules have already been described [19]. MSL chips have already been used for a wide selection of biochemical and molecular biology applications such as DNA sorting and synthesis [20]. In principle, any molecular reaction

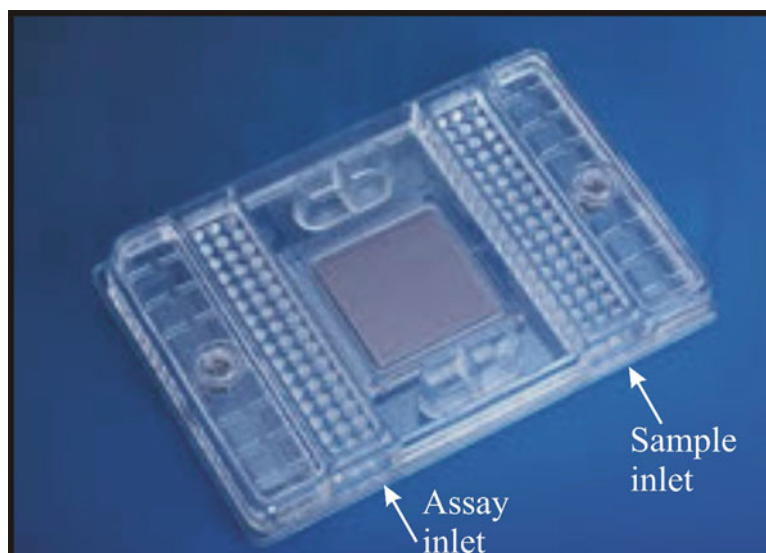


Fig. 4.6 A 48.48CS dynamic array showing the position of the sample inlets and the assay inlets (image courtesy of Fluidigm)

that can be run in a tube can be performed in a microfluidic chip. In 2003, using the concept of a microfluidic matrix, Liu et al. were able to perform 400 independent 3-nl PCR reactions on an MSL chip, at the same time minimizing reagent consumption and the number of pipetting steps necessary [21]. Applying similar logic but a different design, IFC chips have been developed for high-throughput gene expression and SNP genotyping using a real-time PCR assay [22].

In 2007, Fluidigm introduced a new array capable of performing digital PCR (Fig. 4.6). By utilizing microfluidic valves, a single sample can be partitioned into many hundreds to thousands of reactions in an extremely convenient, automated manner to performed digital PCR [23]. In this digital array format, which is a novel nanofluidic biochip, digital PCR reactions can be performed by partitioning DNA molecules, instead of diluting them. This chip utilizes integrated channels and valves that partition mixtures of sample and reagents into reaction chambers that have a volume of 6 nanoliters. DNA molecules in each mixture are randomly partitioned into the 765 chambers of each panel. The chip is then thermocycled and imaged on Fluidigm's BioMark real-time PCR. By simply counting the number of positive chambers, researchers can determine the starting number of molecules in the sample [24]. The concept of digitalizing PCR by spreading a sample is very versatile and has found applications in the quantification of single-cell gene expression [25], absolute quantification of point mutations [26], and in a variety of relative quantifications, e.g., the determination of copy number variations (CNV) [27], loss of heterozygosity (LOH), and aneuploidy detection [28] and SNP genotyping.

4.5.3 Whole-Genome Amplification

All of the in vitro amplification methods discussed thus far amplify specific target sites in the genome. However, for some applications, such as preimplantation genetic diagnosis (PGD), amplification of the whole genome is required for analysis. The technique of whole-genome amplification (WGA) can be used to amplify the entire genome from a single cell generating sufficient DNA to allow diagnosis of any known single gene defect that would have been impossible otherwise [29]. In addition, these

whole-genome PCR products can be analyzed as a template for multiloci, multigene, and genome research during the subsequent DNA analysis. An early approach for amplifying large fragments of a genome was to use primers directed at human Alu repeat element, providing a simple method for the isolation and analysis of specific chromosomal regions [30]. Because of the highly uneven distribution of Alu sequences, this protocol cannot be used reliably in conjunction with molecular analysis of single cells. Later, several other methods have been developed to amplify the whole genome. In 1992, the primer extension preamplification (PEP) method for haplotyping of a single sperm cell was employed [31]. Around the same time, the degenerate oligonucleotide primed-PCR (DOP-PCR) was described for genome-mapping studies [32], and linker-adaptor PCR amplification is being used for next-generation sequencing. Another exciting method was introduced in 2002, multiple displacement amplification (MDA), in an effort to reduce the limitations of PCR-based WGA techniques [33]. In the following section, we describe the principles of WGA of each method and its applications in genetic testing.

4.5.4 Primer Extension Preamplification (PEP)

PEP is a PCR-based amplification method that uses 15-base random primers to initiate DNA synthesis in an ~50-cycle reaction of primer extension to amplify the entire genome [31]. Unlike the conventional PCR, following denaturation, the primers are placed at a low temperature (37°C) to anneal to the DNA genomic template and the temperature is slowly increased to 55°C followed by a 4-min elongation step at 55°C. This low temperature favors nonspecific hybridization with the DNA template allowing a more uniform enrichment of the genome. It is estimated that approximately 96% of the genome can be amplified at least 1,000 times [34]. PEP has been utilized for mutation detection in β -thalassemia, familial adenomatous polyposis (FAP), and Becker/Duchenne muscular dystrophy [35–37]. The PEP protocol has been modified and improved (improved-PEP, I-PEP) and has been shown to have an increased efficiency of amplification compared with PEP [38]. The amplification bias that varies from a low of 10^2 to a high of 10^4 and the absence of amplified fragments >1,500 bp have discouraged the broad implementation of this method in PGD [33].

4.5.5 Degenerate Oligonucleotide Primed-Polymerase Chain Reaction (DOP-PCR)

Another method for WGA is DOP-PCR which has been widely accepted, and it uses partially degenerate oligonucleotides to prime many sites throughout the genome during several low-temperature annealing cycles [32]. Then, more specific fragments will be generated by increasing the annealing temperature. The starting template DNA of DOP-PCR can be as little as 15 pg or as much as 400 ng, and the quantity of DNA products is much greater than that of PEP [39, 40]. DOP-PCR generates relatively short products similar to those generated by PEP. New methods were reported that create long amplification sequences by using the 3'–5' exonuclease proofreading activity of DNA polymerase Pwo and increasing the annealing and extension times. Products from LL-DOP-PCR (long products from low DNA quantities) protocol can range from 0.5 to >10 kb [41]. In comparison with the conventional DOP-PCR method, it provides significantly better coverage for microsatellites and unique sequences. The major advantage of this technique is that it can produce sufficient amplified products from single cells for use in comparative genomic hybridization (CGH) [42], which requires 100 to 1 μ g of test DNA, equivalent to more than 10,000 cells [43].

The use CGH with DOP-PCR-amplified DNA was reported for the diagnosis of chromosomal aneuploidy in single cells [44].

4.5.6 *OmniPlex*

OmniPlex is another useful method for obtaining sufficient DNA from a limited amount of samples for genetic diagnosis [45]. In OmniPlex WGA, the randomly fragmented genomic DNA (mean predictable fragment size of about 0.4 kb) is ligated to universal priming sites and then amplified with universal primers by PCR. The products of OmniPlex WGA are used for genotyping and CGH [46]. The first successful application using the OmniPlex technology of WGA in PGD was for the genetic disorder β -thalassemia in combination with human leukocyte antigen (HLA) typing [47].

4.5.7 *Linker-Adaptor PCR*

An alternative approach is linker-adaptor PCR in which gDNA is digested and short linkers are ligated to the ends, providing a single annealing site for PCR priming [48]. This approach can alleviate some of the amplification bias associated with thermal cycling in which sequence has a large effect on both the denaturation of the template and the T_m of primers. Although linker adapters provide more uniform priming, only a limited subset of sequence is amplified and the method requires considerably more steps. The applications of this technique include the generation of chromosome-painting probes [49] and whole-genome amplification from single cells to provide templates for CGH analysis and other analyses including next-generation sequencing [50].

4.5.8 *Multiple Displacement Amplification*

One important breakthrough in WGA methods is the development of MDA. MDA offers great promise as the first truly comprehensive WGA with minimal bias and no loss of essential sequences [33]. MDA differs from other WGA methods in that it is not based on repeated temperature cycling. Amplifications are achieved entirely at 30°C with random hexamer primers [51]. The template is replicated again and again by a “hyperbranching” mechanism of strand displacement synthesis, with polymerase laying down a new copy as it concurrently displaces previously made copies [52]. MDA is carried out with a DNA polymerase from the bacteriophage ϕ 29 which has exceptionally tight binding to DNA and continuously adds 70,000 nucleotides every time it binds to the primer [53]. This is the highest processivity reported for any DNA polymerase in the absence of cellular multisubunit complexes and accounts for its ability to generate extremely long DNA products, averaging 12 kb in length and ranging up to more than an estimated 100 kb compared with 100–1,000 bp for most PCR-based methods. Long products are an advantage in many applications, including RFLP analysis, generation of probes, and DNA sequencing. Use of exonuclease-resistant primers is required for 10^4 – 10^6 -fold amplifications and high DNA yields [51, 54]. Moreover, because MDA is carried out in an oven or water bath rather than in a thermocycler, it can be used as a preparative method scaled to any volume. More than 1 mg of genomic sequence is generated from a 1.5-ml reaction and 8 mg is obtained from a 10-ml reaction.

Burlet and coworkers used MDA products for fluorescent PCR analysis, which was performed to amplify the nonexpanded CGG repeat with markers linked to *FMR1* and a sequence from the

amelogenin gene for PGD [55]. Another MDA-PGD protocol was developed for X-linked retinoschisis [56]. MDA products were used for PCR analyses of two polymorphic markers flanking the retinoschisis (RS1) gene and a new X/Y marker, X22, to sex embryos in an X-linked retinoschisis PGD program. Moreover, MDA offers a useful alternative to multiple locus-specific PCR preamplification for large single nucleotide polymorphism (SNP) genotyping scoring studies [33]. In comparison with other WGA methods, it was demonstrated that MDA exhibits a significantly higher concordance of SNP genotypes following amplification and suggested that MDA can be used in genome-wide SNP scans [57]. The accuracy of genotyping varied from 89% to 100% (average 94%) from DNA amplified by MDA [58].

4.6 Summary

In summary, the clinical applications of in vitro amplification for genetic testing are many. We started the chapter by reviewing the basic concepts of PCR and gave an example of how targeted PCR is used in a clinical laboratory to test for trinucleotide expansions in Huntington disease. After that, we discussed more advanced methods that utilize the same basic principles such as multiplex PCR, real-time PCR, and massively parallel targeted methods such as microdroplet PCR and digital PCR. At the end of the chapter we describe several whole-genome amplification methods that have been applied in PGD for several genetic disorders. In the past 25 years, significant progress in the development of various in vitro amplification techniques has greatly enhanced the diagnosis of many different diseases; the continued application of these methodologies to both new and old clinical problems holds great promise for the future.

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Chapter 5

Whole Genome Sequencing in the Clinical Laboratory

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Keywords Next generation sequencing • Whole genome sequencing • Clinical • Genetic • Analysis • Quality • Validation • Bioinformatics • Ultrahigh throughput • Genetic testing • Genomics

5.1 Introduction

The practice of analyzing the nucleotide bases contained within DNA and RNA has existed since the 1970s. The first sequencing methodologies were extremely laborious, and only very short stretches of nucleotides could be read. The situation changed in the mid-1970s with the introduction of radioactively labeled nucleotides or probes and size separation by electrophoresis [1–3]. However, even these methods were laborious and involved significant cost and the use of hazardous isotopes. It was not until the mid-1990s that chain termination methods of sequencing (Sanger sequencing) were combined with fluorescently labeled nucleotides in automated capillary electrophoresis (CE) sequencing machines that could detect the fluorescent signals after laser excitation [4, 5]. CE methods separate molecules rapidly when compared to traditional gel electrophoresis by incorporation of electrokinetic sample injection and high voltage gradients through the capillary. This high-throughput method allowed for cost-effective, widespread use of sequencing. Subsequently, the adoption of sequencing techniques into clinical laboratory practice grew rapidly in the 1990s and first decade of the twenty-first century.

Capillary electrophoresis Sanger sequencing is now widely used in clinical diagnostics for over 1,500 conditions [6]. Typically, clinical tests using DNA sequencing first PCR amplify region(s) of interest, or the entire coding regions for genes of interest, followed by an amplicon purification step. Each unique product amplified by PCR then must be sequenced independently, and each is typically sequenced twice (once in the forward and once in the reverse orientation). This leads to a technique that, while markedly cheaper, safer, and faster than previous sequencing techniques, is still relatively expensive and laborious. Sanger-based sequencing tests typically range between \$1,000 and \$3,000 per gene and require 3–6 weeks in turnaround time. Because of the expense and time required, clinical sequencing tests tend to focus on very limited genetic regions or single genes, particularly where the etiology of the disorder is typically due to rare or unique mutations.

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Automated Workflow

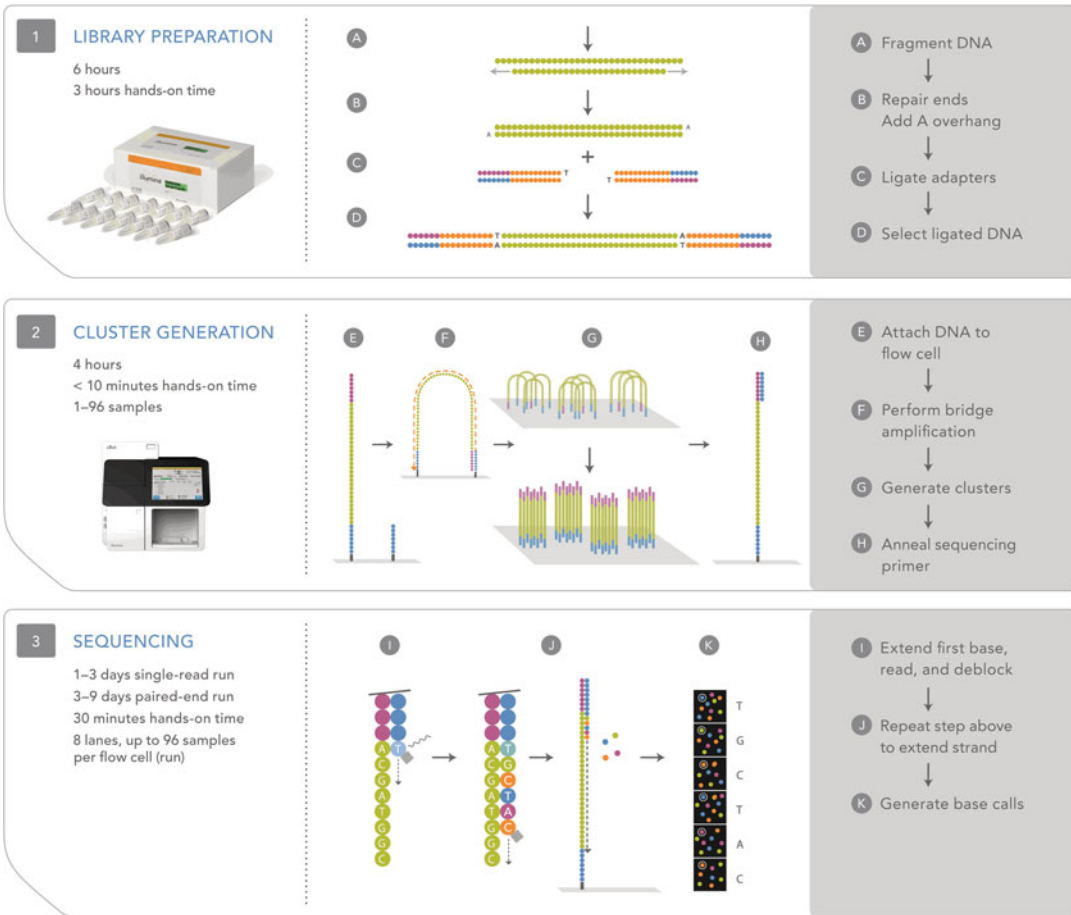


Fig. 5.1 Walk-through illustration of sequencing by synthesis. A representation of the major steps in next generation sequencing using the Illumina protocol. The first section describes the elements involved in generating a whole genome library starting with about 100 mg of high-quality genomic DNA, fragmenting, and ligating the adaptors on to the ends. The second major step is cluster generation, in which the adaptors, now attached to the molecules, bind to their complementary oligos, which are bound to the flowcell, and are then individually amplified into hundreds of thousands of clusters per lane. Finally, the clusters are denatured, primers are attached for sequencing, and the reaction proceeds to step three, in which sequencing is performed

In the last decade, another technical advancement has resulted in so-called next generation sequencing or massively parallel sequencing (MPS) methodologies [7, 8]. In MPS, multiple fragments or amplicons can be simultaneously sequenced. The sequence process utilizes fluorescently labeled nucleotides that are incorporated into the fragments, which are physically fixed in space. Bases are read individually as nucleotides are incorporated (Fig. 5.1). This advancement results in multiple sequences being read in parallel without the need for a capillary channel for each individual sequence. The resulting improved throughput allows for the development of sequencing tests that focus on multiple regions of interest, and even for whole genome sequencing, at prices and turnaround times that are orders of magnitude less than similar efforts would require if performed by Sanger sequencing. Clinical labs can now provide substantially more information for prices that are becoming competitive with single gene testing [9].

5.2 How Massively Parallel Sequencing Works

Sanger sequencing and all MPS technologies have several fundamental aspects in common. They all rely on amplified DNA or RNA, which are then labeled using dyes and visualized using lasers. The signals are all evaluated by the strength of the fluorescent signal relative to noise, and nucleotide bases are called accordingly [10, 11]. In Sanger sequencing, the nucleotide amplification is typically performed using PCR or cloning. This is also possible for targeted MPS applications, but for whole genome sequencing (WGS), a library is constructed from the whole genome and the entire library of DNA is amplified in multiple fragments on the flowcell. The following describes WGS using the Illumina GAIIX MPS platform. Although the general steps are similar across MPS platforms, there are some important differences [12, 13].

After extracting DNA from the clinical sample, the steps for WGS include (1) library preparation, (2) cluster generation, (3) sequencing, and (4) bioinformatic assembly and calling of variants (see Fig. 5.1). For library preparation, intact genomic DNA (gDNA) is first sheared randomly. Prior to adaptor labeling, the sheared gDNA is blunt-ended, and an adenosine overhang is created through a “dATP” tailing reaction. The adaptors are then ligated to the fragment mixture utilizing DNA ligase in a ligation reaction. These adaptors are comprised of the sequencing primer and an additional oligonucleotide that will hybridize to the flowcell. After adaptor ligation, the sample is size-selected by gel electrophoresis, gel extracted, and purified. For WGS, the targeted fragment size is 300 nucleotides. To improve the quantity of purified size-selected fragmented library, the sample is amplified by PCR and finally purified once more before finalizing the library. At this point, the library quality and concentration can be evaluated in a variety of ways, including Q-PCR and quantitative gel analysis. Libraries generated for WGS at the Illumina Clinical Services laboratory (ICSL) are evaluated for the cleanliness of peaks on a bioanalyzer run, the lack of primer-dimers, and must fall within a concentration of 32–400 nM.

After the library has been constructed, it is denatured and loaded on to a glass flowcell where it hybridizes to a lawn of oligonucleotides that are complementary to the adaptors within the cluster station (see Fig. 5.1), an automated liquid-handling instrument that adds appropriate reagents at appropriate times and temperatures. The single-stranded, bound library fragments are then extended, and the free ends hybridize to the neighboring lawn of complementary oligonucleotides. This “bridge” is then grown into a cluster through a series of PCR amplifications. In this way, a cluster of approximately 2,000 clonally amplified molecules are formed; across a single lane of a flowcell, there can be over a 37 million individual, amplified clusters. After amplification, the clusters are denatured so as to free the 5' ends and blocked, and the sequencing primers are hybridized to the free ends. The flowcell is then transferred to the sequencing instrument.

On the sequencing instrument, fluorescently labeled nucleotides compete for hybridization to the single-stranded template. In addition to having a fluorescent label specific for each base, the nucleotides also have a blocker bound to them. After the laser excites the dye and images are captured for each dye, the blocker is cleaved off, allowing the next nucleotide to bind. This process is repeated between 36 and 120 times in a process called sequencing by synthesis. Depending on the target of the sequencing assay, it is possible to perform a paired-end read, where the opposite end of the fragment is also sequenced. To do this, the newly synthesized strand is removed by denaturation, the 3' strand is unblocked and new double-stranded DNA clusters are generated as they were initially in the cluster station by bridge PCR. This time, the forward strands are removed, and the opposite strand is primed for sequencing and sequenced in the same manner. In this way, each fragment is sequenced from opposite ends.

The data captured on the sequencing machine are then processed bioinformatically (see Fig. 5.2). First, the signal strength is assessed against the noise, which is similar to the approach used in generating Phred scores [11] used in Sanger sequencing. At this point, nucleotide calls that do not show high enough quality can be objectively filtered according to the scored quality of the base call. Then, sequence reads that pass the first filter are aligned against the NCBI37 human reference genome. Different alignment programs have different objectives, some permit gaps and slight misalignment,

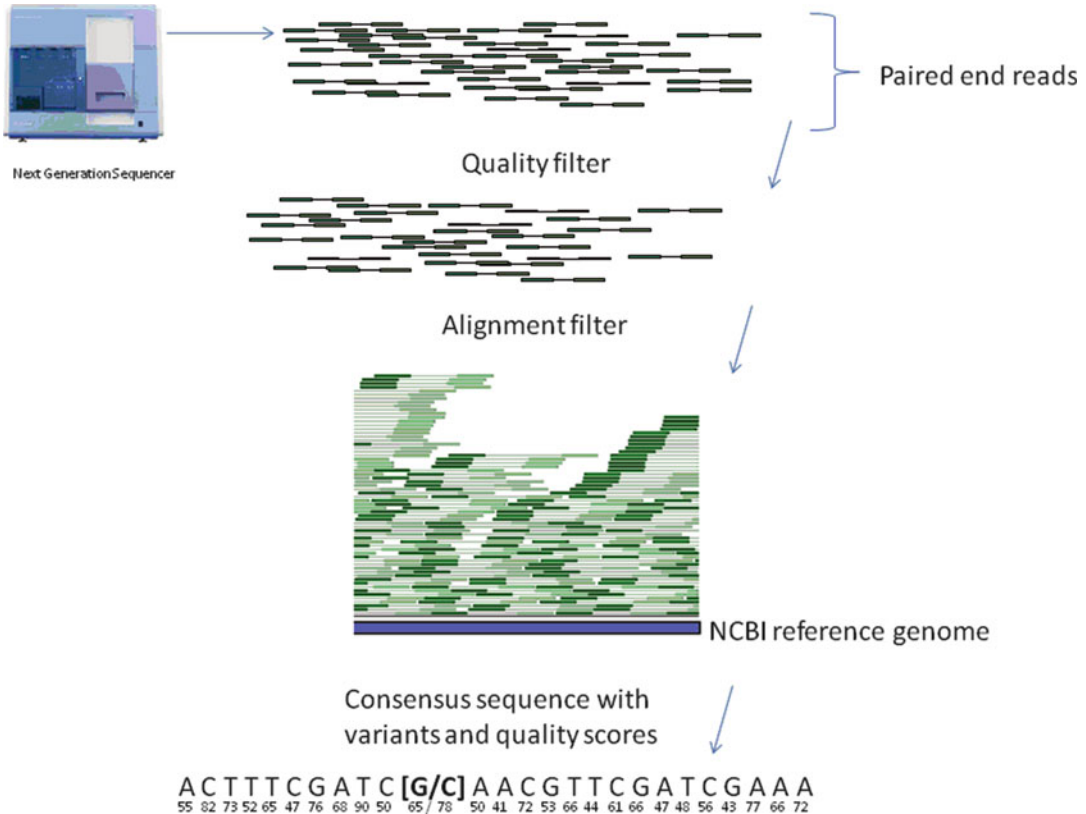


Fig. 5.2 IGS data flow. Raw image data are converted into digital signals which flow off the sequencer and into the bioinformatics pipeline. The first step is calling the bases from the images into raw sequences. A quality filter is applied that eliminates the sequences that do not have high enough signal/noise ratios. Sequence alignment is performed on the filtered sequences using Eland which will filter out reads that do not align uniquely to the genome or do not align with a minimum alignment score. All the aligned reads are then pooled together to build a genome, and the stacks of reads on every position of the genome are used to make a consensus call

and others are more restrictive. The alignment parameters chosen in the clinical setting should be appropriate to the region(s) being sequenced and specific characteristics in that region. For whole genome sequencing, the alignment parameters are strict, and any data not meeting the strict criteria are not included. Finally, once all the reads have passed the filter and have been aligned, consensus bases and variants are called using all the information from all the reads, and again applying rules for what levels of quality and how many independent sequencing events were captured at that position.

The resulting output of MPS is compared to Sanger sequencing in Fig. 5.3. In Sanger sequencing, the strength of the signal and any noise are illustrated in the form of colored peaks, and the calling software evaluates the signal and noise and makes a call. In MPS, the same information is digitized and called with a particular confidence score, which is analogous to the Phred score. One challenge for Sanger sequencing is that each read is comprised of a population of amplicons, so if it is a heterozygous site, both signals are being read at the same time (unless the amplicon is cloned, and the clones are individually sequenced). This results in a loss of signal strength (since there is only approximately half the signal that would be emitted from a homozygous position) and two overlapping signals, which can sometimes be difficult to call with confidence. Because MPS sequences molecules individually (each cluster on the flowcell represents a clonally amplified molecule), there is no mixed signal or reduction of signal strength. However, like with Sanger sequencing cloned molecules,

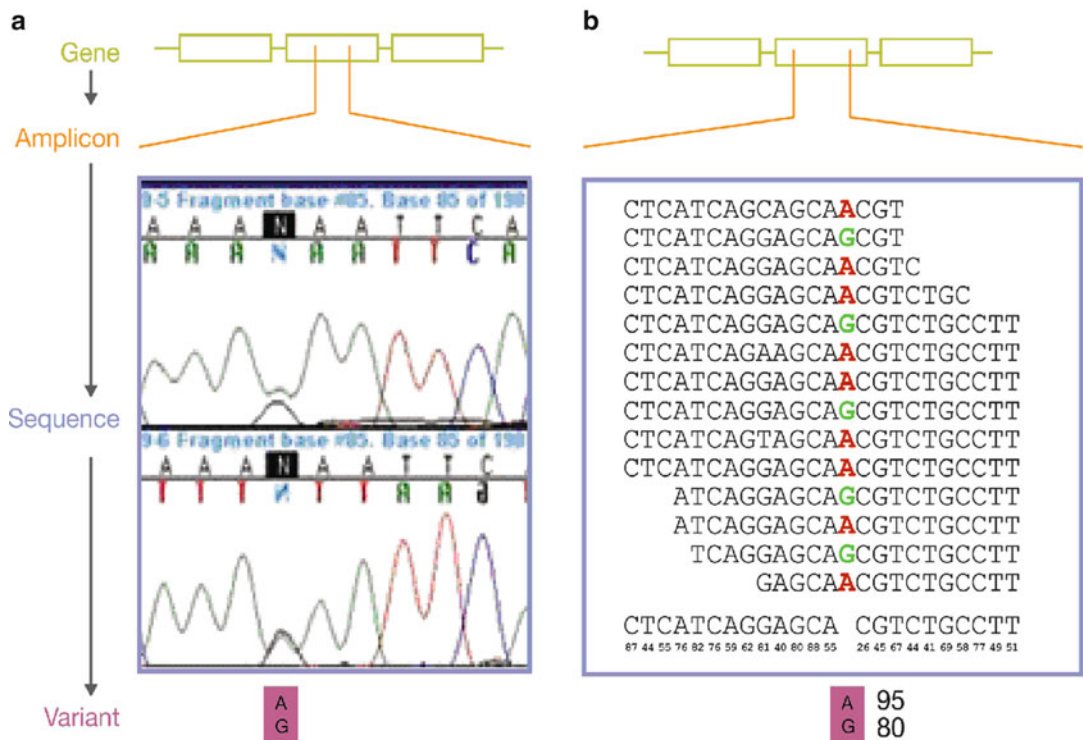


Fig. 5.3 Comparison of Sanger and MPS data. Sanger sequencing and next generation sequencing both rely on imaging of fluorescently labeled nucleotides, and both measure these as signal purity relative to noise. For Sanger sequencing, this is typically visualized as peaks and troughs. Because the amount of information produced by MPS is significantly increased, data are displayed in terms of the call and the quality of that call. However, because each site is sampled (in our lab) at least eight times and on averages 30 times, the quality scores for each of those events are combined to give an overall confidence in the final call. Additionally, in positions where a heterozygote is detected, in Sanger sequencing, both signals are mixed, resulting in a loss of signal strength and, sometimes, ambiguity in the call. In MPS, each molecule is sequenced independently, resulting in high confidence in both calls being made. This is illustrated above, where each call is made for MPS and the corresponding allele score is reflected. For a heterozygous call, both calls and both allele scores are reported, yielding a quantified, standardized measure for the quality of every call

multiple clones must be sequenced for the same position in order to detect a variant when it is present. The need for additional sequencing decreases the likelihood of false positive calls because the final consensus sequence is called based on both the quality of the bases called at that position and the number of bases called. Since the consensus call has an allele score, the quality of a call can be quantitatively measured, thresholds can be established based on validation assays, and the confidence of every call can be reported using an objective scoring system.

5.2.1 Considerations of Sequencing Technologies in the Clinical Laboratory

Regardless of the technique, any clinical test is limited by tissue requirements, turnaround time requirements, and the flexibility of the technology. Massively parallel sequencing shares some characteristics with capillary Sanger sequencing, and therefore can be treated similarly, but the characteristics mentioned above deserve special attention. All MPS technologies share, for example, that the DNA input is typically amplified using a polymerase. Additionally, the sequencing reaction is mediated using an enzyme (polymerase or ligase), and for all but one technology, the base is detected using a

fluorescent-labeling system. These various technologies include Sanger sequencing, the established MPS technologies use pyrosequencing, sequencing using probe ligation events, and sequencing using dye-labeled nucleotides that are added and measured in real time. A new addition to this line up uses a hydrogen ion superconductor approach to detecting nucleotides. These MPS platform differences result in different strengths and weaknesses in performance that should be considered when selecting a platform for testing. For example, if a laboratory is more concerned with specific questions such as knowing the phasing of chromosomal information, then longer reads become important, whereas if regions considered critical to the analysis include homopolymeric regions, then a different technology or chemistry might be most appropriate. While all of these technologies can potentially cover most of the genome, none are able to cover all biochemical regions of the genome equally well, and each has its own bias and strength. As the various technologies are evolving extremely quickly, it is important for a potential user of any MPS approach to carefully consider the specific or most important needs of the intended assay before making a technology decision. Indeed, some labs opt for investing in multiple technologies as a way to supplement each other's weaknesses.

Turnaround time requirements differ according to types of tests. In addition to DNA extraction, next generation sequencing involves three main steps: (1) library preparation, (2) flowcell preparation, and (3) sequencing, and is followed by an intensive bioinformatics analysis. After gDNA has been extracted from tissue and quantified, it can be used to prepare a genomic library or specific regions can be targeted for a "targeted sequencing" panel. For genomic libraries, the preparation time is usually 2–3 days, while targeted sequencing approaches may take up to 5 days. The prepared DNA can then be bound to the flowcell and amplified in the bridge PCR, which typically requires about 4–6 hours. Finally, sequencing can be performed; depending on the length of the read and the specific technique employed, this can take 2–10 days. Not surprisingly, the generation of orders of magnitude of more data requires significantly more posttest data analysis in the form of bioinformatics. The data must be filtered and assembled, then consensus calls made and assessed for quality. Any data annotation must then be made, and of course, if clinical interpretation is provided, that too must be performed. While much of the data analysis and annotation can be automated, it still is both computing and time intensive; it is critical that these requirements be considered when developing clinical applications. Due to the increase in information, data annotation and especially clinical interpretation can require increased time investment by the clinical laboratory team.

A significant advantage to MPS is the flexibility inherent to the technique; this technique can be adapted to short reads or long reads and can sequence at significantly higher depth than traditional methods of sequencing. The fact that each molecule is sequenced independently means that it is possible to detect rare variants when considering a nondiploid situation, such as in infectious disease or cancer samples. Additionally, the bioinformatics associated with the technology allow for users to determine the particular quality thresholds necessary for a test. While this gives users the advantage of quantitatively standardizing the quality of the data that they deliver, it requires a sophisticated understanding of how the bioinformatics filters are applied.

5.3 Moving Whole Genome Sequencing to a Clinical Laboratory

The operation of clinical laboratories is governed by CLIA (the Clinical Laboratory Improvements Amendments). Laboratories are certified under the CLIA provision in which state health departments may inspect and confer certification; these laboratories are required to obtain additional accreditation from an approved professional society. Many clinical laboratories seek third party accreditation, such as that offered by the College of American Pathologists (CAP). CLIA and CAP have published guidelines regarding how a genetic test may be offered in a clinical setting [14–16]. Additional recommendations regarding high-complexity molecular testing have also been written by several groups, sometimes with specific targets in mind [17–20]. Due to the increased complexity and potential for

multiple uses, bringing whole genome sequencing into the clinical laboratory requires special attention to the guidelines and recommendations. Below we discuss some of the recommendations that require special solutions when applied to whole genome sequencing.

5.3.1 *Preanalytical*

The preanalytical component of a clinical test typically focuses on the laboratory infrastructure and document control. This does not differ in any meaningful way for whole genome sequencing. However, other aspects included in the guidance are more critical. Here, we discuss three components in particular: test definition, patient consent, and sample tracking.

Test definition is potentially challenging for whole genome sequencing. Sequencing a whole genome is in many cases more thorough and less prone to ascertainment bias than sequencing a gene panel or exome. A physician may wish to order whole genome sequencing because it provides the most thorough, cost-efficient, high-quality data across the subset of genes he/she believes may be important in a particular case, and a different physician may be focusing on an entirely different set of genes but order a whole genome for the same reason. Therefore, the test is defined in the terms of the information that can be delivered rather than indications for use. Another important component is to define the established analytical performance of the methodology. The analytical performance is measured in terms of accuracy, sensitivity and specificity, limits of detection, and reportable range of the test. In this case, these can all be defined at a very focused and specific level; each base pair that is reported has a score which describes the quality and confidence of that particular call. Reporting can be individualized to the needs of the physician, for example, a subset of genes and specific thresholds for quality of reporting. However, since it is potentially a different test for different physicians under different circumstances, it is necessary to focus on the process and the ability to measure the quality of the data. Because the information must be robust under a variety of uses and conditions, the validation(s) performed for WGS must be more extensive than what is typically performed and must be designed with multiple potential uses in mind.

Informed consent has long been advocated for any kind of genetic testing by professional, regulatory, and patient advocacy groups [17, 19, 21]. In the case of whole genome sequencing, this is particularly important to establish that the patient and physician have a shared understanding of why the test is being ordered, how it will be used in that situation, what will and will not be reported, who will have access to the information and under what circumstances, how data should be managed, and finally what can and cannot be learned. These are issues that must be covered in any informed consent process, but because the scale of information is greater than typical single gene testing, the consenting process must be broader, and anticipate many more possibilities. This is a challenge for the genetic counselor or physician who is counseling the patient, and can require many hours of discussion, often over many meetings. Typically, institutions that use whole genome sequencing are performing 3–6 h of genetic counseling for their informed consent process.

Sample tracking is a challenge for all clinical labs, and most errors in molecular labs involve sample tracking errors [17]. Given the increased expense and level of information provided in whole genome sequencing, a focus on sample tracking is particularly critical. In the Illumina Clinical Services Laboratory (ICSL), we implemented typical sample tracking strategies using documentation and supervision verification of critical steps, but in addition we have designed an additional quality control check. For every patient that is able, saliva and blood samples are collected in matching bar-coded tubes. The saliva must be collected by the physician and therefore has positive identification. The blood can then be collected by a phlebotomist. Both tubes are sent to ICSL, where DNA is extracted, and the DNA from both samples are run on the Human 1M-Duo genotyping chip, which is an array-based test that surveys more than one million variants that are distributed across the genome.

Table 5.1 Quality metrics

Minimum depth of coverage to make a consensus call	≥8 calls with a Q30
Average read depth	≥30-fold with a Q30
Minimum coverage of NCBI37 reference genome by uniquely aligned reads	≥90%

If the DNAs from both samples match, then the genome is sequenced from the DNA extracted from blood. If they do not match, we ask for new samples to be sent. If a patient is not able to contribute a saliva sample, the physician or genetic counselor must attest that the blood sample collected was from the patient. Once the genome has been sequenced and passed internally established quality standards, the calls from the array are compared to the calls made from genome sequencing for those same positions. This allows us to maximize the probability that any contamination or tracking error that occurred internally during laboratory processing would be detected.

5.3.2 Analytical

A major focus of clinical testing guidelines is on understanding and ensuring the quality of the data that is produced. The goal is to ensure that the providing laboratory and ordering medical professionals know how accurate and reliable the results are. In the last 20 years, capillary sequencing has been considered the best method possible for detecting most genetic variants and is generally considered the gold standard. The same rationale must be used for MPS data. Capillary sequencing performed in the clinical space typically validates the process of sequencing by using a few samples with known variants and performing the test on those samples. Once evaluated, the results are assumed to apply with equal fidelity for other variants for which samples might not be readily available but that might occur in that region. Rather than validate a test for detection of a subset of variants, the testing method is validated using a representative subset of samples. Since MPS data include a quantitative measure of the quality of each call, validation testing can be used not only to measure how accurate and precise the method is, but to calibrate where thresholds should be set to pass data for reporting.

Several publications have recently compared Sanger capillary sequencing to MPS [13, 22, 23], showing that the results are comparable. While MPS shares many fundamental principles with capillary sequencing, there are some differences. Figure 5.3 illustrates some important differences. It is important to be aware of the critical differences when designing evaluations of MPS performance for WGS. At this time, using the Illumina technology, fragments are typically about 400 bp long, and 120 bp of either end are read. These reads are then filtered for quality of signal to noise and compared to the reference. Not all regions of the genome can be confidently aligned to the reference using this approach. It is important to assess what regions can and cannot be reliably aligned and to define this as part of the limitations or reportable range for the test. Additionally, it is important to be aware of the signal to noise filter thresholds and the alignment algorithms. Typically, different algorithms are tuned to be more conservative or more permissive (so as to only include regions that are of confidence or so as to maximize the amount of regions to call on); choosing how to set these thresholds and filters is critical to the quality of the data delivered. Tables 5.1 and 5.2 illustrate the quality metrics and corresponding accuracy established at ICSL.

Q30 is a measure of the raw signal:noise ratio (along with other measures of the data) presented as the probability of a miscall: a Q30 means probability of a miscall is 1/1,000; for whole genome sequencing, we require at least 8 independent sampling events for that position, each with a probability of correctness of 99.9%. Across the entire genome, the average number of sampling events per call must be at least 30. At least 90% of the alignable positions in the NCBI37 human reference genome must be called.

Table 5.2 Measured sensitivity and specificity at multiple depths of coverage using known heterozygous samples

Depth of coverage	Sensitivity	Specificity (%)
30×	>99.99%	100%
20×	>99.99%	100%
10×	98%	>99.99%
5×	Not detectable	>99.99%

Depth of coverage refers to the number of times that particular site has been independently sequenced. Sensitivity refers to the rate of detection of correct second allele in known heterozygous position at that particular number of sampling events (depth of coverage) over 10,000 subsampling iterations. Specificity refers to the frequency of detection of correct base call using the number of sampling events (depth of coverage) indicated over 100,000 subsampling iterations

One of the most critical areas to consider when designing validation testing for whole genome sequencing is making the consensus call. Unlike Sanger capillary sequencing methods, where all the products of a PCR reaction are sequenced in a pool of thousands of amplicons, in MPS, each fragment is independently sequenced. This is a benefit in that the signals are never mixed in MPS, but it is also a challenge in that a given position must be sequenced multiple times in order to detect if a variant is present at that position. The number of times a particular position is sequenced is directly related to the probability that a second allele would be detected if it were present, therefore each position that is reported on must be sampled multiple times. The number of sampling events, combined with the quality of each call, can be quantified for every base that is called. While this is a significant advancement in data quality, it requires that the validation be designed not only to assess the quality of the data but also to optimize the bioinformatics software used to filter, align, and call sequence variants for the specific needs of the assay.

There are several commercial software packages available for the analysis of MPS data. These packages range in the tasks they perform. Some only perform primary analysis, such as calling individual reads and assigning quality or Phred-type scores, alignment to a reference or potentially de novo alignments of sequence without a reference, and calling a consensus read/consensus variant. Many of these primary analysis software packages are included in the platform purchase, but there are several others that are commercially available or even available free on the web. All of these analyses software require validation in the CLIA lab for evaluation of their performance. Additionally, most of these can be “tuned” according to the types of questions and regions of focus. Depending on the needs of the laboratory, it might be necessary to perform multiple different analyses or use different primary analysis software packages to make consensus calls or identify variants of different types or that exist in different biochemical regions of the genome. Regions that are known to be challenging include regions with duplication events, such as Alu regions. Types of mutations that can be confounding include insertion-deletion events of different size, mosaicisms and other large structural variation events, and copy number variation detection. Many software packages will allow for “tuning” of the settings and analysis parameters, which is appropriate depending on the function and intended use. It is important for clinical laboratories to consider the intended use of the genomic level information and, in general, to set parameters to more stringent settings in order to ensure the most robust and high-confidence calls. It is far preferable for an assay not to make a call than to make a call with low confidence or low accuracy.

In addition to primary analysis, there are many software packages that are available to provide some level of annotation to genomic level information. That is, these software packages will take data from an aligned genome in which all variants are mapped and called and will then associate with those sites such information as amino acid changes, whether the variants have been detected in dbSNP and other useful information that can aid in navigating the genome and in developing strategies for clinical interpretation of variant information. These software packages also require thorough evaluation with regard to the specific aims of the assay and, in the clinical context, require validation.

5.3.3 *Postanalytical*

The postanalytical guidelines focus on data management, patient privacy, and physician support. Because data management for WGS is a complicated subject, it is discussed separately in section 5.4. The deliverable for a whole genome sequence is currently large and cumbersome. As the technology becomes better established and, in particular, as software becomes more standardized and more validation data becomes available, the deliverable should rapidly shift toward focusing on variants that can provide useful information to physicians in their management of a particular patient. While there are likely to be additional improvements to the software and ability to call variants with known confidence, an additional area of significant concern is that of access to clinically valid variant information. Currently, databases are not curated to clinical grade standards, and geneticists in clinical laboratories are responsible for providing interpretation based on their expertise and reading of available literature. As we progress from limited genetic testing on specific genes to assessing meaningful variants across a whole genome, better guidelines and curation standards need to be developed to aid laboratory professionals in providing the most accurate annotation and interpretation possible.

5.4 Management of WGS

With enough sequence to accurately call variants on large sequences comes the additional complexity of managing more data than many clinical laboratories are accustomed to. MPS data sets are far too large to process by hand and require an automated software pipeline that processes the data according to the established and validated parameters, as well as servers and backup.

5.4.1 *Types of Data*

WGS data come in several forms but largely consist of raw output from the sequencers and downstream analysis of the sequence. The raw data output from the sequencers usually comes in the form of images which can be terabytes in size, but most next generation sequencers have implemented software that runs on the attached machine(s) connected to the sequencing instrument and will process the raw images down to a more condensed text form. The raw data output from the sequencer is then processed into sequence. This sequence is aligned to the reference genome which results in information for each sequence about its alignment position and score. After alignment, a variant caller then produces files detailing the variants found in the sequence relative to the reference. In total, the resulting files include the raw sequencer output, sequence called by the software, alignments, and a consensus sequence for all calls at all positions that passed the various bioinformatic filters. Most clinical tests are concerned with being able to detect either heterozygous or homozygous variants, which in MPS are detected by the software based on a combination of the quality of the raw data, the alignment quality, and the number of times which the position was sequenced.

5.4.2 *Strategies for Data Management*

Clinical laboratories are accustomed to saving and backing up all the data generated in a laboratory. With MPS data sizes, any more than a few samples will quickly make a full IT backup solution much more expensive than regenerating the sequence from DNA. There are two main ways to compensate for the inability to store all MPS generated data. Many labs choose to look at the data as a hierarchy whereby only the raw sequence is really unique to a sample. Everything else can be reproduced at the

expense of computational time. This approach will and store only the sequence, regenerating all downstream alignment and variant data if needed. Another popular solution is to store only the variants, which are relatively small and manageable. This is simple to implement but prevents the reanalysis of any data using improved algorithms, which are constantly evolving.

5.5 Current and Future Uses of WGS

At this time, there are two primary clinical applications for WGS. The first is differential diagnosis. There are many diseases for which no genetic testing is available despite some knowledge of the causative variants because the variants are so rare that it is not cost-effective for a laboratory to develop a specific test. Additionally, many conditions that are phenotypically overlapping or indistinguishable have different causative mutations; in these cases, a differential diagnostic that assesses all potential variants in all genes that are known to play a role in the phenotype can be extremely informative for case management and family planning. In these cases, WGS can also be less expensive than multiple, traditional sequencing tests and can end the stressful, costly diagnostic odyssey for the family and the physician. Furthermore, additional information contained in the genome, such as variants that affect drug metabolism, can be used in patient treatment decisions.

Because MPS has the capacity to sequence very deeply, it is also possible to use this technology to detect rare variants in an individual, such as somatic mutations that occur in tumors. This becomes very useful for assessment of cancer cases, where the tumor genome and the normal genome can both be sequenced and the variants present in the tumor genome but not the normal genome can be assessed for chemotherapeutic indications. This approach has been used successfully [24] to evaluate appropriate chemotherapeutic treatment. The same approach could also be applied to screening for infectious disease, such as HIV strains, within a patient. However, additional challenges exist when detecting variants that no longer exist in the diploid state, and additional validation must be performed around ranges of detection and the software used to detect these variants.

In the future, the challenges to bioinformatics management are expected to be resolved, which will result in increased use of WGS approaches to evaluating and managing cancer care. In addition, other types of variants, such as copy number variants, insertions and deletions, and larger and structural variant analyses, are currently available in the research space and are expected to be robust enough to be used for routine clinical care in the very near future. In addition to applications for cancer, very sensitive detection of copy number variants will be useful for testing in infectious disease monitoring and aneuploidy conditions. Furthermore, advancements in chemistry are expected to yield longer read lengths, which will allow for better coverage across repetitive regions, potential for haplotype resolution, and increased coverage of the genome. These advances will improve testing capacities in areas such as tissue typing. Along with the rapidly falling price and the increase in clinically relevant information that can be delivered, MPS is likely to improve the ability of physicians to manage care of cancer, infectious disease, and multigenic conditions.

5.6 Summary

Whole genome sequencing is currently in its infancy as a clinical application; however, it could potentially affect many areas of medical care. The advantages include: (1) providing nearly comprehensive genetic information for a patient; (2) quality can be established using defined, quantitative thresholds; and (3) the nature and depth of the sequencing allow for greater sensitivity in variant detection. The information hopefully can be useful in the management of the specific condition being tested for, but also can provide additional valuable information, such as pharmacogenetic status and carrier status,

thus reducing both the likelihood of poor outcomes and the need for additional testing. Disadvantages include the expense of the test, the technical complexity of the test in the lab, the computationally intensive analysis, and the bioinformatics support required to apply the software and generate reports appropriately. While there are many challenges listed above, these are being actively addressed and can be expected to progress quickly. More significant challenges will include how regulatory guidance will evolve to address the changes that are inherent in using this new methodology, how and when whole genome sequencing will be reimbursed, and how to provide the clinical infrastructure to support the use of WGS information. The last point is particularly daunting; this will require an ability to manage and transmit large amounts of information appropriately according to physician and patient needs and wishes while still protecting privacy and ensuring that information is not misused.

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Chapter 6

Estimation of Exon Dosage Using Real-Time Quantitative Polymerase Chain Reaction

Applications to Factor VIII Gene in Hemophilia A and Endoglin/ACVRL1 Genes in Hereditary Hemorrhagic Telangiectasia

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Keywords Hemophilia A • Hereditary hemorrhagic telangiectasia (HHT) • Factor VIII gene • ENG • ACVRL1 • Gene dosage • Real-time PCR

6.1 Introduction

Point mutations in the coding region of many different genes account for the majority (~80%) of all genetic diseases. These mutations include single base substitutions and in frame or out of frame insertions and deletions of nucleotides in the coding exonic regions that give rise to missense, nonsense and frameshift mutations. In addition there can be insertion, duplication, or deletion of the whole gene or intragenic segments that can give rise to absent, or altered gene product. Such mutations, also described as exon dosage mutations, account for 10 ~ 15% of all known disease associated changes. The purpose of this chapter is to review the methods used to detect exon dosage changes in two genetic diseases, Hemophilia A and Hereditary Hemorrhagic Telangiectasia.

6.1.1 Clinical Features and Molecular Pathogenesis of Disease

6.1.1.1 Hemophilia A

Hemophilia A is an X-linked recessive bleeding disorder caused by a deficiency in blood coagulation factor VIII. Mutations in the factor VIII gene are responsible for this disease affecting approximately 1 in 5,000 live male births. The clinical findings can include prolonged or renewed bleeding after initial bleeding stops following events such as injury or surgery. Individuals can also present with excessive bruising, hematomas, hemarthrosis, or gastrointestinal bleeding. The severity of disease depends on the factor VIII level present in the blood. The normal level of factor VIII clotting activity in an individual is between 50% and 150%. Individuals with less than 1% factor VIII activity are considered to have severe hemophilia A. Moderately severe and mild forms of hemophilia A have

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factor VIII clotting activities of 1–5% and 5–35%, respectively [1]. Due to its inheritance pattern, hemophilia A most often affects males, with females typically being asymptomatic carriers. However, females have been infrequently reported with hemophilia A. This can be due to skewed X chromosome inactivation in which there is preferential inactivation of the normal X chromosome, resulting in predominant expression of the mutant copy. Other structural abnormalities, such as Turner syndrome, that result in only one functional X chromosome can also cause female hemophilia A [2].

The most common causative mutation found in about 40% of patients with severe hemophilia A is the intron 22 inversion. This can be detected by methodologies such as Southern blot or inverse PCR [3]. An inversion in intron 1 is also found in 2–3% of these individuals [3]. Other mutations such as deletions and insertions, as well as nonsense, missense, frameshift, or splice site changes, can be detected by sequence analysis. A final stage of causative mutation screening is often gene dosage analysis [4].

6.1.2 Hereditary Hemorrhagic Telangiectasia

HHT is an autosomal dominant disease known to be associated with alterations in at least two genes including Endoglin (ENG) on chromosome 9q34 and activin receptor-like kinase 1 (ACVRL1 or ALK1) on chromosome 12q13, which are involved in cell proliferation, differentiation, migration, and adhesion in the vascular system. Affecting between 1 in 5,000 and 1 in 8,000 individuals, HHT most commonly presents with recurrent epistaxis, which can be severe enough to require transfusion. However, this disease is characterized by arteriovenous malformations (AVMs), which are the result of direct connections between arteries and veins without intervening capillaries. The location can range from cutaneous and mucous membrane telangiectasias to AVMs of increased severity including pulmonary, gastrointestinal, and cerebral, which can accumulate fatal complications. An individual can be diagnosed with HHT upon meeting three or more of the following criteria: (1) spontaneous, recurrent epistaxis; (2) mucocutaneous telangiectasias primarily on the tongue, lips, oral cavity, fingers, and nose; (3) pulmonary, cerebral, hepatic, gastrointestinal, or spinal AVMs; and/or (4) a first-degree relative with HHT [5].

The spectrum of mutations found in the ENG and ACVRL1 genes include point mutations such as nonsense, missense, splice site, and frameshift mutations, which can be detected by sequence analysis of both genes [5]. Gene dosage analysis is applied to patient samples without a sequencing mutation in the coding regions of ENG or ACVRL1 to identify any intragenic or entire gene deletions [5].

6.2 Clinical Uses of Testing

6.2.1 Hemophilia A

The clinical implications of hemophilia A testing include diagnostic confirmation, as well as determining the causative mutation to allow further testing for other at-risk family members. Carrier testing is performed for female family members who are at risk. It has been reported that 10% of these females are at risk for bleeding [1]. Carrier females will have a 50% chance of transmitting the factor VIII mutation in each pregnancy. Therefore, 50% of all sons will be affected and 50% of all daughters will be a carrier. Prenatal testing is also possible when the familial mutation is known to establish or eliminate the diagnosis in a male fetus at risk. In the event of the detection of an affected fetus, critical precautions can be taken for the labor and delivery. Prenatal testing is not typically recommended for a female fetus at risk of being a carrier [1].

The type of mutation can predict the severity of the disorder due to the fact that particular mutation types, such as large gene deletions, are more at risk for developing inhibitor antibodies against factor VIII. This is critical when considering response to therapy and is considered the most serious complication of hemophilia A [6]. Genetic counseling is recommended for all families affected by hemophilia A. It is important to be aware of the carrier status of all at-risk female family members before considering pregnancy [1].

6.2.2 *Hereditary Hemorrhagic Telangiectasia*

Genetic testing for HHT can provide clinical diagnostic confirmation, as well as the identification of mutation carrier status in presymptomatic individuals when the familial mutation is known. The identification of a disease-causing mutation in either ENG or ACVRL1 confirms the clinical diagnosis which benefits the proband as well as their family members. Confirmed molecular diagnosis of this disease can be crucial, as many of the complications of HHT can be prevented. This can provide opportunities for early detection of AVMs as well as management of the disease [7]. Due to its dominant inheritance pattern, mutation carriers are presumed to develop symptoms, but the variable expression of the clinical symptoms suggests the absence of a direct genotype-phenotype correlation. It has been suggested that disease severity can be classified by the location of the mutation, with patients carrying ENG mutations having a more severe phenotype than ACVRL1 mutations [7].

6.3 Methodology

As hemophilia A is an X-linked disease, detection of deletions – whole gene or single/multiple exons of factor VIII gene – in male probands is achieved by primary sequencing screens which can detect absence of amplified exonic regions. Large gene deletions in the factor VIII gene in female carriers on the other hand cannot be detected by the primary screen and have been previously detected using multiplex ligation-dependent probe amplification (MLPA) analysis [2]. Quantitative Southern blot analysis has been used to detect whole-gene deletions for ENG and ACVRL1, followed by probing with specific individual exonic clones upon detection of dosage change of one or more exons [7].

Exonic or multiexonic deletions or duplications can be detected by estimating copy number of individual exons in both affected male and carrier female hemophilia A patients, as well as HHT patients, using a real-time quantitative PCR assay.

In this analysis, patient DNA is amplified in a quantitative PCR reaction that uses sequence-specific primers and probes designed to detect an amplicon, which in this case is the exon. The reactions include probe/primer combinations for an internal control amplicon which is known to be present in two copies and the same for specific exons of the gene under interrogation. The ABI StepOne platform is utilized for this assay; however, it can be easily adapted to real-time PCR machines. Applied Biosystems Primer Express software (Applied Biosystems, Inc., Foster City, CA) was used to design Taqman MGB gene expression probes which are Fam-labeled, as well as forward and reverse primers to amplify and detect genomic DNA. At least one assay was designed for each exon with multiple assays designed for longer exons such as F8C exon 14 (see Table 6.1). All assays are run in triplicate to calculate the mean and standard deviation of the three measurements.

When designing the primers and probes, it is critical to avoid any single-nucleotide polymorphisms (SNPs) to eliminate the possibility of false negatives. The presence of a SNP can prevent the primers or probe from binding to the target DNA and can result in the amplification of only one allele or no alleles. This can appear as a false heterozygous or homozygous deletion.

Table 6.1 List of probes for estimating copy numbers of individual exons of factor VIII gene

Factor VIII Exon number	Assay gene location	Assay ID	DGV version	DGV locus location
1	Overlaps exon 1	Hs01657433_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
2	Overlaps intron 1 -Exon 2	Hs00463172_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
3	Within intron 2	Hs04123536_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
4	Within intron 3	Hs04110627_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
5	Overlaps intron 4 -Exon 5	Hs04110057_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
6	Within intron 5	Hs04105171_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
7	Within intron 6	Hs04102010_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
8	Within intron 7	Hs04098648_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
9	Within intron 8	Hs04113873_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
10	Within intron 10	Hs04097468_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
11	Overlaps intron 10 -Exon 11	Hs02323114_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
12	Within intron 11	Hs04507882_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
13	Within intron 12	Hs04119825_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
14	Within exon 14	Hs01936741_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
14	Within exon 14	Hs01497314_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
14	Within exon 14	Hs02082402_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
15	Overlaps intron 14 -Exon 15	Hs01096482_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
16	Within intron 15	Hs04107300_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
17	Within intron 17	Hs04517494_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
18	Within intron 17	Hs04517494_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
19	Within intron 18	Hs04110825_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
20	Within intron 19	Hs04096550_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
21	Within intron 20	Hs04512632_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
22	Within intron 21	Hs04101223_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
23	Within intron 22	Hs04521313_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
24	Within intron 23	Hs04110003_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
25	Within intron 24	Hs04119583_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
26	Within intron 26	Hs04098432_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994
27	Within exon 27	Hs01747877_cn	NCBI Build37,hg19.v10	chrX:154009347-155041994

<http://www.appliedbiosystems.com/absite/us/en/home.html>

6.4 Protocol

6.4.1 Specimen Types

Genomic DNA extracted from peripheral blood leukocytes or from saliva or buccal swab samples can be used. However, DNA extracted from blood using a full isolation protocol has shown to yield more consistent copy-number estimates than DNA from saliva.

6.4.2 Required Reagents

- Nuclease-free H₂O.
- Taqman Fast Universal Master Mix (2×) (Part No. 4352046).
- RNase P Assay Mix (20×) (Part No. 4403328).

- Experimental Assay Mix (20× – see below).
- Experimental assays are made using 75 μL nuclease-free H₂O, 10 μL forward primer, 10 μL reverse primer, and 5 μL probe.

6.4.3 Controls

A negative control containing no DNA template is included in triplicate for each assay, along with a positive control also in triplicate from a reference sample that is known to carry the expected two (or one) copies of the exon or gene under investigation. All reactions are multiplexed with RNase P (Applied Biosystems, Inc.) that is present in two copies and labeled with VIC as a standard endogenous control. All reactions are run in parallel with a reference sample. For assays/genes on the X chromosome, both a male and female control sample are also run in triplicate.

6.4.4 Procedural Steps

1. Design plate setup in StepOne software v 2.1 (Applied Biosystems, Inc.).
2. Prepare a master mix for each assay using the required reagents as follows:

Nuclease-free H ₂ O	1.5 μL
Taqman Fast Universal Master Mix (2×)	5 μL
RNase P Assay Mix (20×)	0.5 μL
Experimental Assay Mix (20×)	0.5 μL

- Aliquot 7.5 μL of master mix to each corresponding well of an Applied Biosystems optical plate.
3. Aliquot DNA using a multichannel pipette into wells in triplicate: 2.5 μL at 2 ng/μL (total 5 ng).
 4. Seal plate and place into StepOne Plus machine.
 5. StepOne Plus Real-Time PCR cycler: 10 μL final volume.
 - (a) 20 s at 95°C
 - (b) 1 s at 95°C
 - (c) 20 s at 60°CRepeat (b) and (c) for 40 cycles.

6.5 Data Analysis

The data analysis is done on the ABI StepOne machine, using the StepOne software version 2.1. The data are analyzed by the comparative Ct method. This is done by measuring Ct (threshold cycle) values, which represent the PCR cycle at which the fluorescence of the reporter dye passes a set threshold. This Ct value has an inverse relationship with the quantity of amplicon in the reaction [8]. The Ct values are then compared with the endogenous control (RNase P) and the reference sample to calculate a ΔΔCt value. Copy number (CN) is then calculated using the formula: $CN = 2^{2 - \Delta\Delta C_t}$. The analysis settings should be set as follows: under the relative quantitation (RQ) settings, the endogenous control should be set to RNase P, the reference sample being used should be specified, and the RQ min/max calculations should be set to standard deviations = 2. All RQ (CN) values outside this range are identified as deletions or duplications [5]. The thresholds for each assay can be set under the Ct settings tab, or the default settings can be used. The threshold lines should be adjusted to be parallel

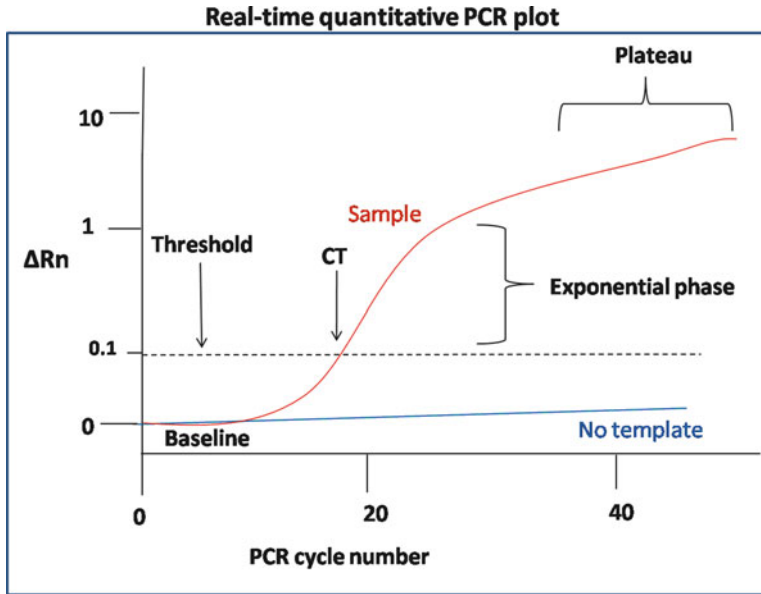


Fig. 6.1 The vertical axis represents copy number (relative quantity) and the horizontal axis shows number of PCR cycles that have occurred. The cycle at which the sample DNA (red line) crosses the threshold, which is arbitrarily set around 0.1, is referred to as the *CT* (cycle threshold). The amplification curve has an exponential phase in which the DNA doubles every cycle and a plateau phase. A negative control is included without template added which shows no amplification (blue line)

to the curve in the portion of exponential amplification across the plot (Fig. 6.1). The threshold level is based on the baseline level of fluorescence in the reaction. A signal above this is used to define the *Ct* of the sample [9]. The software will calculate $\Delta\Delta C_t$ and $RQ = 2^{-\Delta\Delta C_t}$ as well as the standard deviation associated with these values. All values are compared to ranges for individuals who were previously established to carry known copy number of 2, 1, or 3 which corresponds to normal, exon deletion, or duplication, respectively. These ranges were established by running each real-time PCR assay on a panel of at least 16 individuals whose exon or gene copy number was established previously by other gold standard methodologies such as multiplex ligation-dependent probe amplification (MLPA), fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH), or single-nucleotide polymorphism (SNP) array. An *RQ* whose value $\pm 2 \times SD$ falls outside the normal range, which is $CN=2$ for all autosomes and $CN=1$ for X chromosome, is reported as abnormal (duplicated or deleted).

6.6 Interpretation of Results

In the case of male hemophilia A patients, $RQ = CN$ for all X chromosome assays/genes. For assays/genes on all other chromosomes, CN can be calculated by the formula $CN = 2 \times RQ$. The average *RQ* values are considered within normal range when $RQ = \pm 0.33x$, where x is the corresponding normal copy number. For example, if the average *RQ* value is expected to be at 1, all values below 0.67 will be considered a deletion, and values above 1.33 will be considered duplication. A normal male patient will have an average *RQ* value of around 1. Female patients and controls will have an *RQ* value of

around 2. A male hemophilia A patient carrying a deletion on the X chromosome will have an average RQ value of around zero. Autosomal assays, such as in the case of HHT, will have normal RQ values around 1, with heterozygous deletions occurring around 0.5, and homozygous deletions at zero.

6.7 Troubleshooting

The amplification plot for the three replicates in each assay should be examined individually to ensure the correct placement of the threshold, and also that the average RQ value is a true representation of the three experiments. Any outliers can be omitted to obtain a more true RQ average if necessary. However, if at least two out of the three experiments do not match, the assay must be repeated for that patient. The negative controls should also be examined to ensure that there are no amplification lines. If amplification lines occur between cycles 36 and 40 in one or two of the negative controls, it is most likely due to an artifact such as dust. If amplification is present in all three negative controls or earlier than 36 cycles, the reactions must be repeated due to possible assay contamination.

All copy number real-time assays should be aliquoted from the original stock tube and kept frozen hidden from light. In the event of an assay-specific problem, such as all patients on a given plate consistently showing a high or low RQ value with respect to the expected normal copy number, the assay should be repeated using a new aliquot. The particular assay used could have been old, overexposed to light, or experienced too many freeze-thaw cycles.

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Chapter 7

Reporting Clinical Molecular Genetic Laboratory Results

Victor Wei Zhang and Lora J.H. Bean

Keywords Molecular genetics • Laboratory results • Laboratory report • Genetic testing • Results • Interpretation • Mutation • Clinical Laboratory Improvement Amendments • College of American Pathologists • American College of Medical Genetics • American Board of Medical Genetics

7.1 Introduction

The accuracy and comprehensibility with which a molecular genetics test result is reported are as important as the result itself. The goal of molecular genetic testing is to provide a personalized medical service for individuals with features of or at risk for genetic diseases. A molecular test result can be used to confirm a diagnosis, assist in a differential diagnosis, guide disease management, provide information vital for reproductive decisions, and assess risk for other family members.

As of early 2011, the online catalog of human genetic disorders, *Mendelian Inheritance in Man* (OMIM), contained records on more than 20,000 genetic diseases. Currently, more than 12,000 genetic loci spread across the 22 human autosomes, the X and Y chromosomes, and the mitochondrial genome have been implicated in human disease (www.ncbi.nlm.nih.gov/Omim/mimstats.html). Given the impact and complexity of genetic diseases, the interpretation of molecular results requires board-certified experts with a broad range of genetics knowledge, clinical experience, and exceptional skill to manage such highly complex diagnostic challenges.

The purpose of this chapter is to outline the necessary components of a clinical molecular genetics report, based on current federal regulations and professional guidelines. The examples provided here, although not exhaustive, are meant to represent a sufficiently broad range of techniques and testing scenarios as to be adaptable to most situations.

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7.2 Compliance with Regulatory Agencies

To issue a clinical laboratory report, a laboratory must hold a Clinical Laboratory Improvement Amendments (CLIA) Certificate in accordance with federal requirements (details can be found at www.cms.gov/clia). CLIA certification is a minimal standard, and laboratories often hold additional licensure and accreditation. Accreditation by the College of American Pathologists (CAP; www.cap.org), which is widely regarded as an indication of laboratory quality and achievement, is sought by many molecular genetics laboratories. Additional licensure is offered by some states to laboratories that demonstrate compliance with additional guidelines. Notably, the state of New York has an extensive program that licenses clinical laboratories within New York, as well as laboratories that receive clinical samples from New York State residents (described at www.wadsworth.org).

Molecular genetics laboratory reports must include the name (or other unique patient identifier), the name and address of the testing laboratory, the report date, the specimen source, the test result and interpretation, or notification of suboptimal sample condition to comply with CLIA regulations (42CFR493.1291). Accrediting bodies, such as CAP, and professional organizations, such as the American College of Medical Genetics (ACMG; www.acmg.net/StaticContent/SGs/Section_G_2010.pdf), as well as the Centers for Disease Control and Prevention [1], have issued guidelines for reporting molecular genetics results.

To clearly establish that a genetic test has been performed in a clinical laboratory, the name of the laboratory and the laboratory's CLIA certificate number and, if applicable, CAP accreditation number should be provided on the report.

CLIA requirements, CAP, and other regulatory entities also specify the qualifications necessary for laboratory directors. Although there is variability in some qualifications, generally, a laboratory director must hold an M.D., Ph.D., or equivalent degree and be board-certified by an appropriate medical board, such as the American Board of Medical Genetics (ABMG), the American Board of Pathology (ABP), or the American Board of Clinical Chemistry (ABCC). The name and credentials of the laboratory director responsible for the report should appear on it.

Although some genetic tests have been approved by the Food and Drug Administration (FDA), many have not. As stated in the Code of Federal Regulations (21 CFR 809.30), a laboratory reporting a result from an in-house-validated assay must include this statement: "This test was developed and its performance characteristics determined by the (Name of Laboratory). It has not been cleared or approved by the Food and Drug Administration (FDA). FDA approval is not required for clinical use of the test, and therefore validation was done as required under the requirements of the Clinical Laboratory Improvement Act of 1988." The necessity of having FDA approval for genetic tests is a subject of current debate, and regulations are likely to change in the future.

7.3 Patient Information and Demographics

A laboratory report must clearly identify the individual being tested. Name and other identifiers, such as a medical record number, must be presented on the laboratory report as they were reported to the testing laboratory. Other useful information includes date of birth, sex, age, race/ethnicity, referring diagnosis, clinical information, previous test results, and the referring healthcare provider.

Depending on the test being performed, demographic information can have a major impact on the interpretation of genetic test results. For example, mutations in the *CFTR* gene cause cystic fibrosis (OMIM: 219700), an autosomal recessive chronic disease of the lung and endocrine system. The calculated residual carrier risk after testing for a panel of common *CFTR* gene mutations varies greatly for individuals of Caucasian descent versus individuals of African or Asian descent [2]. Knowing an individual's ancestry can guide interpretation of negative test results and recommendations for further testing.

Clinical information can also help to tailor an interpretation to an individual. For instance, knowing whether an individual being tested for an autosomal recessive disease is at risk for being a carrier or has clinical features of the disease substantially changes the interpretation of the result if one gene mutation is identified; in the case of a potential carrier, carrier status has been established, and no further testing is necessary, but in a possible patient, a diagnosis has not been established, and additional testing may be indicated.

7.4 Test Description

The complexity of a test description may range from stating only the gene name or disease being tested for to a full paragraph that includes gene name, disease name, brief description of the disease, and mode of inheritance. A test description need not be lengthy but, at minimum, should give sufficient information using official disease names to direct a provider to additional resources. Comprehensive reviews of Mendelian genetic disorders can be found using such resources such as OMIM (www.ncbi.nlm.nih.gov/omim) or GeneReviews (www.ncbi.nlm.nih.gov/books/NBK1116/).

Gene/Loci. A laboratory report should clearly state what test is being performed using official gene and disease names. Genes should be referred to as defined by the HUGO Gene Nomenclature Committee (HGNC; www.genenames.org/index.html). The mission of the HGNC is to assign a unique gene name to each human gene. Such standardization is critical to avoid confusion over which gene is being referred to. For example, the *PKHD1* gene, mutations in which cause autosomal recessive polycystic kidney disease (OMIM: 263200), has been historically referred to as *ARPKD*, *FCYT*, *TIGM1*, *FLJ46150*, and *DKFZp686C01112*. More confusingly, both *CPT1A* [3, 4] and *CPT2* [5, 6], two unlinked genes associated with autosomal recessive forms of long chain fatty-acid oxidation deficiency (OMIM: 255120 and 255210), as well as *CHPT1* [7, 8], the gene encoding choline phosphotransferase 1, have all been historically called *CPT1*.

Stating the gene or genes being tested for is important when a genetic disorder can be caused by mutations in different genes. For example, a molecular test for Lynch syndrome (OMIM: 120435), a form of hereditary nonpolyposis colon cancer, could include testing for the *MLH1*, *MLH2*, *MSH6*, or the *PMS2* gene, or some combination of these genes.

Mode of Inheritance. Stating the mode of inheritance early in a report can help a reader interpret the findings. For an autosomal dominant disease, the identification of one mutation can confirm a diagnosis, whereas for an autosomal recessive disease, identifying one mutation cannot confirm a diagnosis but could instead establish carrier status. For X-linked disorders, identification of one mutation means that no normal sequence is present in a male, whereas in a female, one mutation indicates the presence of one normal and one mutant chromosome.

Test Being Performed. The test being performed should be clearly stated. Tiered testing strategies are appropriate for some disorders, but test methodology and detection rate vary greatly with the test being performed. For example, the detection rate for Angelman syndrome (AS; OMIM: 105830) varies depending on the methodology used [9]. Deletions of 15q11.2-q13 detectable by FISH or array CGH account for approximately 70% of cases of AS; however, methylation defects, caused by not only deletions but also uniparental disomy for chromosome 15 and imprinting center mutations, detectable by Southern blotting or methylation-sensitive PCR, account for approximately 80% of AS cases. Mutation of the *UBE3A* gene causes approximately 10% of cases of AS. Note that although *UBE3A* gene sequencing is a test for Angelman syndrome, most disease-causing mutations will not be detected by this method. Knowing an individual has had a test for Angelman syndrome that did not detect a mutation is meaningful only if the test performed (deletion detection, methylation testing, *UBE3A* gene sequencing) is also known.

For some disorders, one or more mutations are common in the population. For example, a panel of common mutations in the *CFTR* gene will identify a large percentage of cystic fibrosis alleles [10]. Although testing using a mutation panel is often appropriate and commonly used, full *CFTR* gene sequencing will detect more gene alterations, making it important to specify what test has been performed.

7.5 Methodology

A clear, succinct summary of the methodology used for molecular testing is critical to understanding the intended scope and limitations of an assay. Useful elements of a methodology section vary depending on the test performed. Examples are given in Table 7.1.

Table 7.1 Considerations for the results, interpretation, methodology, and limitations sections of a molecular genetics laboratory report based on the assay used

Report elements	Results and interpretation	Methodology	Statement of limitations
<i>Full gene sequence analysis</i>	<ul style="list-style-type: none"> • Nucleotide changes described using standard nomenclature • Classification of nucleotide changes (i.e. mutation, variant of unknown significance, benign variant) • Justification for classification of nucleotide changes with references (if applicable) • Copy number of nucleotide change 	<ul style="list-style-type: none"> • Gene name • Reference sequence (NM_#) • Assay description 	<ul style="list-style-type: none"> • Large deletion or duplication cannot be detected • Limited to coding and immediate flanking regions
<i>Targeted mutation analysis</i>	<ul style="list-style-type: none"> • Brief statement of result • Table of mutations targeted • Estimated detection rate, if applicable 	<ul style="list-style-type: none"> • Gene name • List of mutations tested • Assay description 	<ul style="list-style-type: none"> • Only targeted mutations detected
<i>Fragment analysis</i>	<ul style="list-style-type: none"> • Size of alleles detected • Reference range for interpretation of allele size • Interpretation 	<ul style="list-style-type: none"> • Assay description • Reference 	<ul style="list-style-type: none"> • Other types of mutations within the gene will not be detected
<i>Southern Blot analysis</i>	<ul style="list-style-type: none"> • Deletion or duplication • Size of event • Location of breakpoints relative to gene structure (i.e. exon, intron) 	<ul style="list-style-type: none"> • Restriction enzyme • Method 	<ul style="list-style-type: none"> • Small changes may not be detected • Point mutations are not detected
<i>Array CGH</i>	<ul style="list-style-type: none"> • Deletion or duplication • Size of event • Location of breakpoints relative to gene structure (i.e. exon, intron) 	<ul style="list-style-type: none"> • Array design • Genome build used as reference 	<ul style="list-style-type: none"> • The location of duplicated regions cannot be determined • Some small and complex rearrangements cannot be detected (inversion, insertion)

A notable example of a mutation type for which methodologies have changed dramatically since clinical testing became available are the methods used to detect large gene deletions and duplications. Available methodologies vary widely in their detection rates and technical limitations. Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are caused by mutations, most frequently deletions and duplications, in the X-linked *DMD* gene (OMIM: 310200 and 300376). Traditionally used multiplex PCR and Southern blotting methods [11] have a lower resolution than more recently described methods, such as multiplex ligation-dependent probe amplification (MLPA) [12, 13] or gene-targeted array comparative genomic hybridization (CGH) [14], particularly when testing for deletion or duplication mutations in females. When an individual with clinical features of DMD/BMD has had negative *DMD* deletion and duplication gene testing, the testing methodology must be known to accurately determine their residual risk for having a *DMD* gene mutation.

7.6 Limitations

Every molecular method has intended uses and limitations (see Table 7.1 for examples). A statement of limitations may be most appropriately given in the test description (e.g., mutations in other genes will not be detected), the methodology (e.g., a test for a targeted mutation panel will not detect other mutations), or the interpretation (e.g., CGH cannot be used to determine the location of a duplication). Clearly communicating the limitations of an assay is just as critical as communicating a result, thus careful consideration should be given to the location and content of a limitations statement.

7.7 Results

Results should be brief, clear, and concise, beginning with whether the result is normal, abnormal, of unknown significance, inconclusive, or incomplete. Precision in wording is critical.

Standardization is also important. When describing DNA sequence changes, whether detected by a targeted assay or full gene sequencing, use of standard nomenclature is critical to uniformly and unambiguously describe DNA variants at the sequence level. Guidelines for naming of sequence variants are available through the HGVS (www.hgvs.org/mutnomen/recs.html). In situations where historical mutation names are well known, for example, the *CFTR* common mutation “deltaF508,” the historical as well as standard name may be given (in this example, c.1521_1523delCTT; NCBI reference NM_000492). When reporting nucleotide changes, stating that a heterozygous (one copy), homozygous (two copy), or hemizygous (one copy on the X chromosome in a male) nucleotide change was observed is better than simply stating that a change was detected.

For repeat expansion disorders, the accuracy with which an allele size can be determined depends on methodology. When an allele size can be accurately determined by fragment analysis, as is critical in differentiating normal, mutable normal, HD with reduced penetrance, and HD alleles in Huntington disease [15], the exact allele size should be given. However, when large allele expansions are detected by Southern blot analysis, as is typical of full CGG expansions in individuals affected with fragile X syndrome (OMIM: 300624), an estimate of allele size consistent with precision of the assay should be given (~1,500 CGG repeats is more accurate than 1,479 CGG repeats since a Southern blot does not have sufficient resolution to determine exact size in the expanded range).

7.8 Interpretation

An interpretation of the results should be given. The expertise offered by a board-certified laboratory director is critical for effective use of the result by a healthcare provider. The purpose of the interpretation is to place the patient's results in the context of expected normal and abnormal results. As appropriate, normal and abnormal reference ranges should be given. References from the literature should be provided, as needed, to support the interpretation.

A summary statement should also be given. When there is no mutation detected, determining the implications for the patient depends upon understanding the limitations of the test; therefore, limitations should be clearly stated. When a test result unequivocally establishes carrier or affected status, this should be clearly stated. Just as importantly, results of uncertain significance should be identified as such. In many cases, references to the peer-reviewed literature or mutation databases are necessary to justify interpretive statements.

For many disorders, interpretation guidelines have been published by experts in the field. Members of the American College of Medical Genetics (ACMG) have published guidelines for the interpretation of repeat expansion disorders, for example, fragile X syndrome [16, 17] and Huntington disease [15], the interpretation of nucleotide changes detected by sequencing [18, 19], and others (www.acmg.net).

7.9 Recommendations

The results of a molecular genetic test can be complex and have significant repercussions for the individual being tested, as well as other family members who have not been tested. When a gene mutation is detected, targeted molecular carrier or diagnostic testing, depending on the mode of disease inheritance, becomes available to other relatives. When a variant of unknown clinical significance is identified, for example, by sequence analysis, further studies of other relatives may be indicated. For example, if a variant is discovered in an X-linked gene in a male, testing his mother to determine whether the change was inherited or occurred *de novo* can provide important information for the interpretation. Similarly, testing both parents of a proband to demonstrate that two sequence variants in a gene associated with an autosomal recessive disorder are *in trans* yields information helpful for the interpretation. Since many genetic disorders are rare, the referring physician may have little experience with the disorder being tested for. A recommendation for genetic counseling or contacting the laboratory for more information is often appropriate.

7.10 Summary

Clear, concise, accurate reporting of molecular genetics laboratory results is vital to proper care and counseling for patients and their families. Compliance with federal and state regulations and professional guidelines can help to ensure quality reporting of genetic results. As the use of genetic testing in medicine becomes more common by both genetics and nongenetics health professionals, continued assessment of reporting standards, such as those already being undertaken [20–22], will help improve current practices.

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Part II
Clinical Molecular Infectious Disease Testing

Chapter 8

HIV-1 Genotyping and Virtual Phenotyping*

Fangling Xu and Jianli Dong

Keywords HIV-1 • Drug resistance testing • Genotyping • Virtual phenotyping

8.1 Introduction

Human immunodeficiency virus (HIV) is the etiologic agent responsible for the development of acquired immune deficiency syndrome (AIDS), a chronic disease leading to immunodeficiency and susceptibility to opportunistic infections. HIV is a member of the genus *Lentivirus* belonging to the family *Retroviridae*. HIV is transmitted as single-stranded, positive-sense, enveloped RNA virus. There are two species, HIV-1 and HIV-2. HIV-1 is the cause of the great majority of HIV infections worldwide. Sequence similarity further classifies HIV-1 into groups, subtypes, and sub-subtypes. Three groups of HIV-1 have been identified, including M (main), O (outlier), and N (non-M/non-O) [1]. Of the three groups of HIV-1, group M dominates the global epidemic and is further classified into subtypes A, B, C, D, F, G, H, J, and K. In addition, circulating recombinant forms (CRFs), mosaic viruses formed between subtypes during co- or superinfection, have also been recognized (<http://www.hiv.lanl.gov/>).

HIV-1 is spread by sexual contact, exposure to infected blood or blood products, and perinatal transmission. Acute HIV-1 syndrome develops approximately 3–6 weeks after initial infection and is associated with high levels of viremia. Following seroconversion, viremia declines dramatically, and most patients enter a phase of apparent latency that may continue for years. Depletion of peripheral blood CD4+ T-cells, the primary target for the virus, occurs during this clinically latent phase, sometimes without large increases in plasma viral loads. Clinically apparent disease or an AIDS-defining illness, coupled with severe immunosuppression, eventually results. HIV diagnostics have been essential in detecting and monitoring infection, and continue to play a major role in saving lives throughout

*Disclaimer: The contents of this chapter represent modified ViroSeq protocol which has been validated at the Department of Pathology, University of Texas Medical Branch

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the world [2]. The extreme replication dynamics of HIV facilitates its escape from the selective pressure exerted by the human immune system and by the applied drug therapy. Viral replication continues in the presence of the selective drug(s). This is called drug resistance, and it is one of the most common reasons for failure of HIV therapy.

Drug resistance testing is recommended for the management of patients with HIV-1 infection (<http://www.aidsinfo.nih.gov/guidelines/>). The ViroSeq HIV-1 Genotyping System (Celaera Diagnostics, Alameda, CA, USA): ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) (ViroSeq) is routinely used clinically in detecting mutations in the protease and reverse transcriptase (RT) that confer resistance to specific types of antiretroviral drugs. This assay is cleared by the US Food and Drug Administration (FDA) and is CE marked for use in detecting HIV-1 subtype B viral resistance in plasma samples with a viral load ranging from 2,000 to 750,000 copies/mL. The assay has also been used in testing HIV-1 non-B subtype sequences and samples with viral load of less than 2,000 copies/mL [3–7].

8.2 Methodology

Materials Provided in ViroSeq™ HIV-1 Genotyping System 2.0 Assay Kit [8].

Pack 1

Boxes	Component	Quantity	Storage (°C)
Sample preparation module	HIV Viral Lysis Buffer	2 × 14.4 mL	–15 to –20
	RNA diluent	3 × 1.6 mL	–15 to –20
RT-PCR module	HIV RT Mix	1 × 384 µL	–15 to –20
	RNase Inhibitor	1 × 48 µL	–15 to –20
	MuLV Reverse Transcriptase	1 × 48 µL	–15 to –20
	DTT (100 mM)	1 × 20 µL	–15 to –20
	HIV PCR Mix	1 × 1.42 mL	–15 to –20
	AmpliTaq Gold DNA polymerase	1 × 24 µL	–15 to –20
	AmpErase UNG	1 × 48 µL	–15 to –20
	RNA diluent	1 × 1.6 mL	–15 to –20
	DNA mass ladder	1 × 36 µL	–15 to –20
	Agarose gel loading buffer	1 × 240 µL	–15 to –20
Sequencing module	HIV SEQ Mix A	1 × 576 µL	–15 to –20
	HIV SEQ Mix B	1 × 576 µL	–15 to –20
	HIV SEQ Mix C	1 × 576 µL	–15 to –20
	HIV SEQ Mix D	1 × 576 µL	–15 to –20
	HIV SEQ Mix F	1 × 576 µL	–15 to –20
	HIV SEQ Mix G	1 × 576 µL	–15 to –20
	HIV SEQ Mix H	1 × 576 µL	–15 to –20
8E5 control module	HIV-1 8E5 positive control	5 × 500 µL	–15 to –20
	HIV-1 8E5 negative control	5 × 950 µL	–15 to –20

Pack 2

Component	Quantity	Storage (°C)
MicroAmp tubes with caps	96 × 2 each	15–30
MicroAmp optical 96-well plate and covers	5 × 2 each	15–30

Pack 3

Component	Quantity	Storage (°C)
PCR cleanup reagent	310 µL	<-15

Materials and equipment required but not provided in HIV-1 RNA 2.0 Assay Kit

- ABI PRISM 3130xl Generic Analyzer with Data Collection software
- PC computer with:
- Microsoft Windows NT v4.0
- 550 MHz Intel Pentium III processor
- 256-MB RAM
- 18-G hard drive
- ABI PRISM Sequencing Analysis software for NT computer
- Thermal cycler, GeneAmp PCR System 9700
- 3,100 capillary array, 50 cm
- 96-well plate base
- 96-well plate retainer
- 96-well plate septa
- MicroAmp base and trays/retainers
- Reservoir septa
- Polymer-reserve glass syringe, 5 mL
- Array fill glass syringe, 250 µL
- O-rings
- ABI 3130xl POP-6 Performance Optimized Polymer
- Genetic Analyzer buffer with EDTA (10×)
- ABI PRISM BigDye Terminator Sequencing Standard
- Hi-Di formamide, 25-mL bottle
- Agarose gel, containing ethidium bromide
- TBE buffer, 10×
- Agarose gel running system
- BioSafety Hood, Laminar Flow
- Centrifuge, refrigerated (21,000–25,000× g) with hermetical-sealed rotor
- Vortex mixer, platform head (2,500 rpm)
- Pipettor, 200–1,000 µL
- Pipettor, 20–200 µL
- Pipettor, 10–100 µL
- Pipettor, 0–20 µL
- Pipettor, 0–10 µL
- Aerosol-resistant tips, RNase-free, 1,000 µL
- Aerosol-resistant tips, RNase-free, 200 µL
- Aerosol-resistant tips, RNase-free, 100 µL
- Aerosol-resistant tips, RNase-free, 20 µL
- Aerosol-resistant tips, RNase-free, 10 µL
- Transfer pipettes, fine-tip
- Screwtop tubes, 1.5 mL (sterile, RNase/DNase-free). Must with stand 21,000–25,000× g
- Gloves, powder-free
- Lab coat/gowns
- Bleach (for cleaning – make a 10% solution prior to use)
- 70% Ethanol (for cleaning)

- 100% Ethanol, non-denatured (Molecular Biology grade)
- 100% Isopropanol, anhydrous (ACS grade)
- Water, sterile, deionized, RNase/DNase-free
- 8-channel pipette capable of dispensing 50–200 μL and 50 mL sterile-packaged, disposable reservoirs
- One four-channel timer or four timers

8.2.1 *Required Controls*

Three controls, high positive control, low positive control, and negative control, must be included with every assay. Treat all control samples the same as specimens. The assay is considered valid if all the following conditions occur:

1. After RT-PCR, the high positive control gets a strong band (>40 ng/5 μL), the low positive control gets a weak band (>20 ng/5 μL), and negative control gets no band.
2. After genotyping, the positive control gets the following expected genotyping results:
 - All seven primers assemble
 - No drug resistance mutations
 - Two novel mutations
 - Protease V31
 - RT L214F
 - No mixed bases

8.3 Protocols

8.3.1 *Sample Preparation (RNA Extraction)*

8.3.1.1 Specimen Type and Handling

1. Collect blood in sterile tubes containing EDTA (K3, 0.15% solution v/v final; or K2 spray coated 1.8 mg/mL final; or K2 Plasma Preparation Tubes (PPT), or ACD solution A, 17.6% solution v/v final).
2. Store whole blood at room temperature (18–30°C) for up to 2 h or according to collection tube manufacturer's instructions. Do not refrigerate.
3. Remove plasma from cells within 4 h of collection. Separate by centrifugation at $1,000 \times g$ for 10–15 min. Do not clarify plasma by filtration or further centrifugation.
4. Samples collected in K2 PPT tubes should be frozen immediately after centrifugation or held at room temperature for up to 24 h after centrifugation. Do not store tubes at 2–8°C after centrifugation prior to freezing.
5. Store plasma at -60°C to -80°C in sterile, screw-capped tubes if viral pellets are not prepared within 30 min of separation. EDTA and ACD solution A plasma may be stored frozen at -60°C to -80°C . K2 PPT tubes may be stored at room temperature for up to 24 h before freezing at -60°C to -80°C . Plasma may also be frozen at -20°C in a non-frost-free freezer for up to 72 h if a -60°C to -80°C freezer is not available.
6. Do not freeze-thaw the plasma more than two times.

8.3.1.2 RNA Extraction

1. Precool a refrigerated centrifuge and rotor to 2–8°C.
2. Thaw plasma samples and one tube each of the positive and negative controls at room temperature (15–25°C).

Note: Keep only one reagent or sample tube open at a time.

3. Vortex the plasma samples 3–5 s to mix.
4. Centrifuge 1–2 s at 2,000 × g to collect the contents at the bottom of the tube.
5. Label tubes according to your laboratory protocol, then aliquot 0.5 mL of the plasma into a fresh, RNase- and DNase-free screwtop tube.
6. Prepare a low viral RNA control.
7. Combine in a microfuge tube:
 - 50 µL 8E5 positive control
 - 450 µL negative control (negative plasma) and label appropriately.
8. Place an orientation mark on the side of the tube and cap.
9. Insert the samples in the rotor with the orientation mark facing the outside rim of the rotor.
10. Centrifuge at 21,000–25,000 × g for 60 min at 2–8°C.
11. While the samples are centrifuging:
 - Thaw the Viral Lysis Buffer at room temperature (RT).
 - Thaw the RNA diluent and store it at 2–8°C.
 - Prepare the 70% ethanol and store it at 2–8°C for use in step 23.
12. Remove the tubes immediately after the rotor stops moving.
13. Carefully remove the supernatant from the tubes, using a fine-tip transfer pipette.

Note: Do not disturb the pellet (note that pellet may not be visible).

14. Add 600 µL of Viral Lysis Buffer to each pellet.
15. Vortex each tube for 3–5 s to mix.
16. Centrifuge 1–2 s at 2,000 × g to collect the contents at the bottom of the tube.
17. Let the samples sit at room temperature (15–25°C) for 10 min to ensure complete lysis of the virus.
18. Add 600 µL of isopropanol to each sample.
19. Vortex each tube for 3–5 s to mix.
20. Insert the samples in the rotor with the orientation mark facing the outside rim of the rotor.
21. Centrifuge the samples 12,500–15,000 × g at room temperature for 15 min.
22. Carefully remove the supernatant with a fine-tip transfer pipette. Do not disturb the pellet (which may not be visible).
23. Add 1 mL of 70% ethanol (prepared at step 11) to each tube.
24. Vortex each tube for 3–5 s to mix.
25. Insert the tubes in the rotor with the orientation mark facing the outside rim of the rotor.
26. Centrifuge 12,500–15,000 × g at room temperature for 5 min.
27. Carefully remove any residual ethanol with another fine-tip transfer pipette.
28. Centrifuge 1–2 s at 2,000 × g to collect the residual fluid at the bottom of the tube.
29. Carefully remove any residual ethanol with another fine-tip transfer pipette. Air-dry the tubes, with the caps off, for 5–10 min.
30. Resuspend each pellet in 50 µL cold (2–8°C) RNA diluents.
31. Vortex vigorously for 10 s to resuspend the pellet. Some insoluble materials may remain.

32. Centrifuge 1–2 s at $2,000 \times g$ to collect contents at the bottom of the tube.
33. When the procedure is complete:

- Proceed to the reverse transcription steps, or
- Stop and store the samples at -65°C to -80°C .

Note: Do not freeze-thaw the RNA more than two times. Do not store the RNA for more than 2 weeks.

8.3.2 Amplification

8.3.2.1 RT-PCR

Reverse transcription

1. If necessary, thaw the RNA samples. Be sure to also thaw the positive and negative control RNAs.
2. Vortex for 3–5 s to mix.
3. Label one 0.2-mL MicroAmp reaction tube for each RNA sample.
4. Thaw the HIV RT Mix and DTT. Vortex for 3–5 s to mix.
5. Remove the RNase Inhibitor and MuLV Reverse Transcriptase from kit.
6. Centrifuge all the reagent tubes and samples for 1–2 s at $2,000 \times g$ to collect the contents at the bottom.
7. Store all reagents and samples at $2-8^{\circ}\text{C}$ until use.
8. Prepare the RT Mastermix according to the following table. When finished, return the stock solutions to -15°C to -25°C .

Reagent	Volume for 1 reaction (μL)	Volume for 5 reactions (μL)	Volume for 15 reactions (μL)
HIV RT Mix	8	40	120
RNase Inhibitor	1	5	15
MuLV Reverse Transcriptase	1	5	15
DTT, 100 mM	0.4	2	6
<i>Final volume</i>	<i>10.4</i>	<i>52.0</i>	<i>156.0</i>

Note: Prepare sufficient volume for 1–2 extra reactions to compensate for pipetting loss.

9. Vortex the RT Mastermix for 2–3 s to mix.
10. Centrifuge 1–2 s at $2,000 \times g$ to collect the contents at the bottom of the tube.

Note: Keep the RT Mastermix at room temperature until you add it to the reaction tubes (≤ 30 min).

11. Add 10 μL of viral RNA to 0.2-mL MicroAmp reaction tubes and return the stock viral RNA to ice.
12. Immediately cap and place the MicroAmp reaction tubes into a thermal cycler that has been set to the conditions below, and begin the program. Allow the samples to ramp, with the instrument, to 65°C . While the RNA is heating, leave the RT Mastermix at room temperature.

Note: Pause the run after the 42°C , 5 min step and proceed to step 13.

Stage	Cycle number	Temp (°C)	Time	Process
Stage 1	1	65	30 s	Relaxes the RNA secondary structure
Stage 2	1	42	5 min	Cools to the optimal enzyme activity temperature
Manually pause and perform step 13, then press Resume				
Stage 3	1	42	60 min	Reverse transcription
Stage 4	1	99	5 min	Inactivates MuLV Reverse Transcriptase
Stage 5	1	4	Hold (>10 min)	Holds until you are ready to proceed

Note: Set the volume on the thermal cycler to 20 µL when prompted.

13. While the instrument is paused, perform the following:

- Immediately remove the RNA samples from the thermal cycler.
- Add 10 µL of the room temperature RT Mastermix to each reaction tube, and cap the tubes.
- Vortex the tray with samples for 3–5 s to mix.
- Centrifuge the sample tray for 1–2 s to at 2,000 × g to collect the contents at the bottom.

14. Add samples to the thermal cycler and press Resume.

15. After completion of the RT program, hold the samples in the thermal cycler for at least 10 min at 4°C, but do not exceed 18 h.

16. When the program is complete:

- Proceed to the PCR steps, or
- Stop and store the samples at –15°C to –25°C until you are ready to perform the PCR, but do not store for more than 2 weeks.

8.3.2.2 PCR

Note: Perform this procedure in different areas to avoid contamination.

1. If the samples are frozen, thaw them at room temperature (15–25°C).
2. Centrifuge the sample tray or tubes for 1–2 s at 2,000 × g to collect the contents at the bottom.
3. Thaw the HIV PCR Mix.
4. Vortex for 3–5 s to mix.
5. Remove the AmpliTaq Gold DNA polymerase and AmpErase UNG reagents from the kit. Thaw and vortex for 1–2 s.
6. Centrifuge all reagents for 1–2 s at 2,000 × g to collect the contents at the bottom.

Note: Use only the GeneAmp 9600 or 9700 thermal cyclers.

7. Prepare the PCR Mastermix.
8. Combine in a sterile 1.5-mL microcentrifuge tube:

Reagent	Volume for 1 reaction (µL)	Volume for 5 reactions (µL)	Volume for 15 reactions (µL)
HIV PCR Mix	29.5	147.5	442.5
AmpliTaq Gold DNA polymerase	0.5	2.5	7.5
AmpErase UNG	1	5	15
<i>Final volume</i>	<i>31</i>	<i>155</i>	<i>465</i>

Note: Prepare sufficient volume for 1 extra reaction to compensate for pipetting loss.

9. Vortex the PCR Mastermix for 3–5 s to mix.
10. Centrifuge 1–2 s at $2,000 \times g$ to collect the contents at the bottom of the tube.
11. Add 30 μL of PCR Mastermix to each RT reaction tube containing HIV-1 cDNA. The final volume is now 50 μL . Cap the tube.

Stage	Temperature ($^{\circ}\text{C}$)	Time	Cycles
1	50	10 min	1
2	93	12 min	1
3	93	20 s	40
	64	45 s	
	66	3 min	
4	72	10 min	1
5	4	Hold (<24 h)	–

12. Proceed with the samples to the thermal cycler in area 3.
13. Transfer the tubes to the thermal cycler and start the program.
14. When the program is complete:
 - Proceed to the purification steps, or
 - Stop and store the samples at -15°C to -25°C .

8.3.3 Quantifying the DNA

1. Thaw the DNA mass ladder and agarose gel loading buffer at room temperature (15 – 25°C). This will take at least 30 min. Vortex 10–15 s to mix before using.
2. Prepare a $1\times$ TBE gel buffer solution.
3. Prepare a 1% agarose gel by mixing 50 mL of $1\times$ TBE gel buffer, 0.5 g agarose, and 5 μL (1/10,000) gel red.
4. Place the prepared gel in the gel box.
5. Add enough $1\times$ TBE gel buffer to cover the gel.
6. For each sample, mix:
 7. 5 μL of agarose gel loading buffer
 8. 5 μL of PCR product
9. Load the DNA mass ladder solution.
 - 6 μL in Lane 1
 - 3 μL in Lane 2

This gives the following results:

Bands			
Location	Size (kb)	6 μL Lane (ng)	3 μL Lane (ng)
<i>Top</i>	2.0	100	50
<i>Second</i>	1.2	60	30
<i>Third</i>	0.8	40	20

10. Load the remaining lanes with 10 μL each of samples.
11. Electrophorese at 10 V/cm until the bromophenol blue has migrated at least 5 cm into the gel.

12. Examine the gel with UV light.
13. Photograph your gel using an exposure time that does not saturate the film and shows the difference in intensity of the mass ladder fragments.
14. Evaluate the quantity of PCR products in each sample by comparing the intensity of each band to the intensities of the low mass ladder bands.

8.3.3.1 To Proceed, Negative Control Needs to Have No Sign of Contamination

Note: Do not dilute at this step.

15. Proceed to purification of PCR products with ExoSAP-IT for samples determined to have intensity equal to or greater than the 20-ng mass ladder band.

Note: The RT-PCR product band must have intensity equal to or greater than the 20-ng mass ladder band to ensure a high-quality sequence.

8.3.3.2 Purifying the PCR Products

Note: Use only the GeneAmp® 9700 thermal cycler.

Note: Program the thermal cycler prior to the reaction setup (refer to step 3). Please refer to the thermal cycler operation manual for detailed programming instructions.

1. Carefully pipette 3 μL of ExoSAP-IT® into each tube that contains 45 μL of PCR product with band intensity greater than or equal to 20 ng/5 μL .
2. Cap the tubes. b. Vortex the tubes or tray with sample for 3–5 s to mix. c. Centrifuge the tubes or sample tray for 1–2 s at $2,000 \times g$ to collect the contents at the bottom. The final volume is now 48 μL .
3. Program the thermal cycler using the following program:

Temperature ($^{\circ}\text{C}$)	Time	Cycles
37	15 min	1
80	15 min	1
4	Hold	<24 h

Note: Set the reaction volume on the thermal cycler to 48 μL when prompted.

4. Transfer tubes or tray with samples to the thermal cycler and start the program.
5. When the program is complete, proceed to the dilution steps.

8.3.3.3 Dilution of Purified Product for Cycle Sequencing

1. Dilute the ExoSAP-IT-purified PCR product for each sample with deionized, distilled water (ddH₂O) according to Table 8.1.
2. Cap and vortex each diluted sample for 3–5 s to mix.
3. Centrifuge 1–2 s at $2,000 \times g$ to collect the contents at the bottom of the tube.
4. When the procedure is complete, proceed to “Cycle Sequencing.”

Table 8.1 Dilution of purified product for cycle sequencing

If the band intensity is...	Then...
20–40 ng	Adjust the sample volume to 60 μ L
40–60 ng	Make a 1:2 dilution of your sample with ddH ₂ O (1 part sample and 1 part water)
60–100 ng	Make a 1:4 dilution of your sample with ddH ₂ O (1 part sample and 3 parts water)
>100 ng	Make a 1:10 dilution of your sample with ddH ₂ O (1 part sample and 9 parts water)

8.3.3.4 Cycle Sequencing

Note: The sequencing mixes are light-sensitive. Do not expose the mixes to the light for extended periods. This step should also take place in another separate area.

To prepare the cycle sequencing reaction

1. Thaw the HIV-1 sequencing mixes at room temperature (15–25°C).
2. A MicroAmp optical 96-well reaction plate (provided with the kit) will be used.
3. A 96-well plate loaded with sample 1 (S1) beginning in column 1 of the 96-well plate, S2 in column 2, and so forth. Row H is not used unless you want to run 13 samples on one plate.
4. In each tube or well, combine as follows:

Component	Volume for one reaction (μ L)
Add one of the following HIV-1 SEQ mixes to each tube or plate well:	12
HIV SEQ Mix A	
HIV SEQ Mix B	
HIV SEQ Mix C	
HIV SEQ Mix D	
HIV SEQ Mix F	
HIV SEQ Mix G	
HIV SEQ Mix H	
Diluted, purified PCR product	8

5. Close the tube caps or place a MicroAmp optical 96-well reaction plate cover over the plate.
6. Centrifuge at room temperature for 5–10 s.
7. Transfer the samples to the thermal cycler.
8. Set the thermal cycler program as follows:

Stage	Temperature (°C)	Time	Cycles
1	96	10 s	25
	50	5 s	
	60	4 min	
2	4	Hold (<24 h)	–

9. Start the thermal cycler.
10. When the program is complete:
 - Proceed to the purification steps, or
 - Stop and store the samples at –15°C to –25°C.
 - Do not store the samples for more than 3 days at –15°C to –25°C.

8.3.3.5 Purifying the Cycle Sequencing Products

Note: Sequencing products are light-sensitive, keep from light.

1. Remove the MicroAmp tray from the thermal cycler, and remove the caps from each tube or the cover off the plate.
2. To each sample reaction, add 5 μL of 125 mM EDTA (prepared from the 0.5 M EDTA).
3. To each sample mixture, add 60 μL of 100% ethanol.
4. Seal the tubes with strip caps.
5. Mix thoroughly by vortexing.
6. Centrifuge the plate at $1,369 \times g$ for 35 min.
7. As soon as the centrifuge stops, carefully remove the caps without disturbing the pellets.
8. Immediately place an absorbent paper towel or lint-free tissue on top of the tray and invert it.
9. Place the tray in the centrifuge in the inverted position, on top of a lint-free tissue or paper towel, and centrifuge at $150 \times g$ for 30 s.
10. Add 150 μL of 70% ethanol to each well, and then seal the tubes with caps.
11. Centrifuge at $1,369 \times g$ for 10 min at room temperature.
12. As soon as the centrifuge stops, carefully remove the caps without disturbing the pellets.
13. Immediately place an absorbent paper towel or lint-free tissue on top of the tray and invert it.
14. Place the tray in the centrifuge in the inverted position, on top of a lint-free tissue or paper towel, and centrifuge at $150 \times g$ for 30 s.

Note: You must perform the inverted spin immediately after centrifugation, or there is a loss of signal.

If the plate...	Then...
Sit for more than 1 min before the inverted spin	Centrifuge it upright for 5 min at $1,369 \times g$ before performing the inverted spin

15. When the centrifuge stops, remove the plate and either:
 - Proceed to “Automated Sequence Detection,” or
 - Seal the plate with attached tape and store at -15°C to -25°C in the dark. Analyze the sample within 1 week.

8.3.4 DNA Sequencing Using ABI PRISM 3130xl Genetic Analyzer [9]

Launch 3130xl Data Collection Software and Flushing and Filling the Water Trap

1. Fill the 20 mL, all-plastic Luer lock syringe with distilled or deionized water. Expel any bubbles from the syringe.

Note: Do not use a syringe smaller than 20 mL. Doing so may generate excessive pressure within the trap.

2. Attach the syringe to the forward-facing Luer fitting at the top of the pump block. Hold the fitting with one hand while threading the syringe onto the fitting with the other hand.
3. Open the Luer fitting by grasping the body of the fitting and turning it and the attached syringe approximately one-half turn counterclockwise.
4. Open the exit fitting at the top left side of the pump block by turning it approximately one-half turn counterclockwise.

5. Hold an empty tube or beaker under the exit fitting to receive approximately 5 mL of waste. Flush the trap by pushing steadily on the syringe plunger.

Note: Do not use excessive force when pushing the syringe plunger as this may damage the trap seals. It should take approximately 30 s to flush 5 mL of either distilled or deionized water through the trap.

6. Close the fittings in this order by turning each clockwise until the fittings seal against the block:

- Luer fitting
- Exit fitting

Note: Do not over-tighten the fittings. Very little pressure develops within the trap during pump operation, so the fittings require only enough tightening to prevent water leaks. Excessive tightening can damage the fittings.

- Removing the syringe from the Luer fitting. Hold the fitting with one hand while turning the syringe counterclockwise with the other hand.

Water wash (do not need before every run)

1. Select Wizards>Water Wash Wizard.
2. Run the Water Wash Wizard to wash the PDP chamber, lower polymer block, channels, and tubing with distilled or deionized water.

Replace or Replenish the polymer

1. Select Wizard>Replenish Polymer Wizard.
2. Follow the directions given in the wizard to put fresh polymer on the instrument.

Remove air bubbles from the polymer block

1. Select Wizards>Bubble Remove Wizard to clear bubbles. Remove bubbles from the interconnect tubing and the channel of the lower polymer block. These areas are part of the electrophoresis current path. Absence of bubbles in the current path is important for problem-free electrophoresis.
2. Replace the buffer if excess polymer is expelled into the anode buffer jar.

Fill Buffer Reservoirs

Note: Applied Biosystems recommends that you change the buffer in the buffer reservoir and tube before each run.

1. Dilute 10× Genetic Analyzer buffer with EDTA with 5-mL buffer and 45 mL of ddH₂O in a 50-mL falcon tube, and mix well.
2. Fill plastic buffer container to the line with the diluted buffer, put on the septa, and place the vial in position 1 on the autosampler (buffer).
3. Fill the anode buffer reservoir to the red line with the diluted buffer, and place it on the pump block.
4. Fill another of the plastic buffer containers to the line with Milli-Q water, put on the septa, and place the vial in position 2 on the autosampler (water).
5. Fill another of the plastic buffer containers to the line with Milli-Q water, put on the septa, and place the vial in position 4 on the autosampler (waste).
6. The diluted buffer water and waste should be replaced before each run.

Perform Spatial Calibration (Do not need before every run, add when is needed)

- You are required to perform a spatial calibration when you:
- Install or replace a capillary array

- Temporarily remove the capillary array from the detection block
 - Move the instrument
1. Select **GA instruments>ga 3130x/**>instrument name>Spatial Run Scheduler.
 2. In the Spatial Protocols section, select **one of the following**:
 - If the capillaries contain fresh polymer, select Protocol>3130SpatialNoFill_1.
 - Otherwise, select Protocol>3130SpatialFill_1.

Note: The capillaries do not have to be filled each time a spatial calibration is performed.

3. Click **Start**.

Note: The spatial profile window will be displayed as black when the spatial calibration is started.

The calibration takes approximately

- 2 min without filling the capillaries
 - 6 min with filling the capillaries
4. Evaluate and save the data.
 - Evaluate the spatial calibration profile.

While viewing the calibration profile in the Details dialog box, use the following criteria to evaluate the data:

Peak attribute	Criteria
Height	Similar height for all peaks
Orange crosses	One orange cross marking the top of every peak. No misplaced crosses To move a cross: (a) Change the value in the Position (pixels) box (b) Click outside of that box (c) Click Enter using the keyboard
Shape	<ul style="list-style-type: none">• Single sharp peak for each capillary• Small shoulders are acceptable
Spacing	The difference between adjacent positions is 13–16 pixels. Theoretical spatial spacing between capillaries is 15

5. Accept or abort a spatial calibration as follows:

If the spatial calibration profile is...	Then...
Passed	Click Accept to write the calibration data to the database and .ini file
Failed	Click Reject , and then refer to “if the spatial calibration fails”

6. If the calibration fails, try one or more of the following corrective actions:

Corrective Actions

- Repeat the calibration.
- Fill the capillaries with polymer, and then repeat the calibration.
- Clean the detection cell (see ViroSeq operator’s manual page 2–10), and then repeat the calibration.
- Reposition the array window in the detection cell, and then repeat the spatial calibration.
- Try another capillary array.
- Contact an Applied Biosystems service engineer to check laser alignment. Refer to the *Applied Biosystems® 3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide*.

Perform Spectral Calibration (Do not need before every run)

- When you use a new dye set on the instrument
 - When you change capillary array length (or polymer type for fragment analysis)
 - After the laser or CCD camera has been realigned/replaced by a service engineer
 - If you begin to see a decrease in spectral separation (pull-up and/or pull-down peaks) in the raw or analyzed data
1. Resuspend a tube of the BigDye version 1.1 Terminator Sequencing Standard with 170 μL of Hi-Di formamide.
 2. Vortex the standard 10–15 s to mix well.
 3. Spin the mixture for 10–15 s at $2,000 \times g$ in a microcentrifuge.
 4. Dispense 10 μL of the standard into the MicroAmp optical 96-well plate, loading wells A1 through H2.
 5. Cover the plate and centrifuge it for 5–10 s at $2,000 \times g$.
 6. Heat the plate at 95°C for 2 min to denature the standards, and quick chill the plate on ice for 2 min.
 7. Immediately load the plate on the autosampler in the 3130xl instrument.

Note: Do not store the plate.

8. Select **GA instruments > ga 3130xl > Protocol Manager** to open the Protocol Manager window. In the Instrument Protocol pane, click **New**. **The Protocol Editor dialog box opens.**
9. Complete the Protocol Editor dialog box by entering information or making selections from drop-down lists:
 - Name: ViroSeq_spectral_instrument_protocol.
 - Description: enter a description for the protocol (optional).
 - Type: Spectral (bold no not).
 - Dye set: E-BigDyeV1.
 - Polymer: POP-6.
 - Array length: 50.
 - Chemistry: Sequencing Standard.
 - Run module: Spect50_POP6_1.
 - Click **OK**.
10. To create a spectral plate record:
 - Select **GA instruments > ga 3130xl > Plate Manager**. Click **New to open the New Plate dialog box**.
 - Complete the New Plate dialog box.
 - Name: enter a name for the plate.
 - Description: enter a description for the plate record (optional).
 - Application: Spectral Calibration.
 - Plate type: 96-well.
 - Owner name: enter a name for the owner.
 - Operator name: enter a name for the operator.
 - Click **OK**.
 - Complete the Spectral Calibration Plate Editor dialog box:
 - Sample name: enter the name for the samples.
 - Comments: enter any additional comments or notations (optional).
 - Priority: the value 100 automatically displays in the column.
 - Instrument Protocol 1: select the ViroSeq spectral instrument protocol. Refer to “To Create a ViroSeq Spectral Instrument Protocol.”
 - Click **OK**.

Note: Verify that you have selected the correct spectral instrument protocol file. Selecting the incorrect instrument protocol file will cause the spectral calibration to fail.

8.3.5 *Preparing and Loading Samples*

1. If your samples are frozen, bring them to room temperature.
2. Resuspend your samples by adding 20 μL of Hi-Di formamide to each sample well; use a fresh aliquot of Hi-Di formamide for each experiment.
3. Vortex for 10–15 s.
4. Centrifuge the plate for 30–40 s at $1,369 \times g$ to collect the liquid at the bottom of the plate.
5. Assemble the plate assembly components from upper layer to lower layer according to the following order: plate retainer, plate septa, sample plate, and plate base:
 - Secure clean and dry plate septa on the sample plate.
 - Place the sample plate into the plate base.
 - Snap the plate retainer onto the plate and plate base.
 - Ensure that the plate retainer holes are aligned with the holes in the septa strip.
 - Place the plate onto the autosampler.
 - Press the Tray button.
 - Place the plate assembly on the autosampler in position A or B.
 - When the plate is correctly positioned (position well A1 at upper-right corner), the plate position indicator on the Plate View page changes from gray to yellow.
 - Close the instrument doors.

Note: Closing the doors returns the autosampler to the home position, placing the tips of the capillaries in buffer.

6. Load samples and link a plate to the plate record:
 - GA instruments > ga3130xl > plate manager.
 - Select **New**.
7. In the New Plate dialog box that opens:
 - Name your plate (i.e., HIV03022007).
 - Specify the application.
 - Specify the plate type.
8. Select **Application > Sequencing Analysis**.
9. From the **Plate Type** list box, select **96 well**.
10. Click **Finish**. The Plate Editor spreadsheet opens.
11. In the sample name column, enter the names of your samples.

Note: Be sure to use the sample naming convention given below, in order for the ViroSeq HIV-1 Genotyping System v2.8 Software to correctly assemble sample projects.

SampleName__PrimerName (separated by double underscore)

12. Select the following setting for each sample in the spreadsheet:

<i>Priority</i>	The value 100 automatically displays in the column
<i>Comments</i>	Enter your comments, if any
<i>Results Group 1</i>	ViroSeq_Results_Group
<i>Instrument Protocol 1</i>	ViroSeq_Instrument_Protocol
<i>Analysis Protocol 1</i>	ViroSeq_Analysis_Protocol

Note: To facilitate text entry, select the appropriate setting for the first sample, then click on the column heading to select the entire column. Then from the **Edit** menu, use the **Fill Down** command. (Or use **Control & D** on the keyboard to copy.)

13. Verify that the plate record is correct, and then click **OK** to return to the main menu.
14. Search for the plate record: GA instruments>ga3130xl>instrument name>Run Scheduler>Plate View.
15. Select the plate record, and then click the plate position indicator that corresponds to the plate being linked. The plate position indicator changes from yellow to green when linked, and the green Run Instrument button is active.
16. After a plate is linked, use the Run View window to verify that the runs are scheduled correctly: GA instruments>ga3130xl>instrument name>Run Scheduler>Run View.

8.3.6 Starting the Run

1. Start the run:
 - Verify that the active spectral calibration matches your dye set, capillary array length, and polymer type for all scheduled runs.
 - Click the green Run Instrument button in the toolbar.
 - The Processing Plates dialog box opens, then click OK.
 - The software automatically performs a run validation: If the validation passes, the run will start; If any of the validation test fails, the run will not start. Check the Event Log for information.
2. Monitor a Run in progress:
 - Select **GA instruments>ga3130xl>instrument name>Instrument Status**.
 - The **Event Messages** lists the instrument's recent actions.
 - The **Error Messages** lists errors that have occurred during the current run.
 - The Electrophoresis Power and Temperature (EPT) Chart displays the real-time chart of EPT status.
 - System Status changes from green to flashing red when errors occur.
3. Monitor the system messages from a run in progress: **GA instruments>ga3130xl>instrument name>Instrument Status>Event Log**.
4. View data during a run:
 - Use the Capillaries Viewer: **GA instruments>ga3130xl>instrument name>Capillaries Viewer**.
 - Use the Cap/Array Viewer: **GA instruments>ga3130xl>instrument name>Cap/Array Viewer**.
5. View **Raw Data** from a completed run in the **Data Collection software**:
 - Select **GA instruments>ga3130xl>Run History** to select a run to view.
 - After choosing a run, click the **Capillary Viewer** or the **Cap/Array Viewer**.
 - The electrophoresis data of the run, such as voltage, current, and oven temperature profiles, can be seen in the **EPT Viewer** under **Run**.

8.4 Data Analysis

Using Sequencing Analysis Software 5.3.1 with KB Basecaller v1.4 and Analyzing the Data

1. Launch the Sequencing Analysis software:

- When the run is complete, launch the **Sequencing Analysis software 5.3.1** by double-clicking the Sequencing Analysis 5.3.1 desktop shortcut or select **Start>Programs>Applied Biosystems>Sequencing Analysis>Sequencing Analysis 5.3.1**.
- In the Login window, enter a valid username and password. Click **OK**. To check the software name and version number, from the main menu select **Help>About Sequencing Analysis**. Click **OK** to close the **About** dialog.
- Set the **Analysis Defaults**.
- Create a **ViroSeq Analysis Protocol** (done by company service person).

2. Add files to the sample manager window:

- Click the **Add files** icon in the toolbar, or select **File>Add sample(s)**.
- Navigate to the location of the sample folders. The individual sample files are located within the sample folders.
- When the desired sample files have been added, click **OK** in the **Add Samples** dialog box to close the dialog box.

3. Start the analysis: Click **Start Analysis** to start the analysis:

- Clear the **P** (print) columns.
- Select **A** (analyze) check box for each sample and click **Start**.
- After analysis, check the **A** column to see if a green box appears for each sample.

Note: If a sample has a red box, review your settings and reanalyze your data.

4. Before proceeding with ViroSeq HIV-1 genotyping system software, review Sequencing Analysis v5.3.1 software checklist for ViroSeq sample files.

Review or correct ending base	Verify that the Ending Base for subset of sample electropherograms is at 580 bases
Review or correct start point	Inspect a subset of samples to verify that the Start point begins after the initial dye blob
Sample names	Verify sample file names are correct
DyeSet/Primer file	KB_3130_POP6BDTv1.mob
BaseCaller	KB.bcp
Sufficient signal strength	Review the signal strength numbers for each base If the total A, C, G, and T signal strength value is below 400, inspect the raw and analyzed data and discard any noisy data
Sequence segments	IMPORTANT! Six of the seven segments must be successfully sequenced in order to proceed with the analysis in the ViroSeq software. One of the missing segments can be A or D

8.4.1 Working with ViroSeq Software v2.8 to Determine Genotype

1. Navigate to the folder (D:\AppliedBio\3130x\Extractor) containing your Sequencing Analysis software files and transfer them to another computer with Internet (C:\Documents and Settings\HIVPheno sequence 2011\from 3130x\ data extractor).
2. Launch ViroSeq and click **New** in the Project Status window. The Open dialog is displayed.

3. If ViroSeq is already opened, select **File>New Project**.
4. Select one sequence data file and click **Open**:
 - The software assembles and aligns the primer sequence files from a sample to generate a consensus sequence and creates a project.
 - A Progress window opens while the software is assembling the project.
 - When complete, the Progress window closes and the Navigation and View*Edit windows open, displaying one of your assembled projects.
5. **View project status** window by going to the Navigation window and selecting **File>Open Project**. The Status column indicates whether or not the software found all the input segments.

If the Status is...	Then...
OK	All the expected segments were found, and the project assembled successfully
?	ViroSeq encountered assembly issues Possible causes include: <ul style="list-style-type: none"> • One or more segments could not be identified • Fewer than 6 segments were assembled <i>Note:</i> Do not proceed if fewer than x segments are assembled into the project (Except one of A or D segment is missing)

6. Verify that the sequences are positioned as expected:
 - Forward direction – primers A, D, B, C
 - Reverse direction – primers F, G, H
7. Click **Navigation Options** to open the Navigation Option window.
8. Make sure all the check boxes for the options in the Navigation Options window are selected.
9. Select and trim areas of poor sequence quality to improve basecalling.
10. Confirm or edit the basecalls:
 - Begin at the leftmost POI (Position of Interest) in the protease gene and edit or confirm the basecall.
 - Press the spacebar to confirm your selection.
 - Proceed to next POI by pressing “>” and edit or confirm the basecall. Repeat until you have edited or confirmed all the POIs in the consensus sequence.
11. In the Navigation Options window, clear all the check boxes except Show Resistance positions and click **OK**.
12. Repeat the edit/confirm procedure, starting at the far left and moving to the right.
General guidelines of editing a project
 - *Trim segment ends to remove poor-quality sequence segments.*
 - *Verify and reconcile mixed basecalls and mismatched bases.*
 - *Compare areas with mobility problems with other sequence segments and with the reference sequence to determine the correct peak position.*
13. After you finished reviewing one project, select **Edit>Edit Report Information**.
14. In the Edit Report Information window, type in the information that you want to appear as a default on all Antiretroviral Drug Resistance Report.
15. Click **Set As Default**.
16. Then type in the patient name, patient accession number, assay operator, and sample information and/or comments.
17. Click **OK** to close the dialog box.
18. Click **File>Save** to save changes.

19. Select **File>Save consensus [FASTA]** to save the sample in the folder (C:\Documents and Settings\HIVPheno sequence 2010\Fasta files 2010).
20. Select **File>Generate Resistance Report** when the report is open; click Save as to the folder C:\Documents and Settings\HIVPheno sequence 2010\Genotype reports 2010.

8.4.2 Working with VircoNET™ Software to Get Phenotype

1. Launch ViroNET™ and select **File>Patients**. The **Patients** window will present.
2. To add a new patient, click the “+ insert” button; the **Input form** window will appear. Type the patient UH # in **Patient ID**, and ACC# in **SSN** and type in the patient’s name.

Note: If the patient has existed in the patient list, use **Space** button on the keyboard to make a space after the UH # to create a new patient data, because FASTA file only could be imported once for each patient.

3. Click on the **Sequence** tab at the top of the page; the list of sequences is presented.
4. To add a sequence, click “+ Add One Sequence” button; the Sequence Data window shall present.
5. Click “**Import from file**” button, and open the FASTA files of this patient you just saved.
6. Click “**Accept**” button to out of the **Input form** window, and repeat steps 2–5 to input other patients’ sequence.
7. After you are done with inputting the patients’ sequence, click “**Done**” button to out of **Patients** window.
8. Select **File>Sending&Receiving** to upload the sequences. The sequences you just imported will be uploaded automatically, and wait for 20–30 min while the company is analyzing the sequences you uploaded.
9. After 20 min, select **File>Sending&Receiving** to download the **Virco reports**.
10. After all the reports were received in your computer, select **File>Reports**: the **Reports** window will present on the display.
11. Select the report you want to print in the reports list, and click **Print** button to print the report as a paper copy and/or a PDF file (for inpatient).
12. Copy and send outpatient reports to client services and appropriate clinics.
13. Upload the PDF file of inpatient reports (saved in C:\Documents and Settings\HIVPheno sequence 2010\Virco report 2010) into MyUTMB.

Save and back up all the folders under HIVPheno sequence to the hard drive marked with “HIVVIRPHEN RECORD ONLY.”

Reference intervals and interpretation of results

- Determination of HIV-1 genotypes is based on analysis of nucleic acid sequence in the reverse transcriptase (RT) up to codon 335 and protease regions of the *pol* genes of HIV.
- Clinical samples with HIV-1 viral load <75 copies/mL will be rejected. Viral loads of 75–2,000 copies/mL performed by Siemens bDNA have been validated analytically in our laboratory and will be accepted for ViroSeq testing [4].
- PCR failure can be caused by reasons including too low a viral load or sequence variation affecting primer annealing. These will be reported as indeterminate.
- Cases of strong PCR amplicon but failed sequencing (including chromatogram high background and missing fragment) will also be reported as indeterminate.
- Decisions regarding the management of HIV-infected patients should be made after a consideration of all clinical and laboratory information, not solely the HIV genotype.

8.5 Troubleshooting

8.5.1 Instrument Start-Up

Observation	Possible cause	Recommended action
No communication between the instrument and the computer. The event viewer is blank	Incorrect Ethernet configuration	Check the configuration IP address: 192.168.0.1
Instrument red light is blinking	Incorrect start-up procedure	Start up in the following sequence: <ol style="list-style-type: none"> 1. Log out of the computer 2. Turn off the instrument 3. Boot up the computer 4. After the computer has booted completely, turn the instrument on. Wait for the green status light to come on 5. Launch the Data Collection software
Data Collection software will not launch	Applications in the service console did not start properly	Restart the computer
Computer screen is frozen	Communication error	There will be no loss of data. However, if the instrument is in the middle of a run, wait for the run to stop. Then, exit the Data Collection software and restart, as described above

8.5.2 Spatial Calibration

Observation	Possible cause	Recommended action
Unusual peaks or a flat line for the spatial calibration	The instrument may need more time to reach stability. An unstable instrument can cause a flat line with no peaks in the spatial view	Repeat the spatial calibration
	Improper installation of the detection cell	Reinstall the detection cell to reposition and make sure it fits in the proper position. If the calibration fails again: <ol style="list-style-type: none"> 1. Fill the capillaries with polymer 2. Repeat the spatial calibration
	Broken capillary resulting in a bad array fill	Check for a broken capillary, particularly in the detection cell area. If necessary, replace the capillary array using the Install Array Wizard
Persistently bad spatial calibration results	Bad capillary array	Replace the capillary array and then repeat the calibration. Call Technical Support if the results do not improve

8.5.3 Spectral Calibration

Observation	Possible cause	Recommended action
If the spectral calibration fails, or if a message displays “No candidate spectral files found”	Blocked capillary	Refill capillary array. You may have to install a fresh array or consider that capillary nonusable for purposes of planning your runs
	Incorrect chemistry file, dye set, and/or run module selected	Correct the files and rerun the calibration
	Insufficient filling of array	Check for broken capillaries and refill the capillary array
	Expired standards or old reagents	Check the expiration date and storage conditions of the standards and/or reagents. If necessary, replace with a fresh lot

8.5.4 Plate Linking

Observation	Possible cause	Recommended action
Plate does not link	Spatial calibration was not performed	Perform a spatial calibration Relink the plate(s) in the Run Scheduler
The plates in the Run Scheduler were linked, but now are unlinked	A wizard was used after linking a plate, but before starting a run automatically unlinking the plate	Relink the plate(s) in the Run Scheduler
The plate links, but System Status changes from green to red	A different length capillary array was installed, and the appropriate active spectral calibration was not selected or does not exist	<ol style="list-style-type: none"> 1. View the error messages in the Event Log 2. In the Spectral Calibration Viewer, set the active spectral calibration for the dye set and array length 3. If one does not exist, create a new spectral calibration for the dye set and array length you are using, then set it as the active spectral calibration 4. Relink the plate(s) in the Run Scheduler
	The capillary array length and/or polymer type selected in the instrument Protocol does not match capillary array length and/or polymer type stored in the database	Correct the Instrument Protocol, or <ol style="list-style-type: none"> 1. Use the wizards to update the information in the database 2. Set or create an active spectral calibration 3. Relink the plate
	The database and/or drive E is full	To check the available space: <ol style="list-style-type: none"> 1. View the error message in the Event Log 2. Refer to “Hard Disk and Database Status: Manually Checking Available Disk Space on Drive E” 3. Make more space 4. Relink the plate(s) in the Run Scheduler

8.5.5 Run Performance

Observation	Possible cause	Recommended action
No peaks in any sample file	Bubbles in the system	Visually inspect the polymer block for bubbles
	No sample injection	Remove bubbles using the Bubble Remove Wizard
		1. If bubbles persist, select the Water Wash Wizard
		2. If necessary, select the Replenish Polymer Wizard to install fresh polymer
Green Start button is disabled	Possible contaminant in the polymer path	Select the Water Wash Wizard
	Possible contaminant or crystal deposits in the polymer bottle	Bring the polymer to room temperature, swirl to dissolve any deposits
		Replace the polymer if it has expired
	Communication failure between the Data Collection software and Sequencing Instrument	Install Software Patch CE_DC_300-002. To download the patch, go to http://www.appliedbiosystems.com , then click the link for Support, then Software Downloads, the Applied Biosystems 3130xl Genetic Analyzer, then Updaters and Patches. To install the patch, follow the instructions in the Read Me pdf file
No signal	Autosampler calibration is not optimal	Check the injection with 20 μ L samples
		If the 20 μ L samples have adequate signal levels, then recalibrate the autosampler using the Autosampler Calibration Wizard
		If the 20 μ L samples still have no signal, then check the other possible causes
	Bubble at bottom of sample tube	Centrifuge the sample tubes
	Bent capillary array tips	Replace the capillary array and recalibrate the autosampler using the Autosampler Calibration Wizard
	Failed reaction	Repeat reaction
	Cracked or broken capillary	Visually inspect the capillary array, including the detector window area for signs of breakage
Signal too high	Blocked capillary	Refill capillary array. You may have to install a fresh array or consider that capillary nonusable for purposes of planning your runs
	Sample concentration is too high	Dilute the sample
	Too much DNA added to the reaction, resulting in uneven signal distribution	Decrease the injection time Optimize reaction conditions
Low signal strength	Degraded formamide	Use a fresh aliquot of Hi-Di formamide
	Pipetting error; not enough samples	Increase the amount of DNA added
		Recalibrate the pipettes
	Sample has high salt concentration	Dilute with distilled or deionized water
	Insufficient mixing	Desalt using a column purification method
		Vortex the sample thoroughly, and then centrifuge the tube to condense the sample to the bottom of the tube
	Autosampler out of calibration	Check the injection with 20 μ L samples. If the 20 μ L samples have adequate signal levels, then recalibrate the autosampler using the Autosampler Calibration Wizard
		Power off and on the instrument to use the new calibration values
	Weak amplification of DNA	Reamplify the DNA
		Check DNA quality

(continued)

Observation	Possible cause	Recommended action
Elevated baseline	Possible contaminant in the polymer path	Select the Water Wash Wizard
	Possible contaminant or crystal deposits in the polymer	Bring the polymer to room temperature, swirl to dissolve any deposits Replace the polymer if it has expired
	Poor spectral calibration	Perform new spectral calibration
Loss of resolution	Too much sample injected	Dilute the sample and reinject
	Poor-quality water	Use distilled or deionized water
	Poor-quality or dilute running buffer	Prepare fresh 1× running buffer from 10×running buffer
	Degraded polymer	Use a fresh supply of polymer
	Capillary array used for more than 100 injections	Replace with new capillary array
	Degraded formamide	Prepare fresh Hi-Di formamide and reprepare samples
	High salt concentration in samples	Use a recommended protocol for salt removal. Dilute salts with water
Poor resolution in some capillaries	Insufficient filling of capillary array	Refill the capillary array and look for polymer leakage. If problem persists, contact Technical Support Reinject the same samples
	Poor-quality samples	Check the sample preparation
No current	Water placed in buffer reservoir position 1	Replace with fresh 1×running buffer
	Not enough buffer in anode reservoir	Add buffer up to the fill line
	Buffer too dilute	Prepare 1×running buffer Add 3 mL of 10× running buffer to 27-mL deionized water
	Bubbles present in the lower polymer block and/or the array and/or tubing	Pause run and inspect for bubbles hidden in the tubing connectors. Select the Bubble Remove Wizard to remove the bubbles
Elevated current	Degraded polymer	Open fresh supply of polymer and use Replenish Polymer Wizard
	Incorrect buffer dilution	Prepare 1× running buffer. Add 3 mL of 10× running buffer to 27-mL deionized water
	Arcing in the lower polymer block	Inspect the lower polymer block for discoloration or damage Replace the lower polymer block if necessary
Fluctuating current	Bubble in polymer block	Pause run and inspect for bubbles hidden in the tubing connectors Select Bubble Remove Wizard to remove the bubbles
	A slow leak may be present in the system	Check polymer blocks for leaks. Tighten all fittings
	Incorrect buffer concentration	Prepare 1× running buffer Add 3 mL of 10× running buffer to 27-mL deionized water
	Not enough buffer in anode reservoir	Add buffer up to the fill line
	Arcing	Check for moisture in and around the septa, the reservoirs, the oven, and the autosampler
Poor performance of capillary array used for fewer than 100 runs	Poor-quality samples, possible cleanup problems	Desalt samples using a recommended purification protocol
	Poor-quality formamide	Prepare fresh Hi-Di formamide and reprepare samples
	Incorrect buffer	Use 10× running buffer to prepare 1× running buffer

(continued)

Observation	Possible cause	Recommended action
Migration time becomes progressively slower	Leak in system	Tighten the tubing connectors and array knob
	Improper filling of the system with polymer	Polymer delivery pump may need to be serviced. Call a service representative
Migration time becomes progressively faster	Expired polymer	Check expiration of polymer. If necessary, change the lot
	Water in polymer system, resulting in diluted polymer	Use Bubble Remove Wizard to add polymer to system
Extra peaks in the electropherogram	Buffer valve leakage	Check the buffer valve pin and see if it closes correctly
	Data off scale	Dilute the sample and reinject the sample
	Possible contaminant in sample	Reamplify the DNA
Error message, “leak detected” appears. The run aborts	Sample renaturation	Heat-denature the sample in good-quality formamide and immediately place on ice
	Bubbles in the polymer system	Select the Bubble Remove Wizard to clear bubbles
Buffer jar overflows very quickly with polymer	Leak in the polymer system	Check for evidence of leaks. Tighten the tubing connectors and array knob
	Buffer valve leakage	Check the buffer valve pin and see if it closes correctly
Detection cell comes out while replacing the capillary array. Replacing the cell in the correct orientation is difficult	Bubbles in the polymer path (overuse of the Bubble Remove Wizard)	Check for bubbles and remove if present
	Improperly placed detection cell	Loosen the array knob. Close the detection block door Retighten the array knob
Detection cell stuck. It is difficult to remove when changing the capillary array		To loosen the detection cell: 1. Undo the array knob and pull the polymer block toward you to first notch 2. Hold both sides of the capillary array around the detection cell area, and apply gentle pressure equally on both sides 3. Remove the capillary comb from the holder in oven 4. Release

8.6 Summary

HIV genotyping (i.e., gene sequencing) has revealed specific point mutations or combinations of mutations in the enzymes targeted by these drugs, i.e., viral protease and reverse transcriptase. HIV phenotyping measures drug resistance by identifying the drug concentration necessary to inhibit virus replications. Results of genotypes have also been used to predict the phenotype by identifying similar genotypes from a large database of other HIV genotypes for which the phenotypes are known.

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Chapter 9

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, Version 2.0

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Keywords COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, Version 2.0 • Selective amplification of target nucleic acid • Viral RNA in plasma • Nucleic acid amplification • AmpErase® enzyme in the COBAS® AmpliPrep/COBAS® TaqMan HIV-1 Test • Master Mix • Amplicon contamination

9.1 Introduction

There are three HIV-1 RNA quantitative assays on the market in the USA which are FDA approved. The three assays are: COBAS® Ampli Prep/COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Molecular Diagnostics Pleasanton, CA); Abbott RealTime™ HIV-1 m2000rt (Abbott Molecular, Des Plains, IL); and VERSANT® HIV-1 3.0 Assay (Siemens Healthcare Diagnostics, Deerfield, IL). For these assays, a listing of the methods and the linear ranges are found in Table 9.1. In this chapter, we will describe our modification of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0.

NOTE: This chapter contains excerpts only from the package insert for the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test, Doc Rev. 4.0 (07/2011). Please note that this test should not be performed without first reviewing the complete and most current version of the package insert that accompanies the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test kit. If you have any questions about this test or the package insert, you should contact Roche Molecular Diagnostics Technical Support at 1-800-526-1247 or visit www.mylabonline.com.

9.2 Intended Use

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, version 2.0 (v2.0) is an in vitro nucleic acid amplification test for the quantitation of human immunodeficiency virus type 1 [HIV-1] RNA in human plasma using the COBAS® AmpliPrep Instrument for automated specimen processing and the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer for automated amplification and detection. The test can quantitate HIV-1 RNA over the range of 20–10,000,000 copies (cp)/mL. One copy of HIV-1

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Table 9.1 FDA-approved quantitative HIV-1 RNA assays

Assay	Company	Method	Amplification target	Linear range (copies/ml)
COBAS® AmpliPrep/ COBAS® TaqMan® HIV-1 Test, v2.0	Roche Molecular Diagnostics Pleasanton, CA	RT-PCR	LTR-region gag	20–10,000,000
Abbott RealTime™ HIV m2000rt	Abbott Molecular Des Plaines, IL	RT-PCR	integrase	1.0 ml 40–10,000.00
				0.5 ml 75–10,000.00
				0.2 ml 150–10,000.00
VERSANT® HIV-1 RNA 3.0 Assay	Siemens Healthcare Diagnostics Deerfield, IL	Signal amplifica- tion (bDNA)	pol	75–500,000

RNA is equivalent to 1.7 ± 0.1 international units (IU) based on the WHO first International Standard for HIV-1 RNA for nucleic acid-based techniques (NAT) (NIBSC 97/656) [1].

This test is intended for use in conjunction with clinical presentation and other laboratory markers of disease progress for the clinical management of HIV-1 infected patients. The test can be used to assess patient prognosis by measuring the baseline HIV-1 RNA level or to monitor the effects of anti-retroviral therapy by measuring changes in EDTA plasma HIV-1 RNA levels during the course of antiretroviral treatment.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 is not intended for use as a screening test for the presence of HIV-1 in blood or blood products or as a diagnostic test to confirm the presence of HIV-1 infection.

NOTE: The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 exhibits a higher level of sensitivity compared with the COBAS® AMPLICOR HIV-1 MONITOR Test, v1.5 and the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, reporting values ≤ 20 cp/mL that were undetectable or < lower limit of quantitation (LLOQ) in these methods [2].

9.3 General Testing Principle

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 is a nucleic acid amplification test for the quantitation of human immunodeficiency virus type 1 (HIV-1) RNA in human plasma. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 is based on three major processes: (1) specimen preparation to isolate HIV-1 RNA; (2) reverse transcription of the target RNA to generate complementary DNA (cDNA), and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide detection probe specific to the target.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 permits automated specimen preparation followed by automated reverse transcription, PCR amplification, and detection of HIV-1 target RNA and HIV-1 Quantitation Standard (QS) Armored RNA. The Master Mix reagent contains primers and probes specific for both HIV-1 RNA and HIV-1 QS RNA. The Master Mix has been developed to ensure equivalent quantitation of group M subtypes of HIV-1 and HIV-1 group O. The detection of amplified DNA is performed using target-specific and QS-specific dual-labeled oligonucleotide probes that permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon.

The quantitation of HIV-1 viral RNA is performed using the HIV-1 QS. It compensates for effects of inhibition and controls the preparation and amplification processes, allowing a more accurate quantitation of HIV-1 RNA in each specimen. The HIV-1 QS is a noninfectious armored RNA construct that contains HIV sequences with identical primer binding sites as the HIV-1 target RNA and a unique probe binding region that allows HIV-1 QS amplicon to be distinguished from HIV-1 target amplicon.

The HIV-1 QS is added to each specimen at a known copy number and is carried through the subsequent steps of specimen preparation, reverse transcription, simultaneous PCR amplification, and detection of cleaved dual-labeled oligonucleotide detection probes. The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer calculates the HIV-1 RNA concentration in the test specimens by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen and control [2].

9.4 Methodology

9.4.1 Target Selection and Specimen Preparation

Selection of the target RNA sequence for HIV-1 depends on identification of regions within the HIV-1 genome that show maximum sequence conservation among the various HIV-1 group M subtypes and HIV-1 group O specimens. In order to address the high genetic variability of the virus, two regions of HIV genome are simultaneously targeted for amplification and detection by the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0. Two target-specific and one QS-specific dual-labeled oligonucleotide probes permit independent identification of the HIV-1 amplicon and of the HIV-1 QS amplicon. Accordingly, the appropriate selection of the primers and the dual-labeled oligonucleotide probes is critical to the ability of the test to amplify and detect the HIV-1 group M subtypes and HIV-1 group O. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 uses reverse transcription and PCR amplification primers that define sequences within the highly conserved regions of the HIV-1 *gag* gene and of the HIV-1 LTR region.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 utilizes automated specimen preparation on the COBAS® AmpliPrep Instrument by a generic silica-based capture technique. The procedure processes 850 µL of plasma. The HIV-1 virus particles are lysed by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HIV-1 RNA from RNases in plasma. Protease and a known number of HIV-1 QS armored RNA molecules are introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture is incubated and the HIV-1 RNA and HIV-1 QS RNA are bound to the surface of the magnetic glass particles. Unbound substances, such as salts, proteins, and other cellular impurities, are removed by washing the magnetic glass particles. After separating the magnetic glass particles and completing the washing steps, the adsorbed nucleic acids are eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HIV-1 RNA and HIV-1 QS RNA, is added to the amplification mixture and transferred to the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer. The HIV-1 target RNA and the HIV-1 QS RNA are then reverse transcribed, amplified, and simultaneously detected by cleavage of two target-specific and one QS-specific dual-labeled oligonucleotide probe[2].

9.4.2 Reverse Transcription and PCR Amplification

The reverse transcription and PCR amplification reaction is performed with the thermostable recombinant enzyme *Thermus specie* Z05 DNA polymerase (Z05). In the presence of manganese (Mn^{2+}) and under the appropriate buffer conditions, Z05 has both reverse transcriptase and DNA polymerase activity. This allows both reverse transcription and PCR amplification to occur together with real-time detection of the amplicon.

Processed specimens are added to the amplification mixture in amplification tubes (K-tubes) in which both reverse transcription and PCR amplification occur. The reaction mixture is heated to allow the downstream primers to anneal specifically to the HIV-1 target RNA and to the HIV-1 QS RNA. In

the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine, and deoxythymidine triphosphates, Z05 polymerase extends the annealed primers forming DNA strands complementary to the RNA target [2].

9.4.3 Target Amplification

Processed specimens are added to the amplification mixture in amplification tubes (K-tubes) in which PCR amplification occurs. Following reverse transcription of the HIV-1 target RNA and the HIV-1 QS RNA, the Thermal Cycler in the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer heats the reaction mixture to denature the RNA:cDNA hybrids and to expose the specific primer target sequences. As the mixture cools, the primers anneal to the target DNA. Z05 in the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine, and deoxythymidine triphosphates, extends the annealed primers along the target template to produce double-stranded DNA molecules termed amplicons. The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 49 Analyzer automatically repeats this process for a designated number of cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer. Amplification occurs only in the two regions of the HIV-1 genome between the primers; the entire HIV-1 genome is not amplified [2].

9.4.4 Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine, but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also, any nonspecific product formed after initial activation of the Master Mix by manganese is destroyed by the AmpErase enzyme. The AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C₁-position. When heated in the first thermal cycling step, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme remains inactive for a prolonged period of time once exposed to temperatures above 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon formed during amplification [2].

9.4.5 Detection Reaction

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 utilizes real-time PCR technology. The use of dual-labeled fluorescent probes allows for real-time detection of PCR product accumulation by monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HIV-1 and HIV-1 QS-specific oligonucleotide probes with a reporter dye and a quencher dye. In the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 the HIV-1 and HIV-1 QS probes are labeled with different fluorescent reporter dyes. When these probes are intact,

the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Forster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and is cleaved by the 5' → 3' nuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplification of HIV-1 RNA and HIV-1 QS RNA are measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HIV-1 RNA and HIV-1 QS RNA. The PCR cycle where a growth curve starts exponential growth is related to the amount of starting material at the beginning of the PCR [2].

9.4.6 HIV-1 RNA Quantitation

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 is inherently quantitative over a very wide dynamic range since the monitoring of amplicon is performed during the exponential phase of amplification. The higher the HIV-1 titer of a specimen, the earlier the fluorescence of the reporter dye of the HIV-1 probes rises above the baseline fluorescence level. Since the amount of HIV-1 QS RNA is constant between all specimens, the fluorescence of the reporter dye of the HIV-1 QS probe should appear at a similar cycle for all specimens. In specimens where the QS fluorescence is affected, the concentration is adjusted accordingly. The appearance of the specific fluorescent signals is reported as a critical threshold value (Ct). The Ct is defined as the fractional cycle number where reporter dye fluorescence exceeds a predetermined threshold (the assigned fluorescence level), and starts the exponential growth phase of this signal. A higher Ct value indicates a lower titer of initial HIV-1 target material. A twofold increase in titer correlates with a decrease of 1 Ct for target HIV-1 RNA, while a tenfold increase in titer correlates with a decrease of 3.3 Ct [2].

9.4.7 Clinical Significance

Viral load measurements of HIV-1 RNA are routinely used in the care of patients infected with HIV-1. When patients have a viral load greater than 100,000 copies/mL within 6 months of seroconversion, they have a high probability of developing AIDS within 5 years. In contrast, a viral load less than 10,000 copies/mL early in infection indicates a decreased risk of developing AIDS [3–5]. HIV-1 RNA levels may also be useful in establishing the diagnosis of HIV infections in neonates and in acute infections [6, 7]. Guidelines from the US Department of Health and Human Services promote the use of plasma viral loads testing when starting antiretroviral therapy, monitoring response to therapy, and instituting a change in drug regimen [8]. Baseline HIV-1 RNA measurements are recommended with repeat testing within a month of starting therapy [9]. There should be a tenfold (1.0 log 10) decrease in the first month and suppression to less than 50 copies/mL by 24 weeks, depending on pretreatment viral load [10]. The viral load should then be repeated every 3 or 4 months.

9.4.8 Specimen Collection and Handling

9.4.8.1 Preferred Specimen

Freshly drawn whole blood in EDTA anticoagulant received from the specimen processing area with patient identification, sample identity, date and time of collection. Plasma must be separated from whole blood by centrifugation at 800–1,600×g for 20 min at room temperature *within 6 h of*

collection. EDTA plasma specimens may be stored at room temperature for up to 1 day and at 2–8°C for up to 5 days or frozen at –20°C to –70°C or colder indefinitely. Plasma specimens may be frozen and thawed up to five times without a loss of HIV-1 RNA. Never return aliquot to the original container. Specimen is saved for 3 days after the result is finalized.

Other Acceptable: None

Minimum Sample Volume: 1 mL of plasma (EDTA)

Optimum Sample Volume: 2 mL of plasma (EDTA)

Criteria for Unacceptable Specimen:

- Not collected in EDTA
- Not processed with 6 h of collection
- Clotted
- Not stored at 2–8°C
- Stored at 2–8°C more than 5 days
- Stored at room temperature (18–25°C) more than 24 h
- Improperly identified (discrepancies in ID number, tube, name)
- Inadequate volume of sample
- Improperly collected test (specimen collected in the wrong collection tube)
- Test requests with incomplete information (i.e., attending physician, time of collection, date of collection)
- Share specimen
- Reject specimen and request recollection.

9.4.8.2 Handling Condition

Plasma must be separated from whole blood within 6 h after collection. Testing must be performed as soon as possible. Transportation of whole blood or plasma must comply with country, federal, state, and local regulations for the transport of etiologic agents [11]. Specimens and controls should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories* [12] and in the CLSI Document M29-A [13]. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water. No aliquot is ever returned to the original container.

9.4.8.3 Reagent Composition

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test includes the reagents in Table 9.2.

9.4.8.4 Special Supplies

The following supplies are needed but not supplied in the COBAS®: AmpliPrep/COBAS® TaqMan® HIV-1 Test (see Table 9.3):

9.4.8.5 Reagent Labeling and Preparation

All reagents are labeled by the manufacturer. The labeling includes contents, lot number, expiration date, and storage instructions. All reagents are liquid and ready to use. Do not freeze reagents or controls. Do not use a kit after its expiration date.

Table 9.2 COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test reagents

Reagents		Compositions
HIV-1 Magnetic Glass Particles Reagent Cassette	HIV-1 CS1	Magnetic glass particles 93% Isopropanol
HIV-1 Lysis Reagent Cassette	HIV-1 CS2,	Sodium citrate dihydrate 42.5% Guanidine thiocyanate <14% Polydocanol 0.9% Dithiothreitol
HIV-1 Multi-Reagent Cassette containing: Proteinase solution	HIV-1 CS3 Pase	Tris buffer <0.05% EDTA Calcium chloride Calcium acetate ≤7.8% Proteinase Glycerol
Elution buffer	EB	Tris-base buffer 0.2% Methylparaben
HIV-1 Test-Specific Reagent Cassette containing:	HIV-1 CS4	
HIV-1 Quantitation Standard	HIV-1 QS	Tris-HCl buffer EDTA <0.005% Poly rA RNA (synthetic) <0.001% Armored HIV-1 RNA construct containing HIV-1 primer binding sequences and a unique probe binding region (noninfectious RNA in MS2 bacteriophage)
HIV-1 Master Mix	HIV-1 MMX	0.05% Sodium azide Tricine buffer Potassium acetate Potassium hydroxide 20% Dimethylsulfoxide Glycerol <0.04% dATP, dCTP, dGTP, dUTP, dTTP <0.003% Upstream and downstream primers to the <i>gag</i> region of HIV-1 <0.003% oligonucleotide aptamer <0.003% Fluorescent-labeled oligonucleotide probes specific for HIV-1 and the HIV-1 QS <0.05% Z05 DNA polymerase (microbial) <0.1% AmpErase (uracil-N-glycosylase) enzyme (microbial) 0.09% Sodium azide
HIV-1 Test-Specific Reagent Cassette containing: CAP/CTM Mn ²⁺	CAP/CTM manganese solution	<0.5% Manganese acetate Glacial acetic acid 0.09% Sodium azide
COBAS® AmpliPrep®/COBAS® TaqMan® Wash Reagent	PG WR	Sodium citrate dihydrate <0.1% N-methylisothiazolone HCl

(continued)

Table 9.2 (continued)

Reagents		Compositions
HIV-1 High Positive Control	HIV-1 H(+) C	<0.001% Armored HIV-1 RNA construct containing HIV-1 sequences (noninfectious RNA in MS2 bacteriophage) Negative human plasma, nonreactive by FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen, and HBsAg; HIV-1 RNA, HCV RNA, and HBV DNA not detectable by PCR methods 0.1% ProClin® 300 preservative
HIV-1 Low (+) Control	HIV-1 L(+) C	<0.001% Armored RNA construct containing HIV-1 sequences (noninfectious RNA in MS2 bacteriophage) at a mean concentration at least 100-fold lower than the mean concentration of Armored HIV-1 RNA in HIV H(+) Negative human plasma, nonreactive by US FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen, and HBsAg; HIV-1 RNA, HCV RNA, and HBV RNA not detectable by PCR methods 0.1% ProClin® 300 preservative
COBAS® TaqMan® Negative Control (Human Plasma)	CTM (–) C	Negative human plasma, nonreactive by FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen, and HBsAg; HIV-1 RNA, HCV RNA, and HBV DNA not detectable by PCR methods 0.1% ProClin® 300 preservative
HIV-1 High Positive Control Barcode Clip	HIV-1 H(+) C Clip	
HIV-1 Low Positive Control Barcode Clip	HIV-1 L(+) C Clip	
HIV-1 Negative Control Barcode Clip	HIV-1 (–) C Clip	

9.4.8.6 Reagent Storage and Use

Store the following reagents at 2–8°C: HIV-1 CS1, HIV-1 CS2, HIV-1 CS3, HIV-1 CS4, HIV-1 H(+) C, HIV-1 L(+)C, and CTM (–) C. Store the following reagents at 2–30°C: HIV-1 H(+)C Clip, HIV-1 L(+)C Clip, HIV-1 (–) C Clip, and PG WR.

Once used, HIV-1 CS1, HIV-1 CS2, HIV-1 CS3, and HIV-1 CS4 are stable for 28 days at 2–8°C or until the expiration date, whichever comes first. They can be used for a maximum of four instrument cycles (up to 64 h cumulative on board the COBAS® AmpliPrep Instrument) and must be stored at 2–8°C between instrument cycles. Once opened, any unused portion of HIV-1 H(+)C, HIV-1 L(+)C, and CTM (–) C must be discarded.

Do not mix reagent cassettes or controls from different kits. Do not pool controls from different lots or from different vials of the same lot. Do not open COBAS® AmpliPrep cassettes and exchange, mix, remove, or add bottles. Dispose of unused reagents, waste, and specimens in accordance with country, federal, state, and local regulations. Handle all reagents with caution and avoid contact with skin, eyes, or mouth. Refer to the package insert for any known toxicity.

Table 9.3 Additional supplies needed for the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test but not supplied in the kit

<i>Instrumentation and software</i>	COBAS® AmpliPrep Instrument COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer Optional: docking station AMPLILINK® software, Version 3.1.x or higher Data station for the AMPLILINK software, with printer COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK software, Version 3.1.x Series COBAS® TaqMan® Analyzer (plus optional docking station) Instrument Manual for use with AMPLILINK software, Version 3.1.x Series COBAS® TaqMan® 48 Analyzer Instrument Manual for use with AMPLILINK software, Version 3.1.x Series AMPLILINK software, Version 3.1 x Series, Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, and COBAS® TaqMan® 48 Analyzer COBAS® TaqMan® Analyzer HIV-1 PCR TEST FILE CD ROM COBAS® TaqMan® 48 Analyzer HIV-1 PCR TEST FILE CD ROM
<i>Disposables</i>	Sample processing units (SPUs) Sample input tubes (S-tubes) with barcode clips Racks of K-tips K-tube Box of 12×96
<i>Other materials required but not provided</i>	Sample rack (SK 24 rack) Reagent rack SPU rack K-tube capper, motorized K-tube capper K-carrier K-carrier transporter K-carrier rack Pipettors with aerosol barrier or positive displacement RNase-free tips (capacity 1,000 µL) ^a Disposable gloves, powderless Vortex mixer

Aerosol barrier RNase-free tips are used where specified to prevent specimen and amplicon cross-contamination

^arequired volume of pipettes

These reagents contain sodium azide: HIV-1 QS, CAP/CTM HIV-1 Mn²⁺, and HIV-1 MMX. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide buildup. These reagents contain thiocyanate: HIV-1 CS2 and liquid waste from the COBAS® AmpliPrep Instrument contain guanidine thiocyanate. Do not allow HIV-1 CS2 or any liquid waste to contact sodium hypochlorite (bleach) solution. These mixtures can produce a highly toxic gas.

Wear eye protection, laboratory coats, and disposable gloves when handling any reagent. Avoid contact of these materials with the skin, eyes, or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

9.4.8.7 New Reagents Verification

All new reagent lots and/or shipments are validated by testing one known negative, one low positive, and one mid- to high positive that were tested with old lot. The results of the new lot must be within 0.5 log of the old lot. The results must be accepted before new reagent being placed in service.

Calibration verification and validation of AMR is performed at least semiannually using QS and reagents which come with the kit by testing, low positive, mid-positive, and high positive. The results must be within 0.5 log of the expected value. *Do not use the reagents if the calibration verification and/or validation of AMR is failed.* Notify the manufacture and supervisor immediately.

9.5 Quality Control

Each of the COBAS® TaqMan® Negative Control, the HIV Low Positive Control, and the HIV High Positive Control must be included in each test batch. Three controls are provided for use: HIV High Positive Control, HIV Low Positive Control, and the COBAS® TaqMan® Negative Control (Human Plasma). The controls are liquid and ready to use. Store the controls at 2–8°C. The controls are stable until the expiration date indicated.

The HIV (–) C must yield a “target not detected” result. The assigned range for HIV L(+)C and HIV H(+)C is specific for each lot of reagents and is provided on the COBAS® AmpliPrep/COBAS® TaqMan® HIV Test reagent cassette barcodes. The HIV RNA copies/mL for HIV L(+)C and HIV H(+)C should fall within their assigned ranges. If one or both of the positive controls are flagged as invalid, then the entire batch is invalid. No result will be reported. Repeat the entire process (specimen and control preparation, amplification, and detection).

If negative control is positive, the decontamination control protocol must be followed. No result will be reported. Repeat the entire process (specimen and control preparation, amplification, and detection). If the HIV RNA titer of one or both of the positive controls is consistently outside the assigned ranges in multiple batches, contact your local Roche office for technical assistance.

9.6 Protocols

Each kit contains reagents sufficient for 48 tests, which may be performed in batches of 12–24 tests. Therefore, it is recommended that processing should be in batches of multiples of 12 for the most efficient use of reagents, specimens, and controls.

9.6.1 Specimen and Control Preparation

9.6.1.1 COBAS® AmpliPrep Instrument Setup

1. Vortex specimens and controls for 3–5 s before use. Controls should be removed from 2°C to 8°C storage and equilibrated to ambient temperature before use.
2. Wear new gloves and a lab coat to perform the following steps.
3. The COBAS® AmpliPrep Instrument is ready for operation in standby mode.
4. Turn the data station for the AMPLILINK software ON. Prepare the data station as follows:
 - Log onto Windows® XP.
 - Double-click the AMPLILINK software icon.
 - Log onto AMPLILINK software by entering the assigned user ID and password.
5. Check the supply of PG WR using the Status Screen and replace if necessary.
6. Perform all maintenance that is listed in the Due Tab.

9.6.1.2 COBAS® AmpliPrep Reagent Loading

All reagent cassettes should be removed from 2°C to 8°C storage, immediately loaded onto the COBAS® AmpliPrep Instrument, and allowed to equilibrate to ambient temperature on the instrument for at least 30 min before the first specimen is to be processed. Do not let reagent cassettes come to ambient temperature outside the instrument.

1. Place **HIV-1 CS1** onto a reagent rack. Place **HIV-1 CS2**, **HIV-1 CS3**, and **HIV-1 CS4** onto a separate reagent rack.
2. Load the reagent rack containing **HIV-1 CS1** onto rack position **A** of the COBAS® AmpliPrep Instrument.
3. Load the reagent rack containing **HIV-1 CS2**, **HIV-1 CS3**, and **HIV-1 CS4** onto rack position **B**, **C**, **D**, or **E** of the COBAS® AmpliPrep Instrument.

9.6.1.3 COBAS® AmpliPrep Loading of Disposables

Determine the number of COBAS® AmpliPrep reagent cassettes, sample processing units (SPUs), input sample tubes (S-tubes), K-tips, and K-tubes needed. One SPU, one input S-tube, one K-tip, and one K-tube are needed for each specimen or control (see Table 9.4).

1. Place the SPUs in the SPU rack(s) and load the rack(s) onto rack position **J**, **K**, or **L** of the COBAS® AmpliPrep Instrument.
2. Depending on the configuration used, load full K-tube rack(s) onto rack position **M**, **N**, **O**, or **P** of the COBAS® AmpliPrep Instrument.
3. Load full K-tip rack(s) onto rack position **M**, **N**, **O**, or **P** of the COBAS® AmpliPrep Instrument.

9.6.1.4 Ordering and Loading of Specimens on Sample Racks

1. Attach a barcode label clip to each sample rack position where a specimen (S-tube) is to be placed.
2. Attach one of the specific barcode label clips for the controls [**CTM (–) C**, **HIV-1 L(+)C**, and **HIV-1 H(+)C**] to each sample rack position where the controls (S-tube) are to be placed.

Table 9.4 Configurations for using COBAS® AmpliPrep Instrument with COBAS® TaqMan® Analyzer

Configuration	Transfer mode to COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer	Racks, carriers, and disposables	Position on COBAS® AmpliPrep Instrument
1. COBAS® AmpliPrep Instrument plus docking station plus COBAS® TaqMan® Analyzer	Automated transfer of K-carrier	K-tubes in full K-tube racks K-tips in full K-tip racks Input S-tubes containing specimens and controls on sample racks SPUs in SPU racks CS1 on cassette rack CS2, CS3, and CS4 on cassette rack	M-P M-P F-H J-L A B-E

3. The barcode label clips for controls should have the same control lot number as the lot number on the control vials in the kit.
4. Take care in assigning the right control to the position with the appropriate control barcode clip. Place one input S-tube into each position containing a barcode label clip.
5. Using the AMPLILINK software, create specimen orders for each specimen and control in the **Orders** window **Sample** folder.
6. The test file name in the **Orders** window will be HIMAPU96 or HIMAPU48. Select the appropriate test file and complete by saving.
7. Assign specimen and control orders to sample rack positions in the **Orders** window **Sample Rack** folder.
8. The sample rack number must be entered for the rack prepared in step 1.
9. Print the **Sample Rack Order** report to use as a worksheet.
10. Prepare specimen and control racks in the designated area for specimen and control addition as follows:
 - (a) Vortex each specimen and control [CTM (–) C, HIV-1 L(+)C, and HIV-1 H(+)C] for 3–5 s.
 - (b) Avoid contaminating gloves when manipulating the specimens and controls.
11. Transfer 1,000–1,050 µL of each specimen and control [CTM (–) C, HIV-1 L(+)C, and HIV-1 H(+)C] to the appropriate barcode-labeled input S-tube using a micropipettor with an aerosol barrier or positive displacement RNase-free tip. *Avoid transferring particulates and/or fibrin clots from the original specimen to the input S-tube.* Specimens and controls should be transferred to tube positions as assigned and recorded on the worksheet in step 4. The barcode label clips for controls should have the same control lot number as the lot number on the control vials in the kit.
12. Assign the correct control to the position with the appropriate control barcode clip. *Avoid contaminating the upper part of the S-tubes with specimens or controls.*
13. Load the sample rack(s) filled with input S-tubes onto rack positions **F**, **G**, or **H** of the COBAS® AmpliPrep Instrument.

9.6.1.5 Start of COBAS® AmpliPrep Instrument Run

1. Start the COBAS® AmpliPrep system using the AMPLILINK software.
2. Check for flags or error messages in the system screen.

9.6.1.6 End of COBAS® TaqMan® Analyzer Run

1. At the completion of the COBAS® TaqMan® instrument run, print Results Report.
2. Check for flags or error messages in the Result section.
3. Specimens with flags and comments are interpreted as described in the Results section.
4. After acceptance, store data in archive.
5. Perform daily maintenance on both COBAS® AmpliPrep and COBAS® TaqMan instruments.

9.7 Calculations

For each specimen and control, the AMPLILINK software automatically:

1. Determines the cycle threshold value (CT) for the HIV-1 RNA and the HIV-1 QS RNA
2. Determines the HIV-1 RNA concentration based upon the CT values for the HIV-1 RNA and HIV-1 QS RNA and the lot-specific calibration coefficients provided on the cassette barcodes
3. Determines that the calculated cp/mL for HIV-1 L(+)C and HIV-1 H(+)C fall within the assigned ranges

9.8 Reporting Results

9.8.1 Reporting Format

The COBAS® TaqMan® Analyzer or the COBAS® TaqMan® 48 Analyzer automatically determines the HIV-1 RNA concentration for the specimens and controls. The HIV-1 RNA concentration is expressed in copies (cp)/mL. For batch validation, check AMPLILINK software results window or printout for flags and comments to ensure that the batch is valid. For control orders, a check is made to determine if the cp/mL value for the control is within its specified range. If the cp/mL value for the control lies outside of its range, a FLAG is generated to show the control has failed. The batch is valid if no flags appear for any of the controls [HIV-1 H(+)C, HIV-1 L(+)C, and CTM (–) C]. The following results are obtained for a valid batch:

Control	Result	Interpretation
Negative control	Target not detected	Control within range
Low positive control	A numeric titer, X.XXE+XX cp/mL	Control within range
High positive control	A numeric titer, X.XXE+XX cp/mL	Control within range

The batch is not valid if any of the following flags appear for the HIV-1 controls:

Flag	Result	Interpretation
<i>Negative Control:</i>		
__N_NC_INVALID	Invalid	An invalid result or a “valid” result that was not negative for HIV-1 target
<i>HIV-1 Low Positive Control:</i>		
__L_LPCINVALID	<4.80E+01 cp/mL	Control below range
__L_LPCINVALID	Target not detected	Control below range
__L_LPCINVALID	A numeric titer, X.XXE+XX cp/mL	Control out of range
__L_LPCINVALID	>1.00E+07 cp/mL	Control above range
__L_LPCINVALID	Invalid	An invalid result
<i>HIV-1 High Positive Control:</i>		
__H_HPCINVALID	<4.80E+01 cp/mL	Control below range
__H_HPCINVALID	Target not detected	Control below range
__H_HPCINVALID	A numeric titer, X.XXE+XX cp/mL	Control out of range
H_HPCINVALID	>1.00E+07 cp/mL	Control above range
__H_HPCINVALID	Invalid	An invalid result

Note: If the batch is invalid, repeat the entire batch including specimen and control preparation, amplification, and detection

Table 9.5 Flagged specimens and their interpretative comments

Titer result	Interpretation
Target not detected	CT value for HIV-1 above the limit for the assay or no CT value for HIV-1 obtained. Report results as “HIV-1 RNA not detected”
<4.80E+01 cp/mL	Calculated cp/mL is below the limit of detection of the assay. Report results as less than “4.8E+01 cp/mL”
≥4.80E+01 cp/mL and ≤1.00E+07 cp/mL	Calculated results greater than or equal to 48 cp/mL and less than or equal to 1.00E+07 cp/mL are within the linear range of the assay
>1.00E+07 cp/mL	Calculated cp/mL is above the range of the assay. Report results as “greater than 1.00E+07 HIV-1 RNA cp/mL.” If quantitative results are desired, the original specimen should be diluted with HIV-1 negative human EDTA plasma, and the test repeated. Multiply the reported result by the dilution factor

Note: Specimens above the range of the assay may also produce an invalid result with a flag “QS_INVALID.” If quantitative results are desired, the original specimen should be diluted with HIV-1 negative human EDTA plasma, and the test repeated. Multiply the reported result by the dilution factor

9.8.2 Data Review

For a valid batch, check each individual specimen for flags or comments on the result printout. Interpret the results as follows:

- For specimens with no flags or comments, report the results shown on the printout.
- A valid batch may include both valid and invalid specimen results depending on whether flags and/or comments are obtained for the individual specimens.
- Specimens with flags and/or comments are interpreted as follows (see Table 9.5):

Materials and Equipment Required:

- 70% ETOH
- Lint-free tissues or gauze
- Deionized water
- DNA AWAY

AmpliPrep

1. Remove K-carriers and soak in DNA AWAY for 10 min.
2. Rinse the K-carriers thoroughly with deionized water followed by 70% ETOH.
3. Wipe the inside of the main cover with a new lint-free tissue of gauze moistened with DNA AWAY.
4. Wipe the initialization post with a new lint-free tissue of gauze moistened with DNA AWAY.
5. Wipe the plastic panel at the initialization post and the plastic panel in the rear with a new lint-free tissue of gauze moistened with DNA AWAY.
6. Wipe the top surface of the rack platform with a new lint-free tissue of gauze moistened with DNA AWAY.
7. Wipe the surface of the rack loading area with a new lint-free tissue of gauze moistened with DNA AWAY.
8. Wipe the SPU incubator surfaces with a new lint-free tissue of gauze moistened with DNA AWAY.

9. Wipe all slots of the output rack platform and the separation area and target release incubator with a new lint-free tissue of gauze moistened with DNA AWAY.
10. Move the transfer head to the middle of the instrument. Wipe the surface of both wash towers with a new lint-free tissue of gauze moistened with DNA AWAY.
11. Wipe the K-carrier Park Positions with a new lint-free tissue of gauze moistened with DNA AWAY.
12. Wipe the reagent tips with a new lint-free tissue of gauze moistened with DNA AWAY.
13. Wipe the tube handler front part with a new lint-free tissue of gauze moistened with DNA AWAY.
14. Wipe the SPU gripper with a new lint-free tissue of gauze moistened with DNA AWAY.
15. Wipe the SPU gripper with a cotton swab a moistened with DNA AWAY.
16. Open the loading panel and wipe it with a new lint-free tissue of gauze moistened with DNA AWAY.
17. Repeat the procedure steps 3–17 once more with DNA AWAY.
18. Wipe the outside of the instrument with a new lint-free tissue of gauze moistened with DNA AWAY.
19. Repeat the procedure steps 3–17 twice with deionized water.
20. Repeat the procedure steps 3–17 with 70% ETOH.

TaqMan

1. Remove K-carriers, including the K-carrier that contains the dummy K-tubes.
2. Discard the dummy K-tubes.
3. Soak the K-carriers in DNA AWAY for 10 min.
4. Rinse the K-carriers thoroughly with deionized water followed by 70% ETOH.
5. Remove the K-tube waste bin and wipe it with a new lint-free tissue of gauze moistened with DNA AWAY.
6. Wipe the inside of the main cover with a new lint-free tissue of gauze moistened with DNA AWAY.
7. Wipe the waste chute and surrounding area with a new lint-free tissue of gauze moistened with DNA AWAY.
8. Wipe the brush at the waste chute with a new lint-free tissue of gauze moistened with DNA AWAY.
9. Wipe the thermal cycler covers and the areas around the thermal cycler segments with a new lint-free tissue of gauze moistened with DNA AWAY.
10. Wipe the sample loading area with a new lint-free tissue of gauze moistened with DNA AWAY.
11. Wipe inside the loading panel with a new lint-free tissue of gauze moistened with DNA AWAY.
12. Wipe the initialization post and surrounding area with a new lint-free tissue of gauze moistened with DNA AWAY.
13. Wipe the K-carrier Park Positions with a new lint-free tissue of gauze moistened with DNA AWAY.
14. Wipe the tube handling front part with a new lint-free tissue of gauze moistened with DNA AWAY.
15. Wipe the bin holder with a new lint-free tissue of gauze moistened with DNA AWAY.
16. Wipe the outside of the instrument with a new lint-free tissue of gauze moistened with DNA AWAY.
17. Repeat the procedure steps 6–16 once more with DNA AWAY.
18. Repeat the procedure steps 6–16 twice with deionized water.
19. Repeat the procedure steps 6–16 with 70% ETOH.
20. Reload the K-carriers to the same positions as before.
21. Replace the K-carriers equipped with new dummy K-tubes and insert in position 2.
22. Place a new waste bag in the K-tube waste bin and reload it to the COBAS TaqMan.

9.9 Procedural Notes

9.9.1 *Interfering Substances*

Elevated levels of triglycerides, bilirubin, albumin, hemoglobin, and human DNA in specimens as well as the presence of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and antinuclear antibody have been shown not to interfere with the quantitation of HIV-1 RNA or impact the specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0.

The following drug compounds tested at three times the peak plasma level (C_{max}) have been shown not to interfere with the quantitation of HIV-1 RNA or impact the specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0: adefovir dipivoxil (nucleotide DNA polymerase inhibitors); lamivudine 3TC, zidovudine, stavudine, 4dT, abacavir sulfate, didanosine, ddl, entecavir, tenofovir DF, telbivudine, and emtricitabine (nucleoside reverse transcriptase and DNA polymerase inhibitors); nevirapine and efavirenz (non-nucleoside HIV reverse transcriptase inhibitors); enfurvitide (HIV fusion inhibitor); maraviroc (HIV entry inhibitor); saquinavir, ritonavir, lopinavir/ritonavir, atazanavir, nelfinavir mesylate, darunavir, tipranavir, and fosamprenavir (HIV protease inhibitors); raltegravir (HIV integrase inhibitor); ribavirin, peginterferon alfa-2a, and peginterferon alfa-2b (immune modulators); and ganciclovir, valganciclovir HCL, and acyclovir (compounds for treatment of herpes viruses).

9.9.2 *Procedural Limitations*

1. This test has been validated for use with only human plasma collected in EDTA anticoagulant. Testing of other specimen types may result in inaccurate results.
2. The performance of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 has neither been evaluated with specimens containing HIV-1 group N, nor with specimens containing HIV-2.
3. Reliable results are dependent on adequate specimen collection, transport, storage, and processing procedures.
4. The presence of AmpErase enzyme in the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 Master Mix reduces the risk of amplicon contamination. However, contamination from HIV-1 positive controls and clinical specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified in this Package Insert.
5. Use of this product should be limited to personnel trained in the techniques of PCR.
6. This product can only be used with the COBAS® AmpliPrep Instrument and the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer.
7. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 demonstrated a linear range from 20 cp/mL to 1.0E+07 cp/mL for HIV-1 group M specimen in EDTA plasma and a linear range from 2.0E+02 cp/mL to 2.0E+05 cp/mL for HIV-1 group O specimen in EDTA plasma.
8. Though rare, mutations within the highly conserved regions of the viral genome covered by the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 primers and/or probes may result in the under-quantitation of or failure to detect the virus.
9. Detection of HIV-1 RNA is dependent on the number of virus particles present in the specimen and may be affected by specimen collection methods and patient factors, (i.e., age, presence of symptoms, and/or stage of the infection).
10. Due to inherent differences between technologies, it is recommended that prior to switching from one technology to the next, users perform method correlation studies in their laboratory to quantify technology differences.

11. This product exhibits a higher level of sensitivity compared to its predecessors, reporting values ≥ 20 cp/mL that were undetectable or $< \text{LLOQ}$ in prior methods. In addition, the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 returns higher titers than the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, except at both the higher range ($> 5 \log_{10}$ cp/mL) and the lower range ($< 2 \log_{10}$ cp/mL) where it returns titers that are lower.

Technical Assistance from Manufacturer

For telephone technical assistance:

Roche Technical Support Center: 1-800-526-1247

Acknowledgment Roche Molecular Diagnostic has granted permission to use a portion of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, Version 2.0 package insert, in the chapter.

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Chapter 10

Hepatitis C Virus (HCV) Genotype Assay (LiPA)

Charles E. Stager and Tavat A. Buraruk

Keywords HCV types and subtypes • Chronic infection • MagNA Pure LC • RNA extraction • Reverse transcription • Amplification • Reverse hybridization • AutoBlot 3000H • Nitrocellulose strips • Banding pattern

10.1 Introduction

HCV genotyping tests that are on the market in the USA are RUOs and include the TRUGENE® HCV genotype assay (Siemens Healthcare Diagnostics), the VERSANT® HCV Genotype 2.0 Line Probe Assay (Siemens Healthcare Diagnostics), and the eSensor® XT-8 (GenMark Diagnostics). The TRUGENE® assay is based on direct sequencing followed by comparison with a reference database. The VERSANT® assay is based on reverse hybridization of a PCR amplicon on a nitrocellulose strip coated with genotype-specific oligonucleotide probes. The eSensor XT-8 involves PCR amplification of the target nucleic acid, and then exonuclease reaction is performed to create single-stranded DNA. The target DNA is then mixed with the signal probe solution in a cartridge and analyzed via electrochemical detection. In this chapter, we will describe our modification of the VERSANT® HCV Genotype 2.0 Line Probe Assay.

10.1.1 General Testing Principle

HCV amplification is designed for use in reverse transcription and amplification of the 5' untranslated region (5' UTR) and core region of the hepatitis C virus (HCV) genome. The reverse transcription (RT) and polymerase chain reaction (PCR) amplification steps are performed sequentially in a single tube. First, genomic HCV RNA is reverse transcribed into complementary DNA (cDNA) using

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HCV-specific primers. Next, the mixture is heated to activate the DNA polymerase for the PCR amplification step and simultaneously inactivate the reverse transcriptase. Portions of the 5' UTR and core regions of the HCV genome are coamplified from the cDNA using two pairs of biotinylated primers to produce two distinct biotinylated DNA fragments of 240 and 270 base pairs, representing the 5' UTR and core HCV regions respectively [1].

HCV genotype assay (LiPA) utilizes reverse hybridization. Biotinylated DNA PCR product generated by RT-PCR amplification of the 5' UTR and core region of HCV RNA is hybridized to immobilized oligonucleotide probes. The probes, which are bound to a nitrocellulose strip by a poly(T) tail, are specific for the 5' UTR and core region of different HCV genotypes. After the hybridization step, unhybridized PCR product is washed from the strip, and alkaline phosphatase-labeled streptavidin (conjugate) is bound to the biotinylated hybrid. BCIP/NBT chromogen (substrate) reacts with the streptavidin-alkaline phosphatase complex forming a purple/brown precipitate, which results in a visible banding pattern on the strip [1].

10.2 Clinical Significance

HCV infection is the most common chronic blood-borne infection in the USA with an estimated seroprevalence of 1.6% [2]. An estimated 3.2 million Americans suffer from chronic HCV infection, making it the leading cause of chronic liver disease [3]. An estimated 85% of those with acute hepatitis C progress to chronic infection, of which 20–25% will develop complications such as cirrhosis within two to three decades of its onset [4]. Of the patients with cirrhosis, a smaller percentage will progress to decompensated liver disease, hepatocellular carcinoma, and death [5]. In the USA, chronic hepatitis C (CHC) is responsible for an estimated 8,000–10,000 deaths per year and is the leading cause of liver transplantation [3]. The burden of HCV-associated disease is likely to increase during the next 10–20 years as the infected cohort reaches an age at which complications of liver disease typically occur [6].

The hepatitis C viral genome is highly variable and is classified into six genotypes groups, or clades, based on the phylogenetic analysis of the genome sequence. These genotype groups differ at 31–34% of their nucleotide sequence positions and about 30% of their amino acid sequence positions [7]. Each genotype contains one or more HCV subtypes or variants.

Subtypes (e.g., a, b, and c) of a specific HCV genotype differ by 20–23% at the nucleotide sequence level from other members of the genotype. An exception is subtype 3k (previously classified as 10a), which differs by 25% at the nucleotide sequence level from the other members of genotype 3. Currently, more than 70 HCV subtypes have been identified [8].

In the past, genotypes 7, 8, 9, 11, and 10 have been described in the scientific literature as separate genotypes, but the current recommendation is that genotypes 7, 8, 9, and 11 should be classified as subtypes 6c–6l of the genotype 6 and genotype 10 should be classified as subtype 3k of genotype 3 [9].

The HCV genome consists of core (C), envelope (E), and nonstructural (NS) domains. The 5' untranslated region (UTR), which is also called the noncoding region (NCR), and core region are mostly highly conserved. The envelope regions are the most variable. The 5' UTR contains multiple genotype-specific motifs distributed over seven small variable regions that can provide accurate genotyping information for the genotypes 1–5 and genotype 6, subtypes a and b. However, due to the high sequence similarity, genotype 6 and subtypes c to l (previously classified as 7, 8, 9, and 11), cannot be distinguished from genotype 1 by analysis of the 5' UTR alone [10]. The accuracy of genotype 1a and 1b subtyping ranges from 80% to 95% by 5' UTR analysis, depending on the specific isolates tested [11]. The LiPA HCV genotype uses sequence motifs from the core region in addition to 5' UTR to identify genotype 6, subtypes c to l. Additional core motifs are included to improve the accuracy of the identification of 1a and 1b.

10.3 Methodology

10.3.1 Specimen Collection and Handling

This protocol was developed at Ben Taub General Hospital.

Preferred specimen: Plasma from freshly drawn whole blood in EDTA anticoagulant received from the specimen processing area with patient identification, sample identity, and date and time of collection. Plasma from EDTA must be separated from whole blood by centrifugation at 800–1,600×g for 20 min at room temperature *within 6 h of collection*. Transfer specimen to a polypropylene tube. EDTA plasma specimens may be stored at 2–8°C for up to 5 days or frozen at –70°C or colder up to 6 weeks. Specimens may be frozen and thawed up to five times without a loss of HCV RNA. Never return aliquot to the original container. Specimen is saved for 3 days at –70°C after the result is finalized.

Other acceptable: None

Minimum sample volume: 2 mL of plasma (EDTA)

Optimum sample volume: 4 mL of plasma (EDTA)

Criteria for unacceptable specimen:

- Not collected in EDTA
- Not processed within 6 h of collection
- Clotted
- Stored at 2–8°C for more than 3 days
- Frozen at –70°C for more than 6 weeks
- Frozen and thawed for more than five times
- Improperly identified (discrepancies in I.D. number, tube, and name)
- Inadequate volume of sample
- Improperly collected test (specimen collected in the wrong collection tube)
- Test requests with incomplete information (i.e., attending physician, time of collection, date of collection)
- Shared specimen

Reject specimen and request recollection.

Handling condition:

Plasma or serum must be separated from whole blood within 6 h after collection. Testing must be performed as soon as possible. *Specimen(s) should be treated as if infectious and should be handled as if they are capable of transmitting infectious disease.* No aliquot is ever returned to the original container.

10.3.2 Materials and Equipment Required

- MagNA Pure LC instrument (Roche #2 236 931)
- Micropipettes capable of 5–200 µL with plug tips
- AutoBlot 3000H from MedTec, Inc.
- Thermal cycler
- Vortex (Allegiance # S8248-1)
- Microcentrifuge
- 1.5-mL screw cap tubes sterile (Sarstedt # 72.692.005)
- Tube rack (Sarstedt # 93.1428)
- 10-mL sterile disposable pipettes

Table 10.1 MagNA Pure LC total nucleic acid isolation kit

Vial	Label	Contents/function
Black cap	Wash buffer I	Two bottles with 100 mL each for removing of PCR inhibitors
Blue cap	Wash buffer II	One bottle with 100 mL for removing of salts, proteins, etc.
Red cap	Wash buffer III	Two bottles with 100 mL each for removing of salts etc.
Green cap	Lysis/binding buffer	One bottle with 100 mL for cell lysis and binding of total nucleic acid
Pink cap	Proteinase K	Six glass vials with lyophilizate for digestion of proteins
Caramel cap	Magnetic glass particles (MGPs) suspension	Six glass vials with 6 mL each for binding of total nucleic acid
Yellow cap	Elution buffer	One bottle with 100 mL for elution of pure total nucleic acid

- Micropipettes capable of 100–1,000 μ L with plug tips
- Deionized water
- 50-mL polypropylene tubes
- Plastic forceps
- Timers
- 20 strip trays

10.3.3 Reagents (See Table 10.1)

Siemens HCV amplification

- HCV amplification mix 2.0 (LiPA) 2×1.4 mL (**Siemens # 00187400**)
- Enzyme mix 2.0 (LiPA) 1×440 μ L (**Siemens #00186226**)

Nucleic acid hybridization pack (Siemens# 06681083)

- Hybridization/stringent wash solution 5×220 mL
- Denatured solution 2×1 mL

Color development pack (Siemens # 06683787)

- Conjugate 100 \times , 1×3 mL
- Conjugate diluent 2×150 mL
- Substrate 100 \times , 1×3 mL
- Substrate buffer 3×180 mL
- Rinse solution $5 \times 2 \times 150$ mL

Probe arrays HCV 5' UTR/CORE strips 2.0 (Siemens# 06724831)

- Nitrocellulose strips coated with 22 DNA probe line (100 strips)

Validation of new reagents

New reagent lot and/or shipments are tested on positive and negative controls, and results must be as expected.

Calibration

Due to the qualitative nature of this test, no calibration is necessary.

10.3.4 *Quality Control*

Control frequency: Include negative and positive controls with each batch. Positive control and negative control will monitor for reagent, extraction, and amplification failure, and negative control monitors for reagent and/or environmental contamination.

Positive control: Known positive sample

Negative control: Nuclease-free water

Tolerance limits: Positive control must be positive and negative control must be negative.

Corrective actions: If controls are not within the specified limits, take the following corrective action:

- If positive controls are flagged as invalid, then the entire batch is invalid. No result will be reported. Repeat the entire process (specimen and control preparation, amplification, and detection).
- If negative control is positive, the decontamination control protocol must be followed (see decontamination protocol). No result will be reported. Repeat the entire process (specimen and control preparation, amplification, and detection).

External controls: CAP Surveys. Include with the routine workload.

10.3.5 *Procedure Steps*

RNA extraction is performed using the MagNA Pure LC.

10.3.5.1 **Reagents**

- MagNA Pure LC total nucleic acid isolation kit (03 038 505 001)

10.3.5.2 **Protocol**

- *Preparation of reagents (All reagents must be at room temperature before use.)*
- *Proteinase K (32 reactions):*
 1. For 32 reactions, dissolve one bottle proteinase K (pink cap) by adding 5.0 mL elution buffer (yellow cap). Close the vial, and then mix well.
 2. Stable at 2–8°C up to 4 weeks.
- *Sample preparation:*
 1. Switch on the MagNA Pure LC instrument, then the computer.
 2. Start the MagNA Pure LC software, then at the Main Menu screen, click on the Sample Ordering button.
 3. At the Sample Ordering screen, select the protocol “Total NA Serum Plasma Blood,” then “Total NA Variable elution_volume” type. Enter the lot numbers of the MagNA Pure and HSV kit.
 4. Enter 200 µL as the sample volume, 50 µL as the elution volume, and 0 µL as the dilution volume.
 5. Type in sample name and I.D. Print sample name function to produce a printout showing the sample names and their position in the sample cartridge.
 6. Click on Stage Setup.

Table 10.2 Preparing the working master mix (ratio of ENZ MIX to AMP MIX is 1 part to 6.5 parts)

Reactions	AMP MIX (μL)	ENZ MIX (μL)
1	13	2
2	26	4
3	39	6
4	52	8
5	65	10
6	78	12
7	91	14
8	104	16
9	117	18
10	130	20

- Manually fill the reagent tubs with the volumes indicated on the Stage Layout Graphic except the magnetic glass particles (MGPs). Cap them with reagent tub lids. Place the filled reagent tubs into the reagent reservoir rack at the position indicated on the Stage Layout Graphic.
- Place the necessary disposable plastics and the prepared reagent reservoir rack on the reagent/sample stage according to Stage Layout Graphic.
- Outside the MagNA Pure, manually transfer 200 μL of sample and controls into the appropriate well of sample cartridge. Place the sample cartridge into the MagNA Pure.
- When the status of the heat unit and the cool unit 1 & 2 displays PASS, vortex the MGP vial and pour into an MGP reagent tub immediately before starting the run.
- Confirm every loaded position on the MagNA Pure via mouse click on the screen. After all items are confirmed, the OK button will appear. Close the lock bar and the door of the MagNA Pure.
- Click OK button to start the run.
- The Result screen will appear after the run is completed. Save and print the Result screen.
- Store the nucleic acids immediately at -70°C to minimize degradation (stable for 1 year when stored at -70°C).

10.3.5.3 Amplification

Reagents

- HCV amplification mix 2.0 (LiPA) $2 \times 1.4 \text{ mL}$ (**Siemens # 00187400**)
- Enzyme mix 2.0 (LiPA) $1 \times 440 \mu\text{L}$ (**Siemens #00186226**)

Reagents preparation for RT-PCR (see Table 10.2)

- Add 15 μL of working master mix to each PCR reaction tube.
- Then add 10 μL of extracted RNA to the PCR reaction tube.
- Place the tubes at room temperature for $5 \pm 1 \text{ min}$ prior to amplification to allow degradation of any contaminating uracil containing DNA.
- Place tray in thermal cycler.
- Select program HCV-LiPA with the following program.
- Remove tubes from the thermal cycler; store the amplicons at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ or proceed immediately to the *nucleic acid hybridization (GPR) step*.

Reverse transcriptase HOLD	50°C	30 min
Initial PCR activation step HOLD	95°C	15 min
Amplification 40 cycles	95°C	30 s
	50°C	30 s
	72°C	15 s
Final extension HOLD	72°C	2 min
Cooling HOLD	4°C	∞
Overnight PCR reaction is acceptable. Keep the samples at 4°C after the last cycle has terminated.		

10.3.5.4 Detection

Nucleic acid hybridization pack (Siemens# 06681083)

- Hybridization/stringent wash solution 5 × 220 mL
- Denatured solution 2 × 1 mL

Color development pack (Siemens # 06683787)

- Conjugate 100×, 1 × 3 mL
- Conjugate diluent 2 × 150 mL
- Substrate 100×, 1 × 3 mL
- Substrate buffer 3 × 180 mL
- Rinse solution 5×, 2 × 150 mL

Probe arrays HCV 5 UTR/CORE strips 2.0 (Siemens# 06724831)

- Nitrocellulose strips coated with 22 DNA probe line (100 strips).
- See Table 10.3 for a list of reagents preparation for the HCV Genotype 2.0 LiPA.

10.3.5.5 Interpretation: Reporting Results

Using the Interpretation Chart

Part A of the interpretation chart is used to interpret genotype 1, subtypes a and b of genotype 1, and those genotype 6 patterns that are identified using both the 5' UTR and the core region. Part B of the interpretation chart is used to interpret genotypes 2–5 and those genotype 6 patterns that are distinguishable from genotype 1 using the 5' UTR only. No calculations are performed. Results are reported as HCV types.

Limit of detection: 1,100 IU/mL (validated at Ben Taub General Hospital using SeraCare Life Sciences HCV genotype performance panel)

Decontamination protocol

Materials and equipment required:

- 70% ETOH
- Lint-free tissues or gauze
- Deionized water
- DNA AWAY

Table 10.3 Reagents preparation for HCV Genotype 2.0 LiPA

Step	Action
1.	Remove the nucleic acid hybridization, color development pack, and LiPA 2.0 probe arrays from the refrigerator and allow to reach room temperature (15–30°C) before use.
2.	Prewarm the determined volume of the hybridization/stringent wash solution (HYB/SW SOLN) needed to complete a run on the AutoBlot 3000H heater from 37°C to 50°C for approximately 60 min, and mix well before use to dissolve the crystals.
3.	Prepare rinse solution, conjugate solution, and substrate solution according to the table below.

No. of strips	Diluted rinse solution (1:5)		Diluted conjugate (1:100)		Diluted substrate (1:100) protect from light		Substrate buffer		Hybridization and stringent wash solution	
	dH ₂ O (mL)	Rinse solution (mL)	CONJ DIL (mL)	CONJ (μL)	SUBS BUFFER (mL)	SUBS BCIP/ NBT (μL)	SUBS BUFFER (mL)		HYB/SW SOLN (mL)	
5	60	15	15	150	15	150	20		55	
6	72	18	17	170	17	170	24		63	
7	84	21	19	190	19	190	28		71	
8	96	24	21	210	21	210	32		79	
9	108	27	23	230	23	230	36		95	
10	120	30	25	250	25	250	40		103	
11	132	33	27	270	27	270	44		111	
12	144	36	29	290	29	290	48		119	
13	156	39	31	310	31	310	52		127	
14	168	42	33	330	33	330	56		135	
15	180	45	35	350	35	350	60		143	
16	192	48	42	420	42	420	64		151	
17	204	51	44	440	44	440	68		159	
18	216	54	46	460	46	460	72		167	
19	228	57	48	480	48	480	76		175	
20	240	60	50	500	50	500	80		183	
21	252	63	52	520	52	520	84		191	
22	264	66	54	540	54	540	88		199	
23	276	69	56	560	56	560	92		207	
24	288	72	58	580	58	580	96		215	
25	300	75	60	600	60	600	100		223	

4.
- Turn the AutoBlot 3000H on by pressing the ON/OFF switch on the back of the unit.
5.
- At the Ready for a New Test prompt, press **YES**.
6.
- At the Run Assay prompt, press **ENTER**.

7.

At the Pump Pads in Place prompt, latch pump pads then press **YES**.

8.

At the Preheat System, press **YES**.

9.

Close the tray cover and press **ENTER**. The system starts preheating the tray platform and the bottle plate for 30 min.

10.

Add a stir bar to the **HYB/SW SOLN** container and place it on the heated bottle plate.

11.

Place reagents in the AutoBlot 3000H reagent holder in the following order from left to right.
- | Tube no. | Reagents |
|----------|----------------------------|
| 1 | Diluted rinse solution |
| 2 | Substrate buffer |
| 3 | Diluted substrate solution |
| 4 | Diluted conjugate |
| 5 and 6 | HYB/SW SOLN |
12.

When the preheat cycle is completed, the AutoBlot 3000H will sound the alarm to indicate the preheat cycle is done. At the Put Tubes in the Bottles prompt, insert tubes in the reagent bottles then press **ENTER**.
13.

At the Prime Pumps prompt, press **YES**.
14.

At the Prime Pump1 Tube prompt, press **YES**. Pump1 will prime.
15.

At the Prime Pump1 again, press **YES**.
16.

At the Prime Pump1 again, press **NO**.
17.

At the Prime Pump2 Tube prompt, press **YES**. Pump2 will prime.
18.

At the Prime Pump2 again, press **YES**.
19.

At the Prime Pump2 again, press **NO**.
20.

At the Prime Pump3 Tube prompt, press **YES**. Pump3 will prime.
21.

At the Prime Pump3 again, press **YES**.
22.

At the Prime Pump3 again, press **NO**.
23.

At the Prime Pump4 Tube prompt, press **YES**. Pump4 will prime.
24.

At the Prime Pump4 again, press **YES**.
25.

At the Prime Pump4 again, press **NO**.
26.

At the Prime Pump5 Tube prompt, press **YES**. Pump5 will prime.
27.

At the Prime Pump5 again, press **YES**.
28.

At the Prime Pump5 again, press **NO**.
29.

At the Prime Pump6 Tube prompt, press **YES**. Pump6 will prime.
30.

At the Prime Pump6 again, press **YES**.
31.

At the Prime Pump6 again, press **NO**.
32.

At the Prime Pumps prompt, press **NO**.
33.

At the Strip Count prompt, enter the strip number by pressing **YES** to increase or **NO** to decrease the number of strips, then press **ENTER**.

(continued)

Table 10.3 (continued)

Step	Action
34.	Allow amplification DNA samples to reach room temperature (15–30°C).
35.	Write the sample identification on the worksheet.
36.	Use clean dedicated plastic forceps to remove the appropriate number of probe arrays strips from the container by gripping each above the green marker (1 strip per sample). DO NOT TOUCH STRIP WITH HAND.
37.	Use a pencil to label the top of strip above the green mark line with an identification number.
38.	Place the labeled strips in the upper portion of the trough, 1 trough per strip, with the green marker lines facing up at the top of the tray.
39.	Load strips 1–10 beginning on the trough immediately to the right of the center trough.
40.	Load strips 11–20 beginning on the trough immediately to the left of the center trough.
41.	Place the negative control strip in a trough between two troughs where sample strips will be processed.
42.	Pipette 10 µL of denatured solution (DENAT SOLN) taken from the nucleic acid hybridization pack into the lower end of each trough. Close vial immediately after use.
43.	Add 10 µL of the appropriate amplified DNA sample to the denatured solution in the trough and mix by pipetting up and down approximately three times.
44.	Incubate the tray at room temperature (15–30°C) for 10 min to denature the amplified DNA.
45.	At the Start Assay prompt, press YES .
46.	At the Enter Load Tray, press ENTER . Open tray cover and quickly load tray.
47.	Close tray. At the Close Tray Cover prompt, press ENTER .
48.	The HCV protocol begins (takes approximately 3.5 h).
49.	At the Aspirate Now prompt, press YES .
50.	Remove the tray from AutoBlot 3000H.
51.	At the Purge Tubing Now prompt, press YES .
52.	At the Put Tubes in the Cleaner, place all tubes in the 2% bleach then press ENTER .
52.	At the Put Tubes in DIH ₂ O, remove all tubes from 2% bleach and place them in deionized water then press ENTER .
53.	At the Remove Tubes from DIH ₂ O prompt, remove all tubes from deionized water and place in the empty container and press ENTER .
54.	At the Clean Drip Tray prompt, open the lid and dry the drip tray by using Kimwipes or gauze then press ENTER .
55.	At the Release Pressure Pad prompt, release all the latches from the back of AutoBlot 3000H then press ENTER . Turn off the AutoBlot 3000H.
56.	Place the blank tray back on AutoBlot 3000H.
57.	Empty all the bottles and rinse with deionized water.
58.	Wipe AutoBlot 3000H with deionized dampened gauze.

MagNA Pure LC system, GeneAmp 9700, and LightCycler system

1. Soak MagNA Pure reagent racks in DNA AWAY for 10 min.
2. Rinse the MagNA Pure reagent racks thoroughly with deionized water followed by 70% ETOH.
3. Wipe the inside of MagNA Pure reagent and sample stage and door handle with a new lint-free tissue or gauze moistened with DNA AWAY.
4. Wipe the GeneAmp 9700 with a new lint-free tissue or gauze moistened with DNA AWAY.
5. Wipe the exterior surface of AutoBlot 3000 with a new lint-free tissue or gauze moistened with DNA AWAY.
6. Repeat the procedure steps 3–5 once more with DNA AWAY.
7. Repeat the procedure steps 3–5 twice with deionized water.
8. Repeat the procedure steps 3–5 with 70% ETOH.

10.4 Limitations of Method: Interfering Substances and Conditions

The controls are for monitoring assay performance and are not for use as calibrators or as a primary reference in the assay. The positive control contains a portion of the HCV viral sequence inserted into an armored RNA construct and may not function for extraction and amplification methods that require the entire HCV viral particle or HCV-specific sequences that are not included in the armored RNA construct correct results. In rare cases, unidentifiable patterns may be produced. These may be due to the sequence heterogeneity of the HCV genome, mixed infections, or cross contamination.

Acknowledgement Siemens Healthcare Diagnostics has granted permission to use a portion of the VERSANT® HCV Genotype (Product 2.0) Line Probe Assay package insert in this chapter.

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Chapter 11

COBAS® AmpliPrep/COBAS® TaqMan® HCV Test

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Keywords Active viremia • Chronic infection • Peginterferons plus ribavirin • Sustained virologic response • COBAS® AmpliPrep • COBAS® TaqMan® • HCV RNA quantitation • Titer result interpretation

11.1 Introduction

There are four HCV RNA quantitative assays on the market in the USA, all of which are FDA approved. The four assays are: VERSANT® HCV RNA 3.0 Assay (Siemens Healthcare Diagnostics, Deerfield, IL); COBAS® AmpliPrep/COBAS® TaqMan® (Roche Molecular Diagnostics, Pleasanton, CA); COBAS® TaqMan® HCV Test, v2.0 with High Pure System (Roche Molecular Diagnostics, Pleasanton, CA); and Abbott RealTime™ HCV m2000rt (Abbott Molecular, Des Plains, IL). For these assays, a listing of the method, limit of detection and linear range is found in Table 11.1. In this chapter, we will describe our modification of the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test.

NOTE: This chapter contains excerpts only from the package insert for the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test, Doc Rev. 4.0 (07/2011). Please note that this test should not be performed without first reviewing the complete and most current version of the package insert that accompanies the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test kit. If you have any questions about this test or the package insert, you should contact Roche Molecular Diagnostics Technical Support at 1-800-526-1247 or visit www.mylabonline.com.

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Table 11.1 FDA-approved quantitative HCV RNA assays

Assay	Method	Lower limit of detection	Dynamic range
Versant HCV RNA 3.0 assay (bDNA)	Signal amplification (bDNA)	615 IU/mL	615 IU/mL to -7.7×10^6 IU/mL
COBAS AmpliPrep/COBAS TaqMan HCV Test	Fully automated RT-PCR	15 IU/mL	43 IU/mL to -6.9×10^7 IU/mL
COBAS TaqMan HCV Test V2.0 with High Pure System	Semiautomated RT-PCR	25 IU/mL	25 IU/mL to -3×10^8 IU/mL
Abbott Real-Time HCV	Semiautomated RT-PCR	12 IU/mL	12 IU/mL to -1×10^8 IU/mL

11.2 Intended Use

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test is an in vitro nucleic acid amplification test for the quantitation of Hepatitis C viral (HCV) RNA in human plasma or serum of HCV-infected individuals using the COBAS® AmpliPrep Instrument for automated specimen processing and the COBAS® TaqMan® Analyzer or the COBAS® TaqMan® Analyzer for automated amplification and detection. Specimens containing HCV genotypes 1–6 have been validated for quantitation in the assay.

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test is intended for use as an aid in the management of HCV-infected individuals undergoing antiviral therapy. The assay measures HCV RNA levels at baseline and during treatment and can be utilized to predict sustained and non-sustained virological response to HCV therapy. The results from the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test must be interpreted within the context of all relevant clinical and laboratory findings.

Assay performance characteristics have been established for individuals treated with peginterferon alfa-2a plus Ribavirin. No information is available on the assay's predictive value when other therapies are used. Assay performance for determining the state of HCV infection has not been established.

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test is not intended for use as a screening test for the presence of HCV in blood or blood products or as a diagnostic test to confirm the presence of HCV infection [1].

11.3 Principles of the Procedure

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test is a nucleic acid amplification test for the quantitation of HCV RNA in human serum or plasma. Specimen preparation is automated using the COBAS® AmpliPrep Instrument with amplification and detection automated using the COBAS® TaqMan® Analyzer or the COBAS® TaqMan® 48 Analyzer.

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test is based on three major processes: (1) specimen preparation to isolate HCV RNA; (2) reverse transcription of the target RNA to generate complementary DNA (cDNA); and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide detection probe specific to the target.

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test permits automated specimen preparation followed by automated reverse transcription, PCR amplification, and detection of HCV target RNA and HCV Quantitation Standard (QS) Armored RNA. The Master Mix reagent contains primers and probes specific for both HCV RNA and HCV QS Armored RNA. The Master Mix has been developed to ensure similar quantitation of HCV genotypes 1 through 6. The detection of amplified DNA is performed using a target-specific and a QS-specific dual-labeled oligonucleotide probe that permit independent identification of HCV amplicon and HCV QS amplicon.

The quantitation of HCV viral RNA is performed using the HCV QS. The HCV QS compensates for effects of inhibition and controls the preparation and amplification processes, allowing a more accurate quantitation of HCV RNA in each specimen. The HCV QS is a noninfectious Armored RNA construct

that contains HCV sequences with identical primer binding sites as the HCV target RNA and a unique probe binding region that allows HCV QS amplicon to be distinguished from HCV target amplicon.

The HCV QS is added to each specimen at a known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification, and detection of cleaved dual-labeled oligonucleotide detection probes. The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer calculates the HCV RNA concentration in the test specimens by comparing the HCV signal to the HCV QS signal for each specimen and control [1].

11.3.1 Target Selection

Selection of the target RNA sequence for HCV depends on identification of regions within the HCV genome that show maximum sequence conservation among the various HCV genotypes. Generic silica-based specimen preparation is used to capture the HCV RNA and HCV QS RNA, and defined oligonucleotides are used as primers in amplification of the HCV RNA and HCV QS RNA. A target-specific and a QS-specific dual-labeled oligonucleotide probe permit independent identification of HCV amplicon and HCV QS amplicon. Accordingly, the appropriate selection of the primers and the dual-labeled oligonucleotide probe is critical to the ability of the test to amplify and detect the HCV genotypes. The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test uses reverse transcription and PCR amplification primers that define a sequence within the highly conserved region of the 5'-untranslated region of the HCV genome. The nucleotide sequence of the primers has been optimized to yield comparable amplification of six HCV genotypes [1].

11.4 Methodologies

11.4.1 Specimen Preparation

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test utilizes automated specimen preparation on the COBAS AmpliPrep Instrument by a generic silica-based capture technique. The procedure processes 850 µL of plasma or serum. The HCV virus particles are lysed by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HCV RNA from RNases in serum or plasma. Protease and a known number of HCV QS RNA molecules are introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture is incubated and the HCV RNA and HCV QS RNA are bound to the surface of the magnetic glass particles. Unbound substances, such as salts, proteins, and other cellular impurities, are removed by washing the magnetic glass particles. After separating the beads and completing the washing steps, the adsorbed nucleic acids are eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HCV RNA and HCV QS RNA, is added to the amplification mixture and transferred to the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer. The HCV target RNA and the HCV QS RNA are then reverse transcribed, amplified, and simultaneously detected by cleavage of a target-specific and a QS-specific dual-labeled oligonucleotide probe [1].

11.4.2 Reverse Transcription and PCR Amplification

The reverse transcription and PCR amplification reaction is performed with the thermostable recombinant enzyme *Thermus specie* DNA polymerase (Z05). In the presence of manganese (Mn²⁺) and

under the appropriate buffer conditions, Z05 has both reverse transcriptase and DNA polymerase activity¹². This allows both reverse transcription and PCR amplification to occur together with real-time detection of the amplicon. Processed specimens are added to the amplification mixture in amplification tubes (K-tubes) in which both reverse transcription and PCR amplification occur. The reaction mixture is heated to allow a downstream primer to anneal specifically to the HCV target RNA and to the HCV QS RNA. In the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxyuridine triphosphates, Z05 polymerase extends the annealed primers, forming a DNA strand complementary to the RNA target [1].

11.4.3 Target Amplification

Following reverse transcription of the HCV target RNA and the HCV QS RNA, the Thermal Cycler in the COBAS® TaqMan® instrument or COBAS® TaqMan® 48 instrument heats the reaction mixture to denature the RNA:cDNA hybrid and to expose the specific primer target sequences. As the mixture cools, the primers anneal to the target DNA. The thermostable *Thermus specie* Z05 DNA polymerase (Z05) in the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxyuridine (in place of thymidine) triphosphates, extends the annealed primers along the target template to produce a double-stranded DNA molecule termed an amplicon. The COBAS® TaqMan® instrument or COBAS® TaqMan® 48 instrument automatically repeats this process for a designated number of cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is pre-programmed into the COBAS® TaqMan® instrument or COBAS® TaqMan® 48 instrument. Amplification occurs only in the region of the HCV genome between the primers; the entire HCV genome is not amplified [1].

11.4.4 Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). The AmpErase enzyme solution recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also, any nonspecific product formed after initial activation of the Master Mix by manganese is destroyed by the AmpErase enzyme. The AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1 position. When heated in the first thermal cycling step, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA nonamplifiable. The AmpErase enzyme remains inactive for a prolonged period of time once exposed to temperatures above 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon formed after PCR reaction [1].

11.4.5 Detection Reaction

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test utilizes real-time PCR technology. The use of dual-labeled fluorescent probes allows for real-time detection of PCR product accumulation by

monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HCV- and HCV QS-specific oligonucleotide probes with a reporter dye and a quencher dye. In the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test, the HCV and HCV QS probes are labeled with different fluorescent reporter dyes. When these probes are intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and is cleaved by the 5'→3' nuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplifications of HCV RNA and HCV QS RNA are measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HCV RNA and HCV QS RNA. The PCR cycle where a growth curve starts exponential growth is related to the amount of starting material at the beginning of the PCR [1].

11.4.6 HCV RNA Quantitation

The COBAS® AmpliPrep/COBAS® TaqMan® HCV Test is inherently quantitative over a wide dynamic range since the monitoring of amplicon is performed during the exponential phase of amplification. The higher the HCV titer of a specimen, the earlier the fluorescence of the reporter dye of the HCV probe rises above the baseline fluorescence level. Since the amount of HCV QS RNA is constant between all specimens, the fluorescence of the reporter dye of the HCV QS probe should appear at the same cycle for all specimens. In specimens, where the QS amplification and detection is affected by inhibition or poor specimen recovery, the appearance of fluorescence will be delayed, thereby enabling the calculated titer of HCV target RNA to be adjusted accordingly. The appearance of the specific fluorescent signals is reported as a critical threshold value (Ct). The Ct is defined as the fractional cycle number where reporter dye fluorescence exceeds a predetermined threshold (the Assigned Fluorescence Level), and starts the beginning of an exponential growth phase of this signal. A higher Ct value indicates a lower titer of initial HCV target material. A twofold increase in titer correlates with a decrease of 1 Ct for target HCV RNA; a tenfold increase in titer correlates with a decrease of 3.3 Ct [1].

11.4.7 Clinical Significance

The World Health Organization estimates that 170 million persons worldwide are infected with hepatitis C virus [2]. In the USA, between the years 1999 and 2002, approximately 4.1 million persons were sero-positive to hepatitis C, of whom 80% were estimated to be viremic [3]. In the USA, HCV is the leading cause of cirrhosis, hepatocellular carcinoma, and liver transplantation [4].

Combination therapy with pegylated interferon and ribavirin has been the standard of care for the treatment of hepatitis C. The HCV genotype is used to determine the required length and potential response to interferon-ribavirin therapy. During the acute phase of genotype 1 infections, treatment has a greater than 90% success rate, with half the treatment time required for those with chronic infection [5]. HCV causes chronic infection in 50–80% of infected persons and the majority will not clear their infection without treatment [6]. Genotype 1 is the most common genotype worldwide and for patients with chronic hepatitis has been the least responsive to therapy with pegylated interferon and ribavirin with less than 50% demonstrating a sustained virologic response (SVR) when given 48 weeks of treatment. If treatment does not provide a 2-log viral load reduction or complete clearance of RNA after 12 weeks, the chance of treatment success is less than 1% [7]. The response of genotype 6 to therapy is

similar to that of genotype 1 [8]. Genotypes 2 and 3 are the most responsive, with an SVR rate of 75% or greater within 24 weeks of treatment [9, 10]. Patients with genotypes 2 or 3 demonstrating HCV viral loads below 1,000 IU/mL by day 7 can be treated for as little as 12 weeks [11]. The Food and Drug Administration, in May, 2011, approved boceprevir and telaprevir for therapy of hepatitis C. Studies with boceprevir in conjunction with peginterferon-ribavirin as therapy for untreated chronic HCV genotype 1 infection [12] and previously treated chronic HCV genotype 1 infection [13] have shown an improved SVR at 44 weeks for boceprevir compared to therapy with peginterferon-ribavirin alone.

11.5 Specimen Requirements

11.5.1 Specimen Collection and Handling

Preferred specimen: freshly drawn whole blood in EDTA anticoagulant or SST® Serum Separation Tubes received from the specimen processing area with patient identification, sample identity, and date and time of collection. Plasma from EDTA or serum from SST must be separated from whole blood by centrifugation at 800–1,600 × *g* for 20 min at room temperature *within 6 h of collection*. Transfer specimen to a polypropylene tube.

EDTA plasma or SST serum specimens may be stored at 2–8°C for up to 3 days or frozen at –70°C or colder up to 6 weeks. Specimens may be frozen and thawed up to five times without a loss of HCV RNA. Never return aliquot to the original container. Specimen is saved for 3 days at –70°C after the result is finalized.

Other acceptable: serum from SST (serum separation tubes).

Minimum sample volume: 2 mL of plasma (EDTA) or serum from SST.

Optimum sample volume: 4 mL of plasma (EDTA) or serum from SST.

Criteria for unacceptable specimen:

- Not collected in EDTA or SST
- Not processed with 6 h of collection
- Clotted (EDTA tube)
- Stored at 2–8°C more than 3 days
- Frozen at –70°C more than 6 weeks
- Frozen and thawed more than five times
- Improperly identified (discrepancies in I.D. number, tube, name)
- Inadequate volume of sample
- Improperly collected test (specimen collected in the wrong collection tube)
- Test requests with incomplete information (i.e., attending physician, time of collection, date of collection)
- Share specimen

Reject specimen and request recollection.

11.5.1.1 Handling Condition:

Plasma or serum must be separated from whole blood within 6 h after collection. Testing must be performed as soon as possible. ⚠ *Specimen(s) should be treated as if infectious and should be handled as if they are capable of transmitting infectious disease.* No aliquot is ever returned to the original container.

11.5.2 Reagent Composition (see Table 11.2)

Table 11.2 COBAS® AmpliPrep/COBAS® TaqMan® HCV Test includes the following reagents

Reagents		Compositions
HCV magnetic glass particles reagent cassette	HCV CS1	Magnetic glass particles 93% Isopropanol Xi 93% (w/w) isopropanol F 93% (w/w) isopropanol
HCV lysis reagent cassette	HCV CS2	Sodium citrate dihydrate 42.5% Guanidine thiocyanate <14% Polydocanol 0.9% Dithiothreitol Xn 42.5% (w/w) guanidine thiocyanate
HCV multireagent cassette containing: Proteinase solution (Pase)	HCV CS3	Tris buffer <0.05% EDTA Calcium chloride Calcium acetate ≤7.8% Proteinase Glycerol Xn ≤7.8% (w/w) proteinase
Elution buffer (EB)		Tris-base buffer 0.2% Methylparaben
HCV Test-specific Reagent cassette containing: HCV quantitation standard (HCVQS)	HCV CS4	Tris-HCl buffer EDTA <0.002% Poly rA RNA (synthetic) <0.001% Armored HCV RNA construct containing HCV primer binding sequences and a unique probe binding region (noninfectious RNA in MS2 bacteriophage) 0.05% Sodium azide
HCV master mix (HCV MMX)		Tricine buffer Potassium acetate Potassium hydroxide <20% Dimethyl sulfoxide Glycerol <0.004% dATP, dCTP, dGTP, dUTP <0.002% Upstream and downstream HCV primers to the 5' UTR region of HCV <0.001% Fluorescent-labeled oligonucleotide probes specific for HCV and the HCV Quantitation Standard <0.001% Oligonucleotide aptamer <0.05% Z05 DNA polymerase (microbial) <0.1% AmpErase (uracil- <i>N</i> -glycosylase) enzyme (microbial) 0.09% Sodium azide
CAP/CTM manganese solution	CAP/CTM Mn2+	<0.5% Manganese acetate Glacial acetic acid 0.09% Sodium azide

(continued)

Table 11.2 (continued)

Reagents		Compositions
HCV high positive control	HCV H(+) C	<0.001% Armored HCV RNA construct containing HCV sequences (noninfectious RNA in MS2 bacteriophage) Lot-specific concentration range is encoded on the COBAS AmpliPrep/ COBAS TaqMan HCV Test reagent cassette barcodes Negative human plasma, nonreactive by US FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HbsAg; HIV-1 RNA, HCV RNA, and HBV DNA not detectable by PCR methods 0.1% ProClin® 300 preservative
HCV low (+) control	HCV L(+) C	<0.001% Armored HCV RNA construct containing HCV sequences (noninfectious RNA in MS2 bacteriophage) Lot-specific concentration range is encoded on the COBAS AmpliPrep/ COBAS TaqMan HCV Test reagent cassette barcodes Negative human plasma, nonreactive by US FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HbsAg; HIV-1 RNA, HCV RNA, and HBV RNA not detectable by PCR methods 0.1% ProClin® 300 preservative
COBAS® TaqMan® negative control (Human plasma)	CTM (–) C	Negative human plasma, nonreactive by US FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HbsAg; HIV-1 RNA, HCV RNA, and HBV DNA not detectable by PCR methods 0.1% ProClin® 300 preservative
HCV high positive control barcode Clip	HCV H(+)C Clip	
HCV low positive control barcode clip	HCV L(+)C Clip	
HCV negative control barcode clip	HCV (–) C Clip	
COBAS® AmpliPrep/ COBAS® TaqMan® wash reagent	PG WR	Sodium citrate dihydrate <0.1% N-methylisothiazolone-HCl

11.5.3 Special Supplies

The following supplies are needed but not supplied in the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test (see Table 11.3):

11.5.4 Reagent Labeling and Preparation

All reagents are labeled by the manufacturer. The labeling includes contents, lot number, expiration date, and storage instructions. All reagents are liquid and ready to use.

Table 11.3 Special supplies needed to run the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test but are not included in the test kit

<i>Instrumentation and software</i>	COBAS® AmpliPrep system
	COBAS® TaqMan® instrument or COBAS® TaqMan® 48 instrument
	Optional: docking station
	AMPLILINK software, version 3.2 series
	Data station for the AMPLILINK software, with printer
	COBAS AmpliPrep System Manual for use with the COBAS® TaqMan® instrument or COBAS®
	TaqMan® 48 instrument, COBAS AMPLICOR analyzer and the AMPLILINK software, version 3.2 series
	COBAS® TaqMan® (plus optional docking station) instrument manual for use with AMPLILINK software, version 3.2 Series
	COBAS® TaqMan® 48 Instrument Manual for use with AMPLILINK Software, version 3.2 series application manual
	AMPLILINK software version 3.2 series application manual for use with the COBAS® AmpliPrep system, COBAS® TaqMan® instrument, and COBAS® TaqMan® 48 instrument and COBAS AMPLICOR Analyzer
<i>Disposables</i>	Sample processing units (SPUs)
	Sample input tubes (S-tubes) with barcode clips
	Racks of K-tips
	K-tube box of 12 × 96
<i>Other materials required but not provided</i>	Sample rack (SK 24 rack)
	Reagent rack
	SPU rack
	K-tube capper, motorized
	K-tube capper
	K-carrier
	K-carrier transporter
	K-carrier rack
	Pipettors with aerosol barrier or positive displacement RNase-free tips (capacity 1,000 µL) ^a
	Disposable gloves, powderless
	Vortex mixer

^aAerosol barrier-free tips are used where specified to prevent specimen and amplicon cross-contamination

11.5.5 Reagent Storage and Use

- Store the following reagents at 2–8°C: HCV CS1, HCV CS2, HCV CS3, HCV CS4, HCV H(+)C, HCV L(+)C, and CTM (–) C.
- Store the following reagents at 2–30°C: HCV H(+)C Clip, HCV L(+)C Clip, HCV (–) C Clip, and PG WR.
- Do not freeze reagents or controls.
- Do not use a kit after its expiration date.
- Once used, HCV CS1, HCV CS2, HCV CS3, and HCV CS4 are stable for 28 days at 2–8°C or until the expiration date, whichever comes first. They can be used for a maximum of four instrument cycles (up to 64 h cumulative on board the COBAS AmpliPrep system) and must be stored at 2–8°C between instrument cycles.
- Once opened, any unused portion of HCV H(+)C, HCV L(+)C, and CTM (–) C must be discarded.

- Do not mix reagent cassettes or controls from different kits. Do not pool controls from different lots or from different vials of the same lot. Do not open COBAS AmpliPrep system cassettes and exchange, mix, remove, or add bottles. Dispose of unused reagents, waste, and specimens in accordance with country, federal, state, and local regulations.
- Handle all reagents with caution and avoid contact with skin, eyes, or mouth. Refer to the package insert for any known toxicity.
- These reagents contain *sodium azide*: HCV QS, CAP/CTM HCV Mn²⁺, and HCV MMX. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide buildup.
- These reagents contain *thiocyanate*: HCV CS2 and liquid waste from the COBAS® AmpliPrep Instrument contain *guanidine thiocyanate*. Do not allow HCV CS2 or any liquid waste to contact *sodium hypochlorite* (bleach) solution. These mixtures can produce a highly toxic gas.

Wear eye protection, laboratory coats, and disposable gloves when handling any reagent. Avoid contact of these materials with the skin, eyes, or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

11.5.6 New Reagents Verification

All new reagent lots and or shipments are validated by testing one known negative, one low positive, and one mid to high positive that were tested with old lot. The results of the new lot must be within 0.5 log of the old lot. The results must be accepted before new reagent being placed in service.

11.6 Quality Control

- Quality Control Information: One each of the COBAS® TaqMan® Negative Control, the HCV Low Positive Control, and the HCV High Positive Control must be included in each test batch.
- Control Material Preparation and Storage: Three controls are provided for use: HCV High Positive Control, HCV Low Positive Control, and the COBAS® TaqMan® Negative Control (Human Plasma). The controls are liquid and ready to use.
- Store the controls at 2–8°C. The controls are stable until the expiration date indicated.
- Acceptable Limits: The CTM (–) C must yield a “Target Not Detected” result. The assigned range for HCV L(+)C and HCV H(+)C is specific for each lot of reagents and is provided on the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test reagent cassette barcodes. The HCV RNA IU/mL for HCV L(+)C and HCV H(+)C should fall within their assigned ranges.
- Corrective Actions: If one or both of the positive controls are flagged as invalid, then the entire batch is invalid. No result will be reported. Repeat the entire process (specimen and control preparation, amplification, and detection).
- If negative control is positive, the decontamination control protocol must be followed (see decontamination protocol). No result will be reported. Repeat the entire process (specimen and control preparation, amplification, and detection).
- If the HCV RNA titer of one or both of the positive controls is consistently outside the assigned ranges in multiple batches, contact your local Roche office for technical assistance.

11.7 Protocols

Each kit contains reagents sufficient for 48 tests, which may be performed in batches of 12–24 tests. Therefore, it is recommended that processing should be in batches of multiples of 12 for the most efficient use of reagents, specimens, and controls. The COBAS® TaqMan® instrument or COBAS® TaqMan® 48 instrument run must be started within 120 min following completion of specimen and control preparation.

Note: DO NOT FREEZE or STORE processed specimens and controls at 2–8°C.

11.7.1 Specimen and Control Preparation

11.7.1.1 COBAS® AmpliPrep System Setup

1. Vortex specimens and controls for 3–5 s before use. Controls should be removed from 2°C to 8°C storage and equilibrated to ambient temperature before use.
2. Wear new gloves and a lab coat to perform the following steps:
3. Turn the COBAS AmpliPrep system on; the system will automatically prime the system and make ready for operation in stand-by mode.
4. Turn the data station for the AMPLILINK software on. Prepare the data station as follows:
 - Log onto Windows® XP.
 - Double click the AMPLILINK software icon.
 - Log onto AMPLILINK software by entering the assigned user ID and password.
5. Check the supply of PG WR using the status screen and replace if necessary.
6. Perform all maintenance that is listed in the due tab.

11.7.1.2 COBAS AmpliPrep System Reagent Loading

All reagent cassettes should be removed from 2°C to 8°C storage, immediately loaded onto the COBAS AmpliPrep system, and allowed to equilibrate to ambient temperature on the instrument for at least 30 min before the first specimen is to be processed. Do not let reagent cassettes come to ambient temperature outside the instrument.

1. Place **HCV CS1** onto a reagent rack. Place **HCV CS2**, **HCV CS3**, and **HCV CS4** onto a separate reagent rack.
2. Load the reagent rack containing **HCV CS1** onto rack position **A** of the COBAS® AmpliPrep Instrument.
3. Load the reagent rack containing **HCV CS2**, **HCV CS3**, and **HCV CS4** onto rack position **B**, **C**, **D**, or **E** of the COBAS® AmpliPrep Instrument (see also Table 11.2 for additional information).

11.7.1.3 COBAS® AmpliPrep Loading of Disposables

Determine the number of COBAS AmpliPrep system reagent cassettes, Sample Processing Units (SPUs), Input Sample tubes (S-tubes), K-tips, and K-tubes needed. One SPU, one Input S-tube, one K-tip, and one K-tube are needed for each specimen or control.

Table 11.4 COBAS® AmpliPrep system with COBAS® TaqMan® instrument configurations

Configuration	Transfer mode to COBAS® TaqMan® instrument or COBAS® TaqMan® 48 instrument	Racks, carriers, and disposables	Position on COBAS® AmpliPrep system
1. COBAS® AmpliPrep system plus docking station plus COBAS® TaqMan® instrument	Automated transfer of K-carrier	K-tubes in full K-tube racks K-tips in full K-tips racks Input S-tubes containing specimens and controls on sample racks SPUs in SPU racks CS1 on cassette rack CS2, CS3, and CS4 on cassette rack K-tips in full K-tips racks Input S-tubes on sample racks SPUs in SPU racks CS1 on cassette rack CS2, CS3, and CS4 on cassette rack K-tips in full K-tips racks	M–P M–P F–H J–L A B–E M–P F–H J–L A B–E M–P

1. Place the SPUs in the SPU rack(s) and load the rack(s) onto rack position **J, K, or L** of the COBAS AmpliPrep system.
2. Load full K-tube rack(s) onto rack position **M, N, O, or P** of the COBAS AmpliPrep system.
3. Load full K-tip rack(s) onto rack position **M, N, O, or P** of the COBAS AmpliPrep system.

11.7.1.4 Configurations for Using COBAS® AmpliPrep System with COBAS® TaqMan® Instrument (Table 11.4)

11.7.1.5 Ordering and Loading of Specimens

1. Prepare sample racks as follows: Attach a barcode label clip to each sample rack position where a specimen (S-tube) is to be placed. Attach one of the specific barcode label clips for the controls [**CTM (–) C, HCV L(+)C, and HCV H(+)C**] to each sample rack position where the controls (S-tube) are to be placed. The barcode label clips for controls should have the same control lot number as the lot number on the control vials in the kit. Take care in assigning the right control to the position with the appropriate control barcode clip. Place one Input S-tube into each position containing a barcode label clip.
2. Using the AMPLILINK software, create specimen orders for each specimen and control in the **Orders** window **Sample** folder. Select the appropriate test file and complete by saving.
3. Assign specimen and control orders to sample rack positions in the **Orders** window **Sample Rack** folder. The sample rack number must be entered for the rack prepared in step D1.
4. Print the **Sample Rack Order** report to use as a worksheet.
5. Prepare specimen and control racks in the designated area for specimen and control addition as follows: Vortex each specimen and control [**CTM (–) C, HCV L(+)C, and HCV H(+)C**] for 3–5 s. Avoid contaminating gloves when manipulating the specimens and controls.
6. Transfer 1,000–1,050 µL of each specimen and control [**CTM (–) C, HCV L(+)C, and HCV H(+)C**] to the appropriate barcode labeled Input S-tube using a micropipettor with an aerosol barrier or positive displacement RNase-free tip. *Avoid transferring particulates and/or fibrin clots from the original specimen to the Input S-tube.* Specimens and controls should be transferred to tube positions as assigned and recorded on the worksheet in step D4. The barcode label clips for controls

should have the same control lot number as the lot number on the control vials in the kit. Assign the correct control to the position with the appropriate control barcode clip. *Avoid contaminating the upper part of the S-tubes with specimens or controls.*

7. Load the sample rack(s) filled with Input S-tubes onto rack positions **F**, **G**, or **H** of the COBAS AmpliPrep system.

11.7.1.6 Start of COBAS® AmpliPrep Instrument Run

1. Start the COBAS® AmpliPrep system using the AMPLILINK software.
2. Check for flags or error messages in the system screen.

11.7.1.7 End of COBAS® TaqMan® Analyzer Run

1. At the completion of the COBAS® TaqMan® instrument run, print Results Report. Check for flags or error messages in the Result section. Specimens with flags and comments are interpreted as described in the Results section. After acceptance, store data in archive.
2. Perform daily maintenance on both COBAS AmpliPrep and COBAS TaqMan instruments.

11.7.2 Calculations

For each specimen and control, the AMPLILINK software automatically:

1. Determines the cycle threshold value (Ct) for the HCV RNA and the HCV QS RNA.
2. Determines the HCV RNA concentration based upon the Ct values for the HCV RNA and HCV QS RNA and the lot-specific calibration coefficients provided on the cassette barcodes.
3. Determines that the calculated IU/mL for HCV L(+)C and HCV H(+)C fall within the lot-specific assigned ranges encoded on the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test reagent cassette barcodes supplied with the kit.

11.8 Reporting Results

11.8.1 Reporting Format

The COBAS® TaqMan® instrument automatically determines the HCV RNA concentration for the specimens and controls. *The HCV RNA concentration is expressed in international units IU/mL.* For batch validation, check AMPLILINK software results window or printout for flags and comments to ensure that the batch is valid. For control orders, a check is made to determine if the IU/mL value for the control is within its specified range. If the IU/mL value for the control lies outside of its range, a flag is generated to show the control has failed. The batch is valid if no flags appear for any of the controls [HCV H(+)C, HCV L(+)C, CTM (–) C]. The following results are obtained for a valid batch:

Control	Result	Interpretation
Negative Control	Target not detected	Control within range
Low Positive Control	A numeric titer, X.XXE+XX IU/mL	Control within range
High Positive Control	A numeric titer, X.XXE+XX IU/mL	Control within range

The batch is not valid if any of the following flags appear for the HCV controls:

11.8.2 Negative Control

Flag	Result	Interpretation
__N_NC_INVALID	Invalid	An invalid result or a “valid” result that was not negative for HCV target

11.8.3 HCV Low Positive Control

Flag	Result	Interpretation
__L_LPCINVALID	<4.3E+01 IU/mL	An invalid result where the control was below the assay range
__L_LPCINVALID	Target not detected	Control below range
__L_LPCINVALID	A numeric titer, X.XXE+XX IU/mL	An invalid result where the control was out of the assigned control range
__L_LPCINVALID	>6.90E+07 IU/mL	An invalid result where the control was above the assay range
__L_LPCINVALID	Invalid	An invalid result

11.8.4 HCV High Positive Control

Flag	Result	Interpretation
__H_HPCINVALID	<4.3E+01 IU/mL	An invalid result where the control was below the assay range
__H_HPCINVALID	Target not detected	Control below range
__H_HPCINVALID	A numeric titer, X.XXE+XX IU/mL	An invalid result where the control was out of the assigned control range
H_HPCINVALID	>6.90E+07 IU/mL	An invalid result where the control was above the assay range
__H_HPCINVALID	Invalid	An invalid result

Note: If the batch is invalid, repeat the entire batch including specimen and control preparation, amplification, and detection.

11.9 Data Review

For a valid batch, check each individual specimen for flags or comments on the result printout. Interpret the results as follows:

- For specimens with no flags or comments, report the results shown on the printout.
- A valid batch may include both valid and invalid specimen results depending on whether flags and/or comments are obtained for the individual specimens.
- Specimens with flags and/or comments are interpreted as follows (see Table 11.5):

Table 11.5 Comments and interpretations for flagged specimens

Titer result	Interpretation
Target not detected	No Ct value for HCV obtained. Report results as “HCV RNA not detected”
<4.3E+01 IU/mL	Below 4.3E+01 IU/mL (lower limit of quantitation, LLoQ); HCV RNA is not quantifiable
≥4.3E+01 IU/mL and ≤6.90E+07 IU/mL	Results greater than or equal to 43 IU/mL and less than or equal to 6.90E+07 IU/mL are within the linear range of the assay
>6.90E+07 IU/mL	Results are above the range of the assay. Report results as “greater than 6.90E+07 HCV RNA IU/mL.” If quantitative results are desired, the original specimen should be diluted 1:100 with HCV-negative human serum or EDTA plasma, depending on the matrix of the original specimen, and the test repeated. Multiply the reported result by the dilution factor

Note: Specimens above the range of the assay may also produce an invalid result with a flag “QS_INVALID.” If quantitative results are desired, the original specimen should be diluted 1:100 with HCV-negative human serum or EDTA plasma, depending on the matrix of the original specimen, and the test repeated. Multiply the reported result by the dilution factor

11.10 Decontamination Protocol

11.10.1 Materials and Equipment Required

- 70% ETOH
- Lint-free tissues or gauze
- Deionized water
- DNA AWAY

11.10.2 AmpliPrep

1. Remove K-carriers and soak in DNA AWAY for 10 min.
2. Rinse the K-carriers thoroughly with deionized water followed by 70% ETOH.
3. Wipe the inside of main cover with a new lint-free tissue of gauze moistened with DNA AWAY.
4. Wipe the initialization post with a new lint-free tissue of gauze moistened with DNA AWAY.
5. Wipe the plastic panel at the initialization post and the plastic panel in the rear with a new lint-free tissue of gauze moistened with DNA AWAY.
6. Wipe the top surface of the rack platform with a new lint-free tissue of gauze moistened with DNA AWAY.
7. Wipe the surface of the rack loading area with a new lint-free tissue of gauze moistened with DNA AWAY.
8. Wipe the SPU incubator surfaces with a new lint-free tissue of gauze moistened with DNA AWAY.
9. Wipe all slots of the output rack platform and the separation area and target release incubator with a new lint-free tissue of gauze moistened with DNA AWAY.
10. Move the transfer head to the middle of the instrument. Wipe the surface of both wash towers with a new lint-free tissue of gauze moistened with DNA AWAY.
11. Wipe the K carrier park positions with a new lint-free tissue of gauze moistened with DNA AWAY.
12. Wipe the reagent tips with a new lint-free tissue of gauze moistened with DNA AWAY.
13. Wipe the tube handler front part with a new lint-free tissue of gauze moistened with DNA AWAY.

14. Wipe the SPU gripper with a new lint-free tissue of gauze moistened with DNA AWAY.
15. Wipe the SPU gripper with a cotton swab a moistened with DNA AWAY.
16. Open the loading panel and wipe it with a new lint-free tissue of gauze moistened with DNA AWAY.
17. Wipe the outside of the instrument with a new lint-free tissue of gauze moistened with DNA AWAY.
18. Repeat the procedure steps 3–17 once more with DNA AWAY.
19. Repeat the procedure steps 3–17 twice with deionized water.
20. Repeat the procedure steps 3–17 with 70% ETOH.

11.10.3 *TaqMan*

1. Remove K-carriers, including the K-carrier that contains the dummy K-tubes.
2. Discard the dummy K-tubes.
3. Soak the K-carriers in DNA AWAY for 10 min.
4. Rinse the K-carriers thoroughly with deionized water followed by 70% ETOH.
5. Remove the K-tube waste bin and wipe it with a new lint-free tissue of gauze moistened with DNA AWAY.
6. Wipe the inside of main cover with a new lint-free tissue of gauze moistened with DNA AWAY.
7. Wipe the waste chute and surrounding area with a new lint-free tissue of gauze moistened with DNA AWAY.
8. Wipe the brush at the waste chute with a new lint-free tissue of gauze moistened with DNA AWAY.
9. Wipe the thermal cycler covers and the areas around the thermal cycler segments with a new lint-free tissue of gauze moistened with DNA AWAY.
10. Wipe the sample loading area with a new lint-free tissue of gauze moistened with DNA AWAY.
11. Wipe inside the loading panel with a new lint-free tissue of gauze moistened with DNA AWAY.
12. Wipe the initialization post and surrounding area with a new lint-free tissue of gauze moistened with DNA AWAY.
13. Wipe the K carrier park positions with a new lint-free tissue of gauze moistened with DNA AWAY.
14. Wipe the tube handling front part with a new lint-free tissue of gauze moistened with DNA AWAY.
15. Wipe the bin holder with a new lint-free tissue of gauze moistened with DNA AWAY.
16. Wipe the outside of the instrument with a new lint-free tissue of gauze moistened with DNA AWAY.
17. Repeat the procedure steps 6–16 once more with DNA AWAY.
18. Repeat the procedure steps 6–16 twice with deionized water.
19. Repeat the procedure steps 6–16 with 70% ETOH.
20. Reload the K-carriers to the same positions as before.
21. Replace the K-carriers equipped with new dummy K-tubes and insert in position 2.
22. Place a new waste bag in the K-tube waste bin and reload it to the COBAS TaqMan

11.10.4 *Limitations of Methods*

11.10.4.1 *Interfering Substances*

Elevated levels of triglycerides, bilirubin, albumin, hemoglobin, and human DNA in specimens as well as the presence of autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and antinuclear antibody (ANA) have been shown not to interfere with the quantitation of HCV RNA by the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test.

The following drug compounds tested at the peak plasma level (C_{max}) and at three times the C_{max} have been shown *not* to interfere with the quantitation of HCV RNA by the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test: tenofovir and adefovir dipivoxil (nucleotide HIV/HBV DNA polymerase inhibitors); zidovudine, didanosine, stavudine, lamivudine, and abacavir (nucleoside HIV reverse transcriptase inhibitors and DNA polymerase inhibitors); indinavir, ritonavir, nelfinavir, saquinavir, amprenavir, and lopinavir/ritonavir (HIV protease inhibitors); nevirapine and efavirenz (nonnucleoside HIV reverse transcriptase inhibitors); interferon alpha-2a, interferon alpha-2b, peginterferon alpha-2a, peginterferon alpha-2a+ribavirin, and interferon alpha-2b+ribavirin (immune modulators); paroxetine HCl, fluoxetine, and sertraline (antidepressants); and ganciclovir, valganciclovir, and acyclovir (compounds for the treatment of herpes viruses).

11.10.4.2 Procedural Limitations

This test has been validated for use with only human serum or plasma collected in EDTA anticoagulant. Testing of other specimen types may result in inaccurate results. Quantitation of HCV RNA is dependent on the number of virus particles present in the specimen and may be affected by specimen collection methods, patient factors (e.g., age, presence of symptoms), and stage of infection. Though rare, mutations within the highly conserved regions of the viral genome covered by the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test primers and/or probe may result in the underquantitation of or failure to detect the presence of the virus in this circumstance. Reliable results are dependent on adequate specimen collection, transport, storage, and processing procedures.

The presence of AmpErase enzyme solution in the COBAS® AmpliPrep/COBAS® TaqMan® HCV Master Mix reduces the risk of amplicon contamination. However, contamination from HCV positive controls and clinical specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified in this package insert. Use of this product should be limited to personnel trained in the techniques of PCR. This product can only be used with the COBAS AmpliPrep system and the COBAS® TaqMan® instrument or COBAS® TaqMan® 48 instrument.

If another assay was initially used for quantitation of HCV viral RNA in order to assess treatment effect on the patient, it is recommended that prior to switching to the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test users perform method correlation studies in their laboratory to quantify assay differences.

Technical Assistance from Manufacturer

For telephone technical assistance:

Roche Technical Support Center: 1-800-526-1247

Acknowledgment Roche Molecular Diagnostics has granted permission to use a portion of the COBAS® AmpliPrep/COBAS® TaqMan® Test, Document Revision 4.0 package insert, in this chapter.

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Chapter 12

Epstein-Barr Virus and Cytomegalovirus Viral Load Monitoring by Quantitative Real-Time Polymerase Chain Reaction

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Keywords EBV • CMV • Real-time PCR • Viral load monitoring • Protocol • Quality control and monitoring

12.1 Introduction

12.1.1 Epstein-Barr Virus and Cytomegalovirus Viral Load Monitoring

Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are double-stranded DNA viruses belonging to the herpesvirus family. These widespread pathogens lead to lifelong latent infection that can be reactivated in immunocompromised states, such as after hematopoietic stem cell or solid organ transplantation and in acquired immunodeficiency syndrome, in which they contribute substantially to morbidity and mortality rates. EBV is the causative agent of infectious mononucleosis and is associated with Hodgkin's lymphoma, Burkitt's lymphoma, posttransplant lymphoproliferative disorder, nasopharyngeal carcinoma, and human immunodeficiency virus–related lymphomas. CMV is known to cause a variety of disorders, including mononucleosis syndrome, congenital deafness, perinatal mortality in premature infants, and disseminated infection in immunocompromised patients, with the most severe effects on the lungs, gastrointestinal tract, and retina.

EBV or CMV viral load, measured by quantitative real-time polymerase chain reaction (qPCR) of the viral DNA, can serve as a biomarker to predict and monitor diseases associated with virus reactivation and is becoming increasingly critical in the management of transplant recipients [1–3]. The EBV qPCR protocol described in this chapter was adapted and modified from descriptions in the published literature [4]. The CMV qPCR protocol was developed and validated using a commercially

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available analyte-specific reagent (ASR) product, Artus CMV PCR ASR reagents-TM (Qiagen [Valencia, CA] catalog number 4503125). For both the EBV and the CMV qPCR described here, the specimen type used is plasma; however, the ideal specimen type depends on the virus-associated disease in question, and analytical validation for each specimen type needs to be done before the specimen type is used.

12.1.2 qPCR

In contrast to the traditional end-point PCR, in which amplification products are analyzed or quantified at the end of PCR, real-time PCR enables monitoring of amplification at every cycle during PCR and thus allows quantitation in the exponential phase of PCR, when the amplification product first becomes detectable. This allows robust detection and quantitation of target DNA templates, with analytical sensitivity reaching between 1 and 10 target molecules per reaction and achieving a dynamic range of more than 10^6 target molecules. Additionally, because no postamplification handling is needed in real-time PCR, the risk of contamination by PCR amplicons is lower than it is in end-point PCR. Several fluorescence chemistries are used for real-time PCR [5]. In this chapter, we describe the TaqMan chemistry from Applied Biosystems, Inc. (Carlsbad, CA). Real-time PCR allows PCR progress to be monitored as it occurs (i.e., in real time).

12.1.3 Analysis of Real-Time PCR Amplification Plots

The results of real-time PCR are displayed as amplification plots. Positive amplification plots usually have an exponential, a linear, and a stationary phase. In the initial cycles of PCR, there is little change in the fluorescence signal. This defines the baseline for the amplification plot. A fixed fluorescence threshold can be set above the baseline; this threshold should be placed at the exponential phase for the most accurate determination of the initial quantity of the target DNA. The parameter threshold cycle (C_t) is defined as the cycle number at which the fluorescence passes the fixed threshold. For absolute quantitation, quantitation standards (QS) are run to generate a standard curve. The optimal slope of the standard curve is -3.3 with a correlation coefficient (R^2) as close to 1 as possible. Removing obvious outliers from the standard curve helps obtain a slope and R^2 close to the optimal values.

12.1.4 Controls Included in Each Run

- Negative control: a sample known to be negative for the target gene, to help rule out a false-positive result
- Positive control: a sample known to be positive for the target gene, to help rule out a false-negative result
- No template control (NTC): a PCR reaction mixture containing all components except the DNA template, to help rule out contamination with the DNA template
- Sensitivity control: a positive control diluted into a negative control close to the analytical sensitivity of the assay, to demonstrate assay sensitivity and help rule out a false-negative result

12.2 EBV qPCR Protocol

12.2.1 EBV qPCR Reagents and Materials

1. QIAamp DNA blood mini kit (Qiagen catalog number 51104 or 51106)
2. TaqMan 2× universal PCR master mix with uracil N-glycosylase (UNG; Applied Biosystems catalog number 4304437)
3. EBV primers and 6-carboxyfluorescein (FAM)-labeled TaqMan probe
4. TaqMan exogenous internal positive control (IPC) reagents (Applied Biosystems catalog number 4308323)

This reagent kit contains IPC DNA with predesigned primers and a VIC-labeled TaqMan probe. Add the IPC into the samples to monitor the DNA extraction procedure and to check for possible PCR inhibition. The VIC-labeled IPC probe and the FAM-labeled EBV probe allow single-tube multiplex reaction and detection of both IPC and EBV.

5. ABI Prism 7900HT sequence detection system (Applied Biosystems)

12.2.2 EBV qPCR Samples

1. Test samples: 3 mL of whole blood in an ethylenediaminetetraacetic acid tube (lavender top).
2. EBV QS 1–5: Namalwa (EBV-positive) cell-line DNA, calibrated to the Acrometrix OptiQuant EBV plasma panel (Life Technologies (Carlsbad, CA) catalog number 94-2017), used to generate a standard curve for quantitation of EBV in test samples.
3. EBV-positive controls: high positive control (HPC) and low positive control (LPC) Namalwa cell-line DNA prepared separately from the quantitation standards, used to monitor the accuracy of EBV quantitation and the sensitivity of the assay.
4. NTC: control with no EBV DNA template, used to rule out a false-positive EBV result.
5. No amplification control (NAC): control with an IPC block (from Applied Biosystems TaqMan exogenous IPC reagents), used to rule out a false-positive IPC result.

12.2.3 Preparation of EBV QS from the Namalwa Cell Line

1. Extract DNA from the Namalwa cell line.
2. After obtaining a homogeneous DNA solution, measure the optical density (OD_{260}) by spectrophotometry and calculate the DNA concentration.

Namalwa cell-line DNA contains 3×10^5 copies of EBV per microgram of DNA. Thus, EBV copies/ μ L DNA = Namalwa cell-line DNA concentration in μ g/ μ L $\times (3 \times 10^5)$. Examples of EBV concentration calculations in stock Namalwa cell-line DNA are shown in Table 12.1. Five microliters of EBV QS is used per PCR reaction mixture, whereas 10 μ L of the test sample is used per PCR reaction mixture; convert the results obtained for test samples to EBV copies/mL plasma by applying the following equation: EBV copies/mL = (EBV copies/ μ L) \times (elution volume [μ L]/initial sample volume [mL]) \times (5 μ L EBV QS/10 μ L test sample), where elution volume = 100 μ L and initial sample volume = 0.2 mL.

Table 12.1 Calculations of Epstein-Barr virus (EBV) genome copies in stock Namalwa cell-line DNA samples

Namalwa cell-line DNA sample	DNA concentration ($\mu\text{g}/\mu\text{L}$)	EBV copies/ μL ($\times [3 \times 10^5]$)	Equivalent to EBV copies/mL plasma ($\times 250$)
1	0.217	65,100	16,275,000
2	0.203	60,900	15,225,000
3	0.197	59,100	14,775,000
4	0.201	60,300	15,075,000

12.2.4 Namalwa QS DNA Aliquoting, Storage, and Average Standard Curve

Store stock Namalwa cell-line DNA at -20°C in 50 μL aliquots. Freshly prepare serially diluted QS from the aliquoted stock DNA each time the QS are to be included in a run. To produce a standard curve, make 10 \times dilutions in water to obtain 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} concentrations of DNA. Assay these, along with the Neat (stock), in duplicate.

The EBV QS may also be calibrated to the Acrometrix OptiQuant EBV plasma panel (Life Technologies catalog number 94-2017). For example, based on calibration to Acrometrix EBV panel, the EBV concentration in Namalwa stock sample 1 in Table 12.1 is equivalent to 4.2×10^7 copies/mL, and thus the serial dilution generates the following QS:

QS1 (stock): 4.2×10^7 copies/mL

QS2: 4.2×10^6 copies/mL

QS3: 4.2×10^5 copies/mL

QS4: 4.2×10^4 copies/mL

QS5: 4.2×10^3 copies/mL

Assay the Namalwa QS with every EBV run. Alternatively, use a stored historical standard curve to calculate the number of EBV copies per milliliter. This stored historical standard curve is an average of 20 standard curve runs, and the following is an example:

Slope = 3.324, intercept = 45.387, $R^2 = 0.997$

12.2.5 EBV qPCR Sample Preparation and DNA Extraction Using the QIAamp DNA Blood Mini Kit (Qiagen)

1. Centrifuge the peripheral blood tube at $1,500 \times g$ for 10 min.
2. Transfer 200 μL of plasma into a 1.5-mL tube (if using frozen plasma, thaw at room temperature, pulse vortex for 10 s, and centrifuge at full speed for 30 s before using plasma).
3. Add 222 μL of a freshly made mixture of 200 μL of buffer AL, 20 μL of proteinase K, and 2 μL of IPC (from the Applied Biosystems TaqMan exogenous IPC reagents).
4. Mix by pulse-vortexing for 15 s, followed by quick spin.
5. Incubate at 56°C for 10 min, followed by quick spin.
6. Add 200 μL of 100% ethanol.
7. Mix by pulse-vortexing for 15 s, followed by quick spin.
8. Transfer the mixture to a spin column with a 2-mL collection tube.
9. Secure the lid and centrifuge at $6,000 \times g$ for 1 min.
10. Place the spin column in a fresh 2-mL collection tube and discard the tube containing the filtrate.
11. Add 500 μL of AW1 buffer to the spin column.
12. Secure the lid and centrifuge at $6,000 \times g$ for 1 min.
13. Place the spin column in a fresh 2-mL collection tube and discard the tube containing the filtrate.

Table 12.2 Thermocycling conditions

Stage 1	50°C for 2 min
Stage 2	95°C for 10 min
Stage 3 (40 cycles)	95°C for 15 sec, 60°C for 1 min

- 8. Centrifuge briefly at no more than 2,000 rotations per minute and remove any bubbles again.
- 9. Perform PCR on the 7900HT sequence detector:
 - (a) Check that the 7900HT is turned on; if not, switch it on.
 - (b) Open the SDS software and open the EBV qPCR assay template, which is set for testing using the absolute quantification (standard curve) assay method, using detector settings for FAM (for EBV) and VIC (for IPC) reporters.
 - (c) Enter the sample identification as set up on the plate.
 - (d) Verify that the thermal cycling conditions are set on the instrument appropriately as shown in Table 12.2, with data collection set for the last (60°C) step.
 - (e) Click on the instrument tab and then click on the **Connect** button. Click **Open**, load the plate into the instrument, and then click **Close**. Click on the **Start** button. Verify that the run has started before leaving the instrument. The run will take approximately 2 h.

12.2.7 EBV qPCR Data Analysis

- 1. After the run is complete, open the run SDS file.
- 2. Omit all empty wells and analyze the run by clicking on the green arrow.
- 3. Check the FAM detector layer to make sure that the QS and positive controls are amplified. The threshold for positive amplification is manually set at 0.20 in the EBV qPCR assay template.
- 4. Check the VIC detector layer to make sure that the IPC is amplified in IPC-positive wells.
- 5. Generate a standard curve using QS 1–5.
- 6. Plot the Ct value of the test sample on the standard curve to determine the number of EBV genome copies per milliliter in the test sample (Fig. 12.2).

12.2.8 Manual Calculation of EBV Genome Concentration

The EBV genome concentration is determined in this assay by running a set of standard samples of known quantity (QS) and measuring their Ct values. Then a graph is plotted using these data, with QS EBV genome concentration on the x-axis and Ct values on the y-axis. The line of best fit is drawn through the points, and the slope and intercept are calculated. (Microsoft Excel software [Microsoft, Redmond, WA] is able to draw the plot and calculate the slope and intercept.) By rearranging the equation for the line of best fit and filling in the known variables, the viral load of the unknown test sample can be calculated. See below:

Straight line equation: $Y = -MX + C$

where

$-M$ = slope

C = intercept

Y = Ct

X = concentration (in \log_{10})

Rearrange equation: $X = (C - Y) / M$

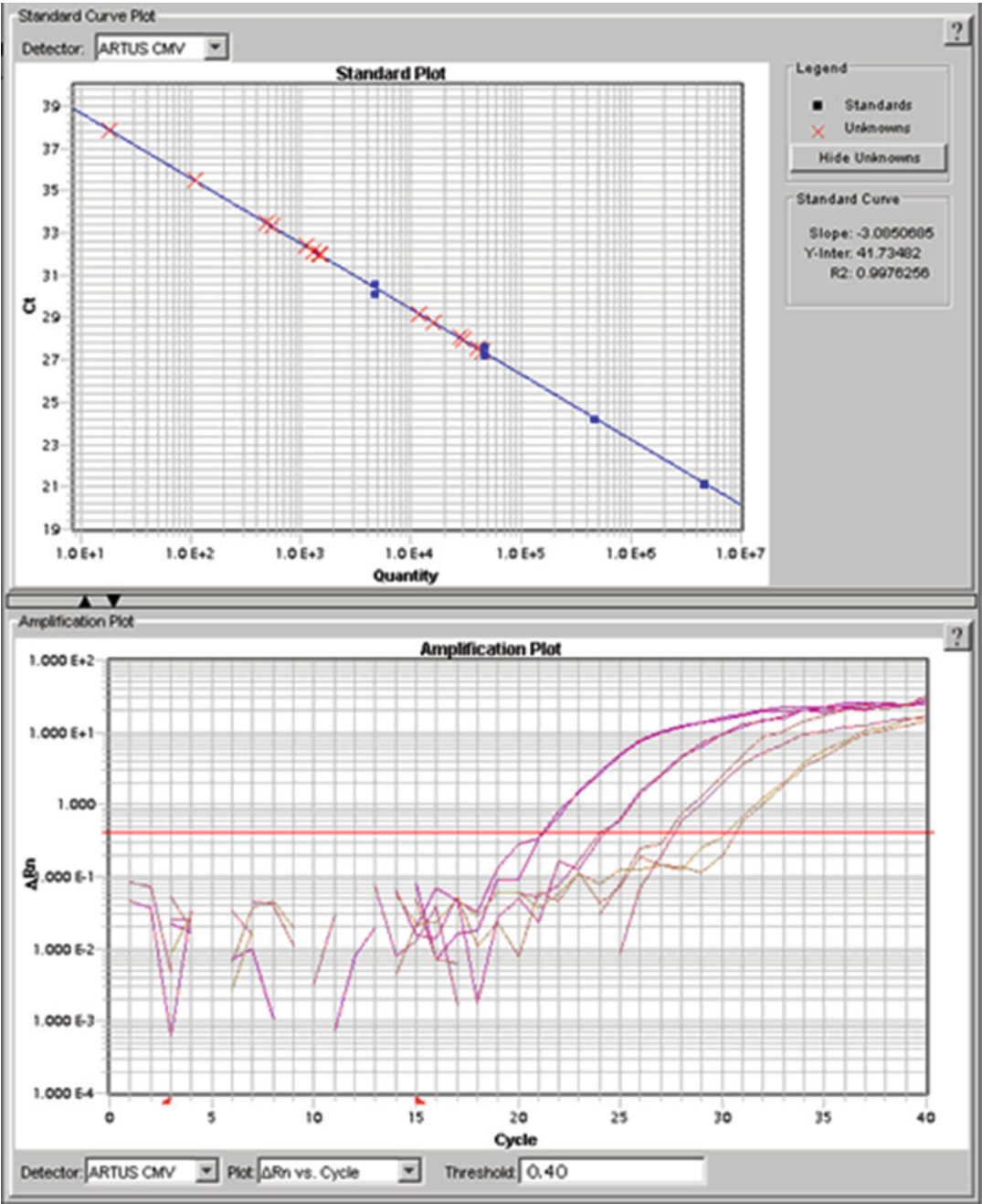


Fig. 12.2 The threshold cycle (*C_t*) value is plotted on the standard curve to determine the initial number of EBV genome copies per milliliter in the test sample

Solving for X gives a log value, so the antilog of X must be derived to obtain the EBV genome concentration.

12.2.9 Test Characteristics

1. Lower limit of detection: 125 EBV genome copies/mL.
2. Lower limit of quantitation: 400 EBV genome copies/mL.
3. Dynamic range: 400–10,000,000 EBV genome copies/mL.
4. Reproducibility: intra- and interassay variability for any particular sample is no more than a 2-Ct difference or a 4× difference in concentration (EBV genome copies/mL).
5. Accuracy: no standardized EBV qPCR assay exists for comparison. The EBV QS in this protocol were calibrated to the Acrometrix OptiQuant EBV plasma panel. The World Health Organization is developing an international calibrator so that test results can be rendered traceable to a community standard reference material.

12.2.10 EBV qPCR Result Interpretation and Reporting

The following results are possible:

1. A FAM fluorescence signal is detected: the result is positive for EBV DNA. In this case, the detection of a VIC fluorescence signal from the IPC is not required because a high concentration of EBV DNA may lead to reduced or absent fluorescence of the IPC.
2. No FAM fluorescence signal is detected, but the VIC fluorescence signal is detected: the result is negative for EBV DNA. In the case of a negative result for EBV DNA, the detection of IPC is required to rule out the possibility of PCR inhibition leading to a false-negative result.
3. Neither a FAM nor a VIC fluorescence signal is detected: no conclusion regarding EBV DNA can be reached. The absence of a VIC signal may indicate poor DNA recovery during extraction or inhibition of amplification during PCR.

The report for an EBV-positive result text:

Epstein-Bar virus (EBV) is detected by quantitative PCR. The viral load is ____ EBV copies/mL plasma.

The report for a low positive result text:

Low levels of Epstein-Barr virus (EBV) detected. The viral load is less than 400 EBV copies/mL plasma.

The report for an EBV-negative result text:

No Epstein-Barr virus (EBV) is detected by quantitative PCR.

The methodology description:

Method: EBV viral load in plasma was measured, following DNA extraction, using quantitative real-time PCR. The lower limit of detection (LOD) is 125 EBV copies/mL plasma. The lower limit of quantitation (LOQ) of this assay is 400 EBV copies/mL plasma. EBV detected below the LOQ is reported as “low positive.”

12.2.11 EBV qPCR Quality Control/Quality Monitoring and Troubleshooting

12.2.11.1 General Quality Control/Quality Monitoring Guidelines

1. Daily monitoring

- (a) Review control results.
- (b) Document problems and corrective actions in a quality control log.

2. Periodic monitoring (monthly/quarterly)

- (a) Review quality control log.
- (b) Review case log: parameters include total number of cases, percentage of positive/negative results, range of EBV concentrations (EBV genome copies/mL) of positive cases, turnaround time, number of repeats/failed cases, unusual/interesting cases.

12.2.11.2 Troubleshooting Guide

- If IPC fails, repeat DNA extraction of the sample.
- If duplicate samples vary by >2 Ct, repeat PCR reaction mixture for the sample.
- If NTC is positive for EBV, reject the whole run and repeat.
- If NAC is positive for IPC, reject the whole run and repeat.
- HPC and LPC: the mean concentrations and standard deviations (SDs) for the HPC and the LPC are preestablished by running them 20 times (4 times in 5 assays). These controls are then included in each run, and the copy number obtained for each level is recorded. The values should fall within 2 SDs of the preestablished mean for each level. Use the following modified Westgard rules for troubleshooting HPC and LPC:
 - 1 2S: any value $> \pm 2$ SD, $< \pm 3$ SD—it is a warning if it occurs on only one level within the last 10 values; accept the run. Reject the run if $> \pm 2$ SD values occur on two levels in the last 10 values; repeat the run. If repeat controls fail, verify calibration.
 - 1 3S: any value $> \pm 3$ SD—reject the run; repeat the run.
 - Both high and low controls $> \pm 2$ SD—reject the run; repeat the run.
 - 10x: 10 values are on same side of the mean (indicates a trend/shift)—review; investigate the cause and perform corrective action.

12.2.11.3 Calibration Verification

- Perform calibration verification using the Namalwa cell-line DNA QS once every 6 months (if it is $> \pm 2$ SD from historical slope and intercept averages, investigate the cause and perform corrective action).
- Perform calibration verification using the Acrometrix OptiQuant EBV plasma panel every 6 months (if it is more than a 3x difference, investigate the cause and perform corrective action).

12.2.11.4 Proficiency Testing

Perform the CAP survey twice per year.

12.3 CMV qPCR Protocol

12.3.1 CMV qPCR Reagents and Materials

1. QIAamp DNA blood mini kit (Qiagen catalog number 51104 or 51106)
2. Artus CMV PCR ASR reagents-TM (Qiagen catalog number 4503125). The kit contains CMV-TM master mix, magnesium solution, QS 1–4, internal control (IC), and PCR-grade water
3. ABI Prism 7900HT sequence detection system (Applied Biosystems)

12.3.2 CMV qPCR Samples

1. Test samples: 5 mL of whole blood in an ethylenediaminetetraacetic acid tube (lavender top). Specimens can be refrigerated for up to 48 h prior to delivery to the lab
2. CMV QS 1–4, used to generate a standard curve for quantitation of CMV in test samples
3. CMV-positive controls: HPC and LPC prepared by dilution of Acrometrix OptiQuant CMV DNA quantification panel (Life Technologies catalog number 94–2014), used to monitor the accuracy of CMV quantitation and the sensitivity of the assay
4. No CMV template control (NCMVC): control with no CMV DNA template but with IC, used to rule out a false-positive CMV result
5. NTC: control with no CMV DNA or IC, used to rule out a false-positive CMV and a false-positive IC result

12.3.3 CMV QS (From the Artus CMV PCR ASR Reagents-TM Kit)

- QS1: 1×10^4 copies/ μ L
- QS2: 1×10^3 copies/ μ L
- QS3: 1×10^2 copies/ μ L
- QS4: 1×10^1 copies/ μ L

Assay the QSs in duplicate in every run to generate a standard curve for quantitation of CMV in the test samples. Obtain the number of CMV genome copies per microliter for test samples using the standard curve and convert to CMV copies/mL by applying the following equation: $\text{CMV copies/mL} = ([\text{CMV copies}/\mu\text{L}] \times \text{elution volume } [\mu\text{L}]) / \text{initial sample volume (mL)}$, where elution volume = 60 μ L and sample volume = 0.2 mL. The QS then become:

- QS1: 3×10^6 copies/mL
- QS2: 3×10^5 copies/mL
- QS3: 3×10^4 copies/mL
- QS4: 3×10^3 copies/mL

Table 12.3 CMV PCR master mix formulation

	Final concentration	CMV	
		$\mu\text{L}/\text{rxn}$	1 rxn
CMV TM master	1×	12.50	12.50
CMV Mg-Sol	1×	2.50	2.50
Total		15.00	15.00
Sample		10	

CMV indicates cytomegalovirus; rxn, reaction; Mg-Sol, magnesium solution

12.3.4 CMV qPCR Sample Preparation and DNA Extraction

Set the temperature of the dry bath to 56°C. Remove the samples and IC from storage and let them come to room temperature.

1. Centrifuge the peripheral blood tube at 1,500× g for 10 min.
2. Transfer 200 μL of plasma into a 1.5-mL tube.
3. Add 226 μL of a freshly made mixture of 200 μL of buffer AL, 20 μL of proteinase K, and 6 μL of IC (from Artus CMV PCR ASR reagents-TM).
4. Mix by pulse-vortexing for 15 s, followed by quick spin.
5. Incubate at 56°C for 10 min, followed by quick spin.
6. Add 200 μL of 100% ethanol.
7. Mix by pulse-vortexing for 15 s, followed by quick spin.
8. Transfer the mixture to a spin column with a 2-mL collection tube.
9. Secure the lid and centrifuge at 6,000× g for 1 min.
10. Place the spin column in a fresh 2-mL collection tube and discard the tube containing the filtrate.
11. Add 500 μL of AW1 buffer to the spin column.
12. Secure the lid and centrifuge at 6,000× g for 1 min.
13. Place the spin column in a fresh 2-mL collection tube and discard the tube containing the filtrate.
14. Add 500 μL of AW2 buffer to the spin column.
15. Secure the lid and centrifuge at 6,000× g for 1 min.
16. Place the spin column in a fresh 2-mL collection tube and discard the tube containing the filtrate.
17. Centrifuge at 20,000× g for 1 min.
18. Place the spin column in a 1.5-mL tube and discard the tube containing the filtrate.
19. Add 60 μL of AE buffer and incubate at room temperature for 1 min.
20. Centrifuge at 6,000 × g for 1 min.
21. Store the eluted DNA at 4°C or –20°C.

12.3.5 CMV qPCR Protocol

1. Prepare a worksheet and a 96-well optical PCR plate for the scheduled reactions (each sample is tested in duplicates).
2. Indicate the number of reactions in the CMV PCR master mix table as shown in Table 12.3 and prepare the master mix in a level 2 biosafety cabinet.
3. Prepare a 1:10 dilution of the IC by adding 10 μL of IC to 90 μL of water.
4. Add 15 μL of master mix to the appropriate wells of a 96-well optical PCR plate.

Table 12.4 Cycling conditions

95°C	10 min	
95°C	15 s	Repeat 40 times
55°C	1 min	

5. Add 10 µL of test sample DNA, or for the controls, add the following accordingly:
 - (a) QS 1–4: add 10 µL of QS + 1 µL of IC (each QS separately).
 - (b) NCMVC: add 9 µL of water + 1 µL of IC.
 - (c) NTC: add 10 µL of water.
6. Cover plate using adhesive plate cover.
7. Vortex briefly to mix and then remove any bubbles in the wells.
8. Centrifuge briefly at no more than 2,000 × g and remove any bubbles again.
9. Perform PCR on the 7900HT sequence detector:
 - (a) Check that the 7900HT is turned on; if not, switch it on.
 - (b) Open the SDS software and open the CMV qPCR assay template, which is set for testing using the absolute quantification (standard curve) assay method, using detector settings for FAM (for CMV) and VIC (for IC) reporters.
 - (c) Enter the sample identification as set up on the plate.
 - (d) Verify that the thermal cycling conditions are set on the instrument appropriately as shown in Table 12.4, with data collection set for the last (55°C) step.
 - (e) Click on the instrument tab and then click on the **Connect** button. Click **Open**, load the plate into the instrument, and then click **Close**. Click on the **Start** button. Verify that the run has started before leaving the instrument. The run will take approximately 2 h.

12.3.6 CMV qPCR Data Analysis

1. After the run is complete, open the run SDS file.
2. Omit all empty wells and analyze the run by clicking on the green arrow.
3. Check the FAM detector layer to make sure that the QS and positive controls are amplified. The threshold for positive amplification is manually set at 0.40 in the CMV qPCR assay template.
4. Check the VIC detector layer to make sure that the IC is amplified in IC-positive wells.
5. Generate a standard curve using QS 1–4.
6. Plot the Ct value of the test sample on the standard curve to determine the initial number of CMV genome copies per milliliter in the test sample (Fig. 12.3).

12.3.7 Manual Calculation of CMV Genome Concentration

The CMV genome concentration is determined in this assay by running a set of control samples of known quantity (QS) and measuring their Ct values. Then, a graph is plotted using these data, with QS CMV genome concentration on the x-axis and Ct values on the y-axis. The line of best fit is drawn through the points, and the slope and intercept are calculated. (Microsoft Excel software is able to draw the plot and calculate the slope and intercept.) By rearranging the equation for the line of best fit and filling in the known variables, the viral load of the unknown test sample can be calculated. See below:

Straight line equation: $Y = -MX + C$ where

$-M$ = slope

C = intercept

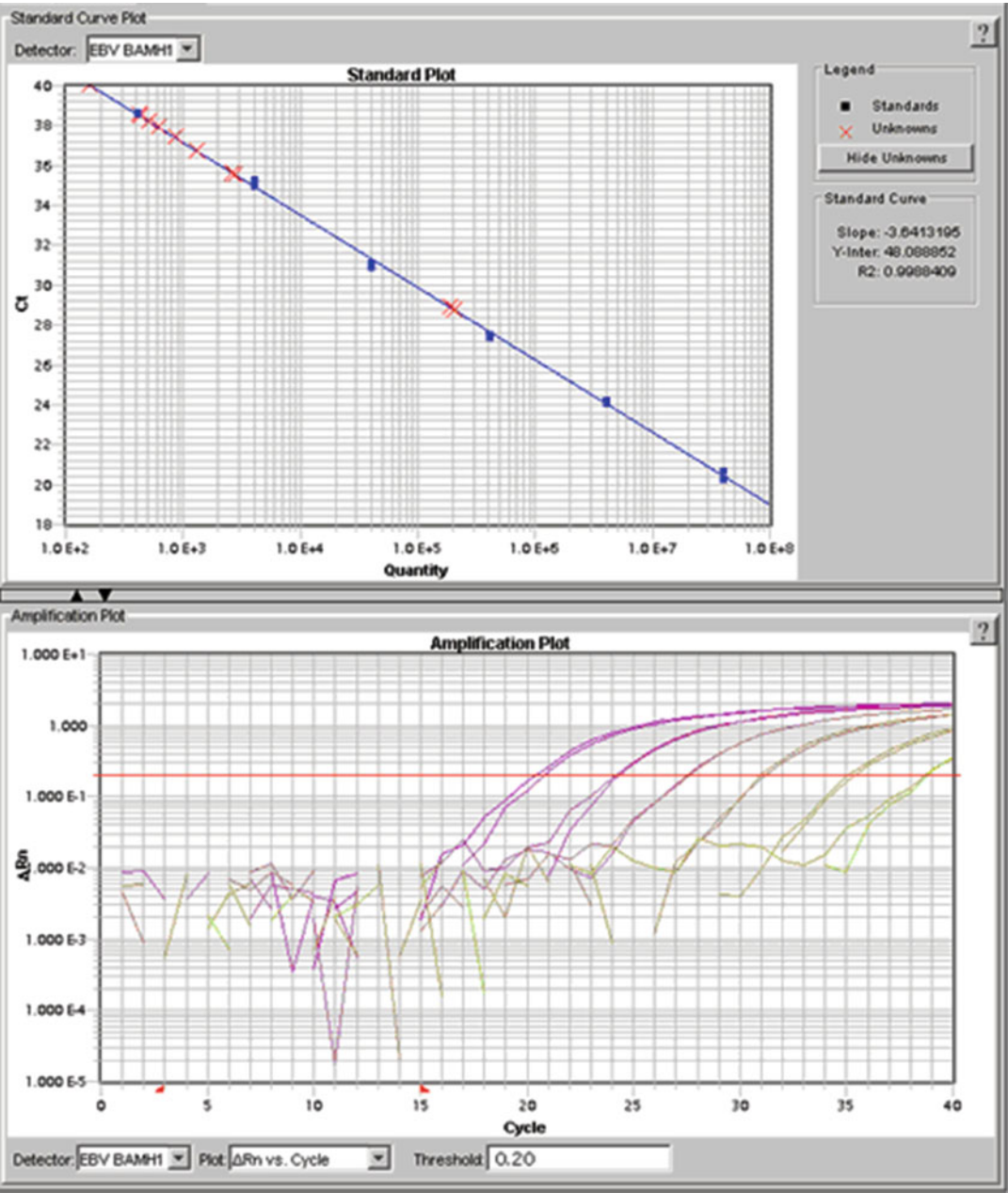


Fig. 12.3 The threshold cycle (*C_t*) value is plotted on the standard curve to determine the initial number of CMV genome copies per milliliter in the test sample

$Y = C_t$
 $X = \text{concentration (in } \log_{10})$
Rearrange equation: $X = (C - Y)/M$
Solving for X gives a log value, so the antilog of X must be derived to obtain the CMV genome concentration.

12.3.8 Test Characteristics

1. Lower limit of detection: 125 CMV genome copies/mL
2. Lower limit of quantitation: 250 CMV genome copies/mL
3. Dynamic range: 250–3,000,000 CMV genome copies/mL
4. Reproducibility: intra- and interassay variability for any particular sample is no more than a 2-Ct difference or a 4× difference in concentration (CMV genome copies/mL).
5. Accuracy: no standardized CMV qPCR assay exists for comparison. The CMV QS in this protocol were not calibrated to any reference standard. However, the World Health Organization has recently released the International Standard for Human CMV [6], which can serve as a reference for developing standardized CMV qPCR assays.

12.3.9 CMV qPCR Result Interpretation and Reporting

The following results are possible:

1. A FAM fluorescence signal is detected: the result is positive for CMV DNA. In this case, the detection of a VIC fluorescence signal from the IC is not required because a high concentration of CMV DNA may lead to reduced or absent fluorescence of the IC.
2. No FAM fluorescence signal is detected, but the VIC fluorescence signal is detected: the result is negative for CMV DNA. In the case of a negative result for CMV DNA, the detection of IC is required to rule out the possibility of PCR inhibition leading to a false-negative result.
3. Neither a FAM nor a VIC fluorescence signal is detected: no conclusion regarding CMV DNA can be reached. The absence of a VIC signal may indicate poor DNA recovery during extraction or inhibition of amplification during PCR.

The report for a CMV-positive result text:

Cytomegalovirus (CMV) is detected by quantitative PCR. The viral load is __ CMV copies/mL plasma.

The report for a low positive result text:

Low levels of cytomegalovirus (CMV) detected. The viral load is less than 250 CMV copies/mL plasma.

The report for a CMV-negative result text:

No cytomegalovirus (CMV) is detected by quantitative PCR.

The methodology description:

Method: CMV viral load in plasma was measured, following DNA extraction, using quantitative real-time PCR. The lower limit of detection (LOD) is 125 CMV copies/mL plasma. The lower limit of quantitation (LOQ) of this assay is 250 EBV copies/mL plasma. CMV detected below the LOQ is reported as “low positive.”

12.3.10 CMV qPCR Quality Control/Quality Monitoring and Troubleshooting

12.3.10.1 General Quality Control/Quality Monitoring Guidelines

1. Daily monitoring
 - (a) Review control results.
 - (b) Document problems and corrective actions in a quality control log.

2. Periodic monitoring (monthly/quarterly)

- (a) Review quality control log.
- (b) Review case log: parameters include total number of cases, percentage of positive/negative results, range of CMV concentrations (CMV genome copies/mL) of positive cases, turnaround time, number of repeats/failed cases, unusual/interesting cases.

12.3.10.2 Troubleshooting Guide

- If IC fails, repeat DNA extraction of the sample.
- If duplicate samples vary by >2 Ct, repeat PCR reaction mixture for the sample.
- If NCMVC is positive for CMV, reject the whole run and repeat.
- If NTC is positive for CMV or IC, reject the whole run and repeat.
- HPC and LPC: the mean concentrations and SDs for the HPC and the LPC are preestablished by running them 20 times (4 times in 5 assays). These controls are then included in each run, and the copy number obtained for each level should be recorded. The values should fall within 2 SDs of the preestablished mean for each level. Use the following modified Westgard rules for troubleshooting HPC and LPC:
 - 1 2S: any value $> \pm 2$ SD, $< \pm 3$ SD—it is a warning if it occurs on only one level within the last ten values; accept the run. Reject the run if $> \pm 2$ SD values occur on two levels in the last ten values; repeat the run. If repeat controls fail, verify calibration.
 - 1 3S: any value $> \pm 3$ SD—reject the run; repeat the run.
 - Both high and low controls $> \pm 2$ SD—reject the run; repeat the run.
 - 10 \times : 10 values are on same side of the mean (indicates a trend/shift)—review; investigate the cause and perform corrective action.

12.3.10.3 Calibration Verification

- Perform calibration verification using the CMV QS 1-4 once every 6 months (if it is $> \pm 2$ SD from historical slope and intercept averages, investigate the cause and perform corrective action).
- Perform calibration verification using the Acrometrix OptiQuant CMV DNA quantification panel every 6 months (if it is more than a $3\times$ difference, investigate the cause and perform corrective action).

12.3.10.4 Proficiency Testing

Perform the CAP survey twice per year.

12.4 Summary

The protocols described here for EBV and CMV viral load testing using qPCR include steps from sample processing to result reporting and quality control and troubleshooting. The QS used for the EBV assay are calibrated to the Acrometrix OptiQuant EBV plasma panel. The QS for the CMV assay are from the Artus CMV PCR ASR reagents-TM and are not calibrated to any reference standard. However, with the release of the World Health Organization International Standard for CMV [6], calibration of

the QS for CMV qPCR is soon expected to become routine. Calibration to such a reference standard will facilitate comparison of viral load measurements among different assay methods or laboratories and thus will facilitate uniform patient management strategies at multiple care centers. Because the protocols described here were developed and validated using ASR, as opposed to reagents labeled “for in vitro diagnostic use,” federal regulations require that the following disclaimer accompany the test results on the patient reports: “This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the US Food and Drug Administration.”

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Chapter 13

Quantification of BK Viral Load

Hai Wu and Jianli Dong

Keywords BK virus • BK virus nephropathy • Viral load • Real-time polymerase chain reaction

13.1 Introduction

Kidney transplants were established in the early 1960s, and BK virus (BKV) was discovered in 1971 (named after a patient's initials) [1]. BKV has no recognized clinical significance until transplant centers experienced outbreaks of BKV infection [2–5]. The virus is a nonenveloped double-stranded DNA virus of the polyomavirus group closely related to SV40 and JC virus. BK viral DNA is approximately 5.13 Kb and functionally divided into three regions: early, late, and noncoding control region (NCCR) (Fig. 13.1). The early region encodes large T and small t antigens, and the late region encodes three capsid structural proteins VP1, VP2, VP3, and agnoprotein [6]. Primary infection with BK generally occurs during childhood without specific symptoms and is widespread in the population, with approximately 80% of adults infected globally. BK virus associated allograft nephropathy (BKVN) is caused by reactivation of latent intra-graft viruses when transplant recipient is under strong immune suppression. BKVN has emerged as a major problem affecting up to 10% of all kidney grafts within the first 6–18 months of transplantation. From this 10%, up to 50% will have total graft failure [2–5]. Renal biopsy is considered the gold standard in the diagnosis of BKVN. In addition to renal biopsy, quantitation of BK viral DNA in blood and urine samples has been increasingly used in the monitoring and management of BKVN [7]. Decreased immunosuppression is often required to prevent the development and progression of BKVN [8].

We describe here a quantitative real-time PCR method to determine BK viral load in plasma and urine specimens. Extraction of viral DNA from plasma and urine is performed using BioRobot M48 (QIAGEN, Valencia, CA). Real-time PCR is performed using minor groove binder (MGB) Alert® real-time PCR reagents, http://epochbio.com/innovation/real_time_pcr_technology/ (Epoch Biosciences, San Diego, CA). Specific primers pertaining to an internal control (IC) and to BK viral DNA are used for amplification. A 72 nucleic acid sequence from the VP1 region of BK virus is amplified (<http://epochbio.com/files/pdf/141075.A.pdf>). The advantage of the MGB probe is that it utilizes the minor groove binding species of DNA to provide a hyperstability of the hybridization

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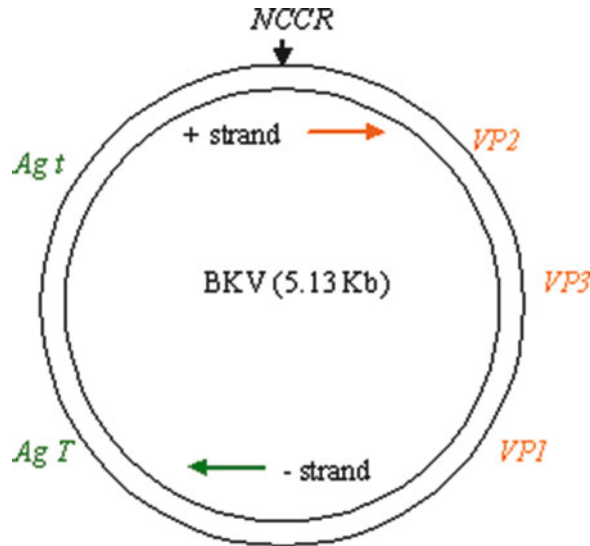


Fig. 13.1 Schematic representation of the gene organization in the BKV genome. The double circle represents the double-stranded DNA genome of 5.13 Kb. The genome is divided into three regions. The early region encodes *T* and *t* antigens. The late region encodes *VP1*, *VP2*, and *VP3* structural proteins. The noncoding control region (*NCCR*) contains the elements for the control of viral DNA replication (*ori*) and viral gene expression. The arrows indicate the positive and negative strands according to the direction of viral transcription [6]

between the DNA probe and target. The BK probe is labeled at 5' end with a nonfluorescent quencher and MGB molecule. The 3' end of the probe is labeled with FAM. The IC probe is labeled similarly at the 5' end, and the 3' end of the IC is labeled with TET. The MGB molecule folds back into the minor groove of the DNA duplex to stabilize hybridization. Therefore, short DNA probes can be designed to obtain a higher melting temperature and improve mismatch discrimination. During the denaturing phase of PCR, fluorescence is quenched because the probe is in solution and the quencher is in close proximity of the reporter dye. When the probe anneals to the target sequence, the quencher becomes spatially separated from the reporter dye allowing fluorescence. The probe becomes released during the extension phase when amplification occurs (http://epochbio.com/innovation/real_time_pcr_technology/).

13.2 Methodology of BKV Real-Time PCR

13.2.1 Samples Types

Based on our validation study, both plasma and urine are accepted to measure BK virus load. Serum specimen is not rejected but not preferred. Separate plasma by centrifugation at $1,000 \times g$ for 15 min within 4 h of blood draw. After centrifugation, store specimen at room temperature. Deliver blood or urine specimens to sample management within 24 h of sample collection. Insufficient sample collection, incorrect blood collection tube, and gross hemolysis specimen will be rejected. Specimen is stored at -70°C in laboratory.

13.2.2 Real-Time PCR Reagents

MGB Alert BK virus (BKV) probe Mix ASR, MGB Alert BK virus (BKV) Primer Mix ASR, and real-time PCR internal control 2B set are purchased from Epoch Biosciences (San Diego, CA). LightCycler FastStart DNA Master HybProbe is purchased from Roche (Pleasanton, CA).

13.2.3 Equipment and Materials

- BioRobot M48 Workstation and extraction reagents, QIAamp DNA Mini Kit (QIAGEN, Valencia, CA)
- SmartCycler II; SmartCycler Reaction Tube, 25 μ L; cooling block (Cepheid, Sunnyvale, CA)
- Tubes with screw caps, 1.5 mL or 2 mL (Sarstedt, Newton NC)
- Pipettes (20, 50, 100, 200, 400, 600, and 1,000 μ L) with aerosol barrier or positive displacement RNase-free pipette tips
- Mini centrifuge Model C1201 (LabNet, Woodbridge, NJ)
- Tube puller

13.3 Protocol

13.3.1 Preparation of MGB Alert Internal Control for BK Viral DNA Extraction

1. Pipette 990 μ L of Buffer AVE taken from the M48 QIAGEN kit and dispense into a 2.0-mL tube.
2. Repeat the same process using a second 2.0-mL tube.
3. Bring the IC to room temperature, vortex briefly, then quick spin.
4. Pipette 10 μ L of the IC and dispense into the first tube and vortex.
5. Pipette 10 μ L from the first tube and add it to the second tube with 990 μ L AVE and vortex.
6. The second tube now has a concentration of 1,000 copies/ μ L of IC for the extraction procedure.

Take 60 μ L of the 1,000 copies/ μ L IC dilute and add it to well number 6 of the preparation plate to be used with the M48 QIAGEN Robot for the extraction process.

13.3.2 DNA Extraction Using M48 Robot or QIAamp DNA Mini Kit

DNA Extraction Using M48 Robot

On the M48 Bio Robot software interface, select extraction volume of 400 μ L (plasma or urine) and DNA elution volume of 50 μ L.

MGB Alert IC must be added to the preparation plate in well number 6. The IC comes at a concentration of 10^7 copies/ μ L and must be diluted to a final concentration of 1,000 copies/ μ L. (Internal control is used to validate the extraction and real-time PCR procedures for each specimen. If IC is not added in the extraction portion, it must be added to the PCR mixture. Otherwise, the reporter dye for the internal control will not work and the run will be invalid.)

DNA Extraction Using QIAamp DNA Mini Kit

Preparation of Buffer AW1

No. of Preps Per Kit	AW1 (concentrate) (mL)	Ethanol (mL)	Final volume (mL)
50	19	25	44
250	95	125	220
1,000	175	230	405

Preparation of Buffer AW2

No. of Preps Per Kit	AW2 (concentrate) (mL)	Ethanol (mL)	Final volume (mL)
50	13	30	43
250	66	160	226
1,000	127	300	427

Tips for Handling the QIAamp Spin Columns

- Carefully apply the sample or solution to the center of the QIAamp spin column membrane.
 - Pipet sample into the QIAamp spin column without wetting the rim of the column.
 - Avoid touching the QIAamp membrane with the pipet tip.
 - Change pipet tips between liquid transfer steps.
1. Equilibrate the samples to room temperature (15–25°C).
 2. Pipet 60 μ L of internal control, 40 μ L of QIAGEN Protease and 400 μ L of Buffer AL to the bottom of a 1.5-mL microcentrifuge tube.
 3. Add 400 μ L of plasma sample to the microcentrifuge tubes.
 4. Mix by pulse-vortexing for 15 s.
 5. Incubate at 56°C for 10 min.
 6. Quick spin the tubes to remove drops from the inside of the lid.
 7. Add 400 μ L of 96–100% ethanol to each sample. Change tips after each sample.
 8. Mix by pulse-vortexing the mixture for 15 s.
 9. Quick spin the tubes to remove drops from inside of the lid.
 10. Carefully pipet 650 μ L of the solution from step 9 to the QIAamp spin column (in a 2-mL collection tube) WITHOUT WETTING THE RIM. Close the cap (do not splash).
 11. Centrifuge the QIAamp spin column and collection tube at $6,000 \times g$ (8,000 rpm) for 1 min.
 12. Place the QIAamp spin column into a clean 2-mL collection tube. Discard the tube containing the filtrate.
 13. Carefully uncap the QIAamp spin column and repeat steps 11, 12, and 13 until all lysate has been loaded onto the spin column.
 14. Carefully uncap the QIAamp spin column and add 500 μ L of Buffer AW1. Close the cap (do not splash).
 15. Centrifuge the QIAamp spin column and collection tube at $6,000 \times g$ (8,000 rpm) for 1 min.
 16. Place the QIAamp spin column in a clean 2-mL collection tube. Discard the tube containing the filtrate.
 17. Carefully uncap the QIAamp spin column and add 500 μ L of Buffer AW2. Close the cap (do not splash).
 18. Centrifuge at full speed $20,000 \times g$ (14,000 rpm) for 3 min.
 19. Place the QIAamp spin column in a new 2-mL collection tube. Discard the collection tube with filtrate.
 20. Centrifuge again at $20,000 \times g$ (14,000 rpm) for 1 min.

21. Place the QIAamp spin column in a clean 1.5-mL microcentrifuge tube. Discard the collection tube containing the filtrate.
22. Carefully open the QIAamp spin column and add 50 μ L of Buffer AE (room temperature).

Note: For better DNA recovery

Ensure that the spin column is in an upright position.

Do not wet the rim with Buffer AE.

Slowly add the Buffer AE to the center of the filter.

23. Close the cap and incubate at room temperature for 5 min.
24. Centrifuge at $6,000 \times g$ (8,000 rpm) for 1 min.
25. Collect eluate. Discard column.
26. Deliver to real-time PCR step directly or freeze at -20°C .

13.3.3 Preparation of Controls

- Negative control: Extract one tube of 400 μ L AVE Buffer with internal control; final elution volume is 50 μ L. Add 10 μ L extraction elute per PCR reaction.
 - Low and high positive control: BK plasmid DNA pBKV (33–1) is purchased from American Type Culture Collection (ATCC, Manassas, VA). It consists of a 5.13 Kb BK genomic DNA insert in vector pBR322 (ATCC). Transform 1 ng of the plasmid DNA containing an antibiotic resistance gene to bacterial competent cells. Plate cells on LB agar plates containing the relevant antibiotic(s). Incubate plates upside down at 37°C for 12–24 h until colonies develop. Extract and purify plasmid DNA using QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA).
1. Pick a single colony from a LB agar plate and inoculate in a culture of 10 mL LB medium containing the appropriate antibiotics. Grow the culture at 37°C with vigorous shaking (~ 240 rpm) for approximately 4–6 h until $\text{OD}_{600} = 0.8\text{--}1.2$.
 2. Harvest cells by centrifugation at $3,000 \times g$ for 15 min at 4°C .
 3. Resuspend pelleted bacterial cells in 250 μ L Buffer P1 containing lysozyme at a final concentration of 1 mg/mL.
 4. Incubate at 37°C for 10 min.
 5. Add 250 μ L Buffer P2 and gently invert the tube four to six times to mix.
 6. Add 350 μ L Buffer N3 and invert the tube immediately but gently four to six times.
 7. Centrifuge at $\geq 10,000 \times g$ for 10 min. During centrifugation, place a QIAprep Spin Column in a 2-mL collection tube.
 8. Apply the supernatant from step 7 to the QIAprep Spin Column by decanting or pipetting.
 9. Centrifuge at $\geq 10,000 \times g$ for 30–60 s. Discard the flow-through.
 10. Wash the QIAprep Spin Column by adding 0.5 mL Buffer PB and centrifuge at $\geq 10,000 \times g$ for 30–60 s. Discard the flow-through.
 11. Wash the QIAprep Spin Column by adding 0.75 mL Buffer PE and centrifuge at $\geq 10,000 \times g$ for 30–60 s.
 12. Discard the flow-through, centrifuge at $\geq 10,000 \times g$ for an additional 1 min to remove residual wash buffer.
 13. Place the QIAprep Spin Column in a clean 1.5-mL microcentrifuge tube. To elute DNA, add 50 μ L Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0) to the center of each QIAprep Spin Column, let stand for 1 min, and centrifuge for 1 min.
 14. The purified plasmid DNA is adjusted to 5,000 copies/mL and 117,000 copies/mL and used as low and high positive controls.

Table 13.1 Serial dilutions of BK DNA to generate standard curve

BK test controls (copies/ μ L)	BK copies/10 μ L (per PCR reaction)	Dilution factor	Copies/mL	Replicates
Positive cont. 8×10^7	Positive cont. 8×10^8	12.5	1×10^{10}	1
BK control A 8×10^6	BK control A 8×10^7	12.5	1×10^9	2
BK control B 8×10^5	BK control B 8×10^6	12.5	1×10^8	1
BK control C 8×10^4	BK control C 8×10^5	12.5	1×10^7	2
BK control D 8×10^3	BK control D 8×10^4	12.5	1×10^6	1
BK control E 8×10^2	BK control E 8×10^3	12.5	1×10^5	2
BK control F 8×10	BK control F 8×10^2	12.5	1×10^4	1
BK control G 8	BK control G 80	12.5	1×10^3	2
BK control H 4	BK control H 40	12.5	5×10^2	3
BK control I 2	BK control I 20	12.5	2.5×10^2	4
Negative	0			1

13.3.4 Standard Curve

A 10^{10} copies/mL BK virus plasmid (Epoch Biosciences) is serially diluted from 8×10^8 copies/10 μ L down to 20 copies/10 μ L to make standard curve.

Ten concentrations are used to generate BK standard curve.

- To dilute Nanogen BK positive control DNA (4×10^{10} copies/ μ L) 1:500 in AVE Buffer (QIAGEN) to reach 8×10^8 copies/10 μ L, add 5 μ L of the BK positive control DNA to 2,495 μ L of AVE Buffer.
- Dilute the above 8×10^8 copies/10 μ L BK control as 1:10 or 1:2 with AVE Buffer (QIAGEN) to make 8×10^7 copies/10 μ L to 20 copies/10 μ L concentration series (Table 13.1., A to I). Combine 10 μ L or 40 μ L of BK control with 90 μ L or 40 μ L of AVE Buffer.
- Take 10 μ L of each per PCR reaction.

13.3.4.1 Dilution Factor

400 μ L of starting sample is used for extraction.

Elution volume is 50 μ L, of which 10 μ L (1/5) is used for PCR.

To generate unit of copies/mL, divide 400 μ L/5 to yield 80 and 1 mL/80 = 12.5.

Note: Multiply BK copies/ μ L by dilution factor of 12.5 to obtain final copies/mL for reporting.

13.3.4.2 Preparing the Standard Curve Samples for PCR

Note: Since the standard curve samples are prepared without internal control, it must be added to the PCR mixture.

Prepare the following reactions for 11 standard controls (Table 13.1):

- Pipette 15 μ L of the mix into the tubes loaded on the cooling rack.
- Pipette the standards into the appropriate tube.
- Mix the reaction with pipette.

Table 13.2 MGB Alert BK PCR reaction for standard curve

	1×	13×
Water PCR grade, vial 3 colorless	6.25 μ L	81.25
MgCl ₂ , vial 2 blue cap	4.0 μ L	52.0
20× MGB Alert BK virus 2.0 detection reagent ASR	1.25 μ L	16.25
10× LightCycler FastStart DNA Master HybProbe	2.5 μ L	32.5
<i>Internal control at 1,000 copies/μL</i>	1.0 μ L	13.0
DNA template	10.0 μ L	
<i>Total for one reaction</i>	25.0 μ L	

Table 13.3 MGB Alert BK PCR reaction for standard curve (40 copies/10 μ L)

	1×	3×
Water PCR grade, vial 3 colorless	6.25 μ L	18.75
MgCl ₂ , vial 2 blue cap	4.0 μ L	12.0
20× MGB Alert BK virus 2.0 detection reagent ASR	1.25 μ L	3.75
10× LightCycler FastStart DNA Master HybProbe	2.5 μ L	7.5
<i>Internal control at 1,000 copies/μL</i>	1.0 μ L	3.0
DNA template 40 copies/10 μ L	10.0 μ L	30.0
<i>Total for one reaction</i>	25.0 μ L	

Table 13.4 MGB Alert BK PCR reaction for standard curve (20 copies/10 μ L)

Water PCR grade, vial 3 colorless	6.25 μ L \times 4 =	25.0
MgCl ₂ , vial 2 blue cap	4.0 μ L	16.0
20× MGB Alert BK virus 2.0 detection reagent ASR	1.25 μ L	5.0
10× LightCycler FastStart DNA Master HybProbe	2.5 μ L	10.0
<i>Internal control at 1,000 copies/μL</i>	1.0 μ L	4.0
DNA template 20 copies/10 μ L	10.0 μ L	40.0
<i>Total per reaction</i>	25.0 μ L	

The standard curve in our laboratory has a range of 8×10^8 to 20 copies/10 μ L (see Table 13.2). Two additional PCR mixes are prepared to replicate the low concentrations (three replicates of 40 copies/10 μ L and four replicates of 20 copies/10 μ L) (see Tables 13.3 and 13.4).

- Pipette 15 μ L of the mix into the tubes loaded on the cooling rack.
- Then pipette 10 μ L of the standards into the appropriate tube.
- Mix the reaction with pipette.

The following table represents the PCR amounts for the lower limit of the curve at 20 copies/10 μ L:

- Pipette 15 μ L of the mix into the tubes loaded on the cooling rack.
- Then pipette 10 μ L of the standards into the appropriate tube.
- Mix the reaction with pipette.
- After adding all samples, bring the tubes into PCR and post-PCR area (Area 3) and spin the tubes for 1 min on the mini centrifuge designed for the SmartCycler tubes. When placing the tube on the mini centrifuge, make sure that the reaction tube is as horizontal as possible.
- Remove the tubes from the centrifuge and place into the I-Core slot of the SmartCycler and close the cover.

In our laboratory, the high and low positives, but not the standard curve samples, are included in every run.

Table 13.5 MGB Alert BK PCR reaction

Reagents	Negative control and patient sample	Positive controls
Water PCR grade, vial 3 colorless	7.25 μL	6.25 μL
MgCl ₂ , vial 2 blue cap	4.0 μL	4.0 μL
20× MGB Alert BK virus 2.0 detection reagent ASR	1.25 μL	1.25 μL
10× LightCycler FastStart DNA Master HybProbe	2.5 μL	2.5 μL
DNA template*	10.0 μL	11.0 μL
Total	25.0 μL per reaction	25.0 μL per reaction

13.3.5 Preparation of the PCR Mix

MGB Alert BK virus 2.0 detection reagent ASR mix is an analyte-specific reagent (ASR) that contains synthesized DNA oligonucleotide primers and probes designed to detect BK nucleic acid sequences. The mix contains internal control primers and probes. The reagent should be protected from light and stored at $\leq -20^{\circ}\text{C}$. This product is limited to five freeze thaws (see Table 13.5).

Set up PCR reaction in a 1.5-mL tube following the listed reagent order in pre-PCR area (Area 1) (except adding DNA samples in sample preparation area, Area 2).

1. *10 μL extracted elute is added to each tube of negative control and patient samples.
2. 10 μL high/low positive control (see positive control preparation in the part three of procedure) and 1 μL 10^3 copies/ μL internal control without extraction is added in each tube of the two positive controls.
3. Calculate the amount of reagent to use based on the above PCR reaction mix and include two positive controls and negative control plus one for pipetting loss.
4. Take out the cooling block from 4°C in sample preparation area (Area 2). Place appropriate numbers of SmartCycler tubes into slots on the block. A blue bracket will fit on top of the block to secure the tubes.

Note: The cooling block provides even dry cooling which allows for more stable and consistent results. It also supports the tubes to make capping easier. Always place a tube rack on top of the block and store the cooling block in 4°C . Clean the cooling block with 70% ethanol and rinse in distilled water and dry well. To disinfect the cooling block, soak it briefly in 10% bleach for only 5 min and rinse with copious amounts of water.

5. Label the tubes accordingly.

Note: The polypropylene SmartCycler reaction tube is designed for optimal thermal and optical characteristics. The optical detection windows are along the bottom edges of the tube. Handle the reaction tube by the ribbed upper portion of the tube and avoid touching the sides of the diamond shape area. The cap must be seated properly in the tube before the reaction tube is closed. When closed correctly, the tube becomes pressurized, and the sides of the diamond shape area bulge slightly. Amplification may not be efficient if the tube fails to pressurize. A distinct snap will occur when closing a reaction tube seated on the cooling block.

6. Aliquot 15 μL of the prepared PCR mix into each of the SmartCycler tubes.
7. Then aliquot 10 μL of the template DNA and mix up and down with the pipette.
8. After adding the samples, bring the tubes into Area 3 and spin the tubes for 1 min on the mini centrifuge designed for the SmartCycler tubes. When placing the tube on the mini centrifuge, make sure that the reaction tube is horizontal as possible.
9. Remove the tubes from the centrifuge and place into the I-Core slot of the SmartCycler and close the cover.

Note: The tube puller can make it easier to remove reaction tubes from the I-Core module. Place the prongs of the puller between the ribs of the tube near the bottom. Pull the tube straight up and do not rock the tube from side to side instead rock it gently front to back.

13.3.6 *Opening the SmartCycler Software and Creating a New Run*

1. Turn ON the SmartCycler II processing block from the back on top.
2. Double click on the SmartCycler icon on the desktop of the computer.
3. Click the Create Run icon to create a new run.
4. Enter today's date on the Run Name field.
5. Click the arrow in the Dye Set box and select FTTC25 (represents FAM, TET, TX red, and Cy5) for 25- μ L reaction tubes.
6. Under the heading of Usage, click the field next to the box representing TX red and Cy5 and select Unused.
7. Most importantly, click the field next to the TET dye and select internal control.
8. Click the Add/Remove Sites button to open the Select Protocols and Sites dialog box.
9. Highlight the protocol named Epoch Biosciences-BKV.
10. Then highlight sites to be used.
11. Click the right arrow to transfer the selected site and protocol to the Selections field.
12. Click OK to save the selections.
13. Review the run set up and click Start Run.
14. After the start run is clicked, the orange LEDs on the SmartCycler II will turn on.

The cycling conditions for amplification and detection are as follow:

1. 1 \times of
95°C 10min
2. 50 \times of
95°C 15 s
56°C 30 s
76°C 30 s
3. Melting curve analysis, 40°C, -80°C, 0.5°C/s.
4. The software will switch to the View Results screen.
5. Click Result Table, enter the names of the samples, and define sample type as UNKN (unknown) or STD (standard).
6. The Epoch Biosciences-BKV protocol will take 1 h and 36 min to complete.
7. Figure 13.2. is an example of a real-time PCR.

13.3.7 *Process the Results*

1. After the run is completed and data generated, the next step is to import the standard curve (Fig. 13.3).
2. Select the icon Import Std Curve at the bottom of the computer screen.
3. Highlight the Std Curve name (e.g., STCUR07202009), click OK.
4. A yellow screen will appear indicating the standard curve. Scrolling below the yellow screen to reveal the results.
5. Review the results: Work Status OK; Internal Control PASS; Melt Peak Temp.
6. Select Export to transfer the table with test results to an excel format.

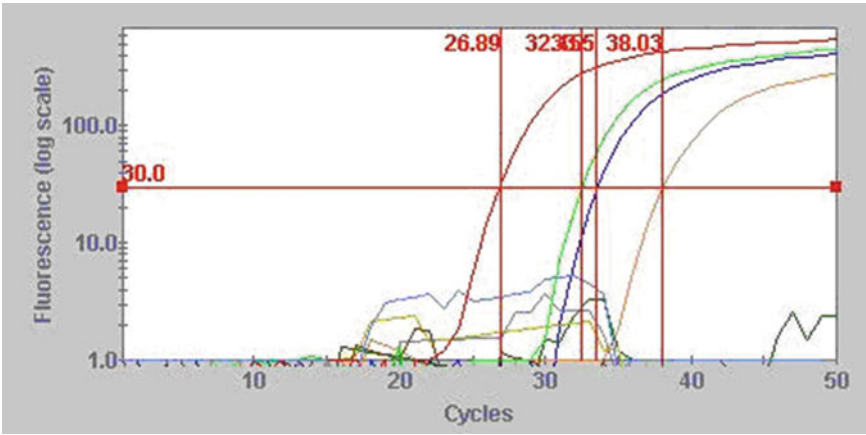


Fig. 13.2 *Graph of real-time PCR results.* The horizontal red line in the middle of the graph is the “threshold” set by user. The point at which the amplification plot of a sample crosses this threshold is the cross threshold (*C_t*) value. The lower the *C_t* value of a sample, the greater the starting amount of DNA in the sample. The concentrations of sample DNA are calculated based on comparison of *C_t* values of samples vs. standard curve samples. Included in the figure are amplification plots of two positive controls, two positive samples, one negative control, two negative samples, and some background fluorescent signals

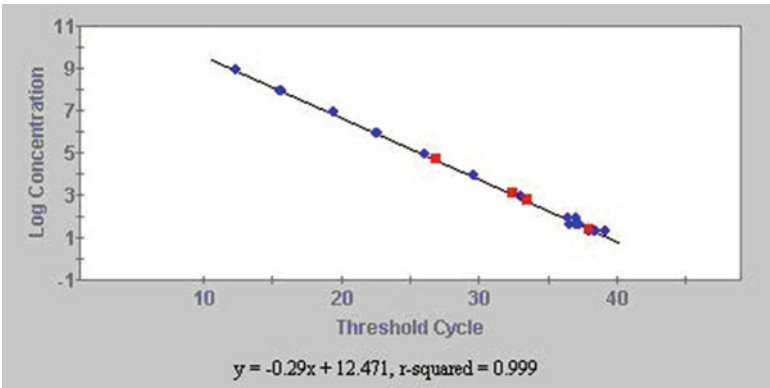


Fig. 13.3 *Standard curve and the location of the two positive controls and two positive samples (red spots)*

7. The Export Data dialog box appears.
8. Select the type of data to export.
9. Choose Export Results Table and Analysis Settings.
10. Select a file name.
11. Minimize the software screen and open the exported file by right clicking the Start icon and choosing Explore.
12. Open the folder named SmartCycler.
13. Click on the Export folder and open the file (dated) that was exported.
14. Open the folder TEMPLATE and double click the file named BKQUANT-REPORT.
15. The BKQUANT-REPORT file contains three tabs on the bottom.
16. Go to the tab labeled RAWDATA.
17. Copy the entire exported file with the results and paste it to RAWDATA file, making sure the fields match.
18. Once the exported file is copied over the RAWDATA file, the calculations will be made instantly for the copies/mL and the log copies/mL values.

13.3.8 Reference Intervals and Interpretation of Results

After the data processing and analyzing, the concentrations of BK DNA copies/mL and log copies/mL are calculated for each control and patient sample. The reportable range for this assay is 2.6–8.6 log₁₀ copies/mL or 390–3.9 × 10⁸ copies/mL. Patient's results are reported as below:

1. When values are below 390 copies/mL or 2.6 log copies/mL, results are reported as “<390 copies/mL” or “<2.6 log copies/mL.”
2. When values are between 390 and 3.9 × 10⁸ copies/mL or 2.6 and 8.6 log copies/mL, report the viral load value.
3. The values are above 3.9 × 10⁸ copies/mL or log 8.6 log copies/mL, report “>3.9 × 10⁸ copies/mL” or “>8.6 log copies/mL.”

A negative result of BK virus DNA quantitation (less than 2.6 log copies/mL or less than 390 copies/mL) does not rule out the presence of PCR inhibitors in the patient specimen or BK virus DNA concentrations below the level of detection by the assay. Inhibition may also lead to underestimation of viral quantitation.

13.4 Troubleshooting

1. If the copy numbers of positives are out range (high positive 5.1 ± 1 log copies/mL, low positive 3.7 log copies/mL), do not report patient results. Check the Ct of IC, if Ct of IC is correct (<45, >32), repeat the positive controls and samples.
2. If the positive control is correct, but the Ct of IC is incorrect, do not report the results. Check the content of PCR mix (primer, probe, and LightCycler FastStart) and repeat the test.
3. It is known that urine has PCR inhibitors [7]. If IC Ct of a urine specimen is >45, use 200 µL of sample to repeat testing, the dilution factor will change from 12.5 to 25. Report indeterminant (IND) if IC again is Ct >45.
4. When both urine and plasma samples are collected from the same patient at the same time, urine should have higher BK copy number than plasma. Otherwise both samples need to be repeated, starting from sample preparation.
5. The assay is validated as a quantitative test. Test limit of detection (LOD) is not determined. In rare cases, when BK result is <2.6 log copies/mL but result is clearly positive based on PCR slope and cycle cutoff (e.g., 2.5 log copies/mL), clinics prefer to know that the sample is BK positive.
6. The amplicon T_m is 62 ± 2°C with control “wild-type” strain of BK DNA. If the T_m of a sample is out of the range, it is possible that the probe binding site has sequence variations due to genotype/subtype difference or mutations. Subcloning and sequencing of PCR amplicons should help distinguish the cause.

13.5 Summary

Here we described the use of Epoch Biosciences MGB Alert BK real-time PCR ASR in our laboratory to monitoring BK viral load in plasma and urine samples as a surrogate marker of BKV nephropathy. BKV is a nonenveloped, double-stranded DNA virus of the polyomavirus family that primarily affects immunocompromised people. BKV may cause nephropathy in renal transplant recipients receiving immunosuppressive therapy, resulting in renal dysfunction and, possibly, graft loss. Specific and

potent antiviral drugs to treat active BKV infections are not available, thus requiring patient screening and early diagnosis of BKVN. Measurement of BKV loads in the plasma and urine is a powerful clinical tool for identifying patients at risk for developing BKVN and for monitoring response to therapy. Real-time quantitative PCR has become an increasingly popular method in the diagnosis and management of BKVN. However, assay sensitivity and viral load magnitude of quantitative PCR vary from laboratory to laboratory [7]. Hence, each testing laboratory will need to establish its own cutoff values for the purposes of clinical management.

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Chapter 14

Molecular Diagnosis and Epidemiology of Rabies

Rodney E. Rohde and Bonny C. Mayes

Keywords Rabies • Rabies virus variants • Rhabdoviridae • FDA • Nucleic acid amplification test • CNS viruses • Rabies virus typing • RT-PCR • Restriction digest • DNA sequencing analysis

14.1 Introduction

Rabies is a viral disease that causes acute inflammation of the brain (encephalitis) in warm-blooded animals. It is a disease, primarily transmitted by the bite of animals (zoonotic), which is almost always fatal if treatment (post-exposure prophylaxis) is not administered prior to onset of severe symptoms. The disease occurs in more than 150 countries and territories and is responsible for thousands of deaths worldwide each year. Antigenic and genetic typing (and diagnostic) methods are powerful laboratory tools that help shed light on the often difficult identification and epidemiology which surrounds a rabies case.

14.2 Rabies

14.2.1 *Clinical Features and Molecular Pathogenesis of Disease*

At the present time, the genus *Lyssavirus* contains seven viral genotypes; one of these defines rabies virus genotype 1, serotype 1 (RV), and the other six represent specific rabies-related lyssaviruses. Rabies is a zoonotic disease caused by viruses of the *Lyssavirus* genus. The virus is a single-stranded RNA, nonsegmented, negative-sense virus [1]. Transmission of rabies occurs when saliva containing rabies virus is introduced into an opening in the skin, usually via the bite or scratch of a rabid animal. Although rare, transmission can also occur through contamination of mucous membranes or

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transplantation. RV has the highest case fatality ratio of any infectious disease when intervention is not initiated prior to symptoms. Typical treatment for RV exposure consists of wound care, passive immunization with RIG, and a series of four doses of rabies vaccine [2, 3].

A differential diagnosis of rabies should be suspected for individuals with signs or symptoms of encephalitis or myelitis. Patients with these symptoms who respond to treatment do not require rabies testing. The absence of an exposure history does not provide evidence to terminate any suspicions of a rabies diagnosis because most patients in the USA have no definitive exposure history [4]. Indeed, several recent cases of rabies in humans in the United States have been diagnosed either retrospectively or after the clinical course of the disease has progressed despite compatible clinical observations. A heightened awareness is needed among the medical community of possible rabies infections in cases where clinical signs are compatible with a diagnosis of rabies. In addition, medical personnel must be aware of appropriate methods for sample collection for antemortem diagnosis and must know how to interpret the test results.

14.2.2 Specimen Submission and Laboratory Procedures

A patient history form detailing the clinical history of the patient should accompany the specimen, complete with the name and phone number of the physician who should be contacted with the test results. Likewise, animal specimens should also be accompanied by appropriate contact information. All samples should be considered potentially infectious. Test tubes and other sample containers must be securely sealed (tape around the cap will ensure that the containers do not open during transit). If immediate shipment is not possible, all samples, except brain tissues, should be stored frozen at -4.0°F or below. Brain tissues should be refrigerated at $45\text{--}32^{\circ}\text{F}$. Samples should be shipped frozen on dry ice (brain tissues on wet ice or frozen gel packs) by an overnight courier in watertight primary containers and leakproof secondary containers that meet the guidelines of the International Air Transport Association. The state health department, regional reference laboratory, and/or rabies laboratory at the Centers for Disease Control and Prevention (CDC) should be telephoned at the time of shipment and given information on the mode of shipment, expected arrival time, and courier tracking number [5]. In most states, it is preferable to ship by bus or other reliable carrier; contact the entity that will be receiving the shipment for their recommendations (http://www.cdc.gov/rabies/specific_groups/laboratories/index.html).

The single most important standard diagnostic test for rabies in animals is the DFA test [6, 7]. In cases where individuals are aware of an animal exposure with a known or suspected rabid animal, rapid and accurate laboratory testing of the animal, if it is available, allows hospital physicians to begin timely postexposure prophylaxis (PEP). It is equally important to know that an animal is not rabid because one is able to eliminate the need for expensive and extended rabies prophylaxis treatment. Even in instances where a laboratory diagnosis is delayed, once a negative rabies result is obtained, the PEP can be halted, preventing any further unnecessary medical treatment and associated costs [7]. For recommendations with respect to the testing of animals suspected of rabies, individuals should consult the annual Compendium of Animal Rabies Prevention and Control [8].

Lyssavirus samples can be discriminated (typed) by both antigenic and genetic methods. Antigenic methods commonly rely on monoclonal antibodies (MAbs) to identify unique epitopes or recognition sites. The resolving power of an antigenic analysis is typically determined by a direct relationship to the number of MAbs used to type a virus. Genetic typing methods detect viral RNA mutations at the nucleotide level, and different genetic typing methods can be ranked in their discriminatory power. Nucleotide sequencing (entire RV genome is approximately 12,000 nucleotides) has the highest resolution. Following sequencing, digestion with restriction enzymes follows in resolution. Finally, this is followed by using specific oligonucleotide probes to differentiate virus populations. The focus of this chapter will be that of genetic typing methods, which are more reproducible and readily quantified [9].

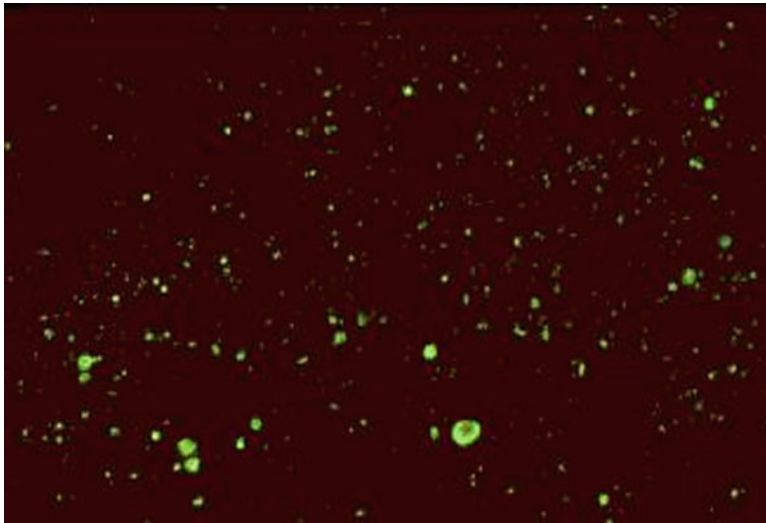


Fig. 14.1 Immunofluorescence (DFA) of positive rabies antigen

14.2.3 Antemortem Testing Procedures

If rabies is considered as a diagnosis, a variety of samples should be sent for antemortem study. These include nuchal skin biopsy, saliva, serum, and cerebrospinal fluid (CSF). As with any disease of unknown origin, personal protective equipment and barrier protection should be considered for the collection of these samples. This will not only protect the technologist from exposure to rabies if the patient is rabid, but it will also protect the technologist against any other potential pathogens that have yet to be identified. The following instructions should be used to collect samples only after consultation with your state health department, with regional reference laboratory or with the rabies laboratory at the CDC [5].

Skin biopsies (5–6 mm in diameter) should be taken from the posterior region of the neck at the hairline. A minimum of 10 hair follicles, biopsied at a depth to include the cutaneous nerves at the follicle base, should be contained in the specimen. The specimen should be placed on a piece of sterile gauze moistened with sterile water and placed in a sealed container. Do not add preservatives or additional fluids. Laboratory tests to be performed include reverse transcription-polymerase chain reaction (RT-PCR) of extracted nucleic acids and DFA (see Fig. 14.1) for viral antigen in frozen sections of the biopsy [5].

Saliva should be collected using a sterile eyedropper pipette and placed in a small sterile container that can be sealed securely. Preservatives or additional material should not be added. Laboratory tests to be performed include detection of rabies RNA using RT-PCR and isolation of infectious virus in cell culture. Tracheal aspirates and sputum are not suitable for rabies tests [5].

A minimum of 0.5 ml of serum or CSF should be collected; no preservatives should be added. Whole blood should not be submitted because it contains various inhibitors against nucleic acid amplification techniques. If no vaccine or rabies immune serum has been given, the presence of antibody to rabies virus in the serum is diagnostic and testing of CSF is unnecessary. Antibody to rabies virus in the CSF, regardless of the immunization history, suggests a rabies virus infection. Laboratory tests for antibody include DFA and virus neutralization [5].

The rarity of rabies and the lack of an effective treatment make the collection of a brain biopsy unwarranted; however, biopsy samples negative for herpes and other types of encephalitis should be

tested for evidence of rabies infection [5, 10]. The biopsy is placed in a sterile sealed container; preservatives or other fluids should not be added. Laboratory tests to be performed include RT-PCR and DFA for viral antigen in touch impressions [5].

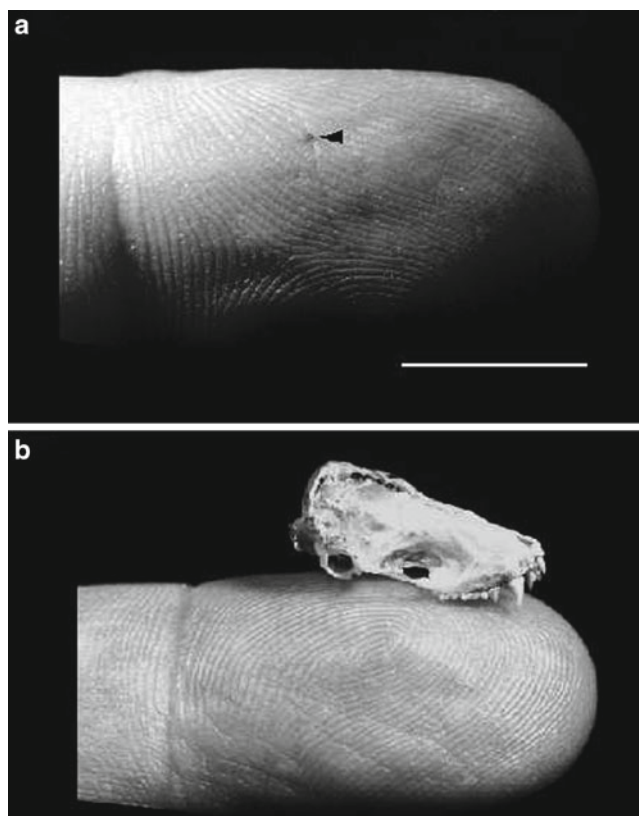
Integrity, type, and time of collection of antemortem specimens are critical to the correct diagnosis of a rabies infection. The neurotropic nature of the virus makes it important to collect a variety of samples early and intermittently during the course of a differential diagnosis. Due to the properties of rabies pathogenesis, one can assume limited success of antemortem diagnostic tests for rabies. For example, following primary infection, the virus enters an eclipse phase in which it cannot be easily detected within the host. This phase may last for several days or months. Investigations have shown both direct entry of virus into peripheral nerves at the site of infection and indirect entry after viral replication in nonnervous tissue (i.e., muscle cells). During the eclipse phase, the host immune defenses may confer cell-mediated immunity against viral infection because rabies virus is a good antigen. The uptake of virus into peripheral nerves is important for progressive infection to occur. After uptake into peripheral nerves, rabies virus is transported to the central nervous system (CNS) via retrograde axoplasmic flow. Typically, this occurs via sensory and motor nerves at the initial site of infection. The incubation period is the time from exposure to onset of clinical signs of disease. The incubation period may vary from a few days to several years, but it is typically 1–3 months. Dissemination of virus within the CNS is rapid and includes early involvement of limbic system neurons. Active cerebral infection is followed by passive centrifugal spread of virus to peripheral nerves. The amplification of infection within the CNS occurs through cycles of viral replication and cell-to-cell transfer of progeny virus. Centrifugal spread of virus may lead to the invasion of highly innervated sites of various tissues, including the salivary glands. During this period of cerebral infection, the classic behavioral changes associated with rabies develop and any virus present in the saliva is intermittent [11].

14.2.4 Postmortem Testing Procedures

Postmortem diagnosis of rabies in humans is made by the standard test DFA staining of viral antigen in touch impressions of brain tissue. Portions of the medulla (brain stem), the cerebellum, and the hippocampus should be kept refrigerated and shipped to a public health laboratory for rabies testing [5]. Preservation of tissues by fixation in formalin is not recommended if rabies diagnosis is desired. However, if tissue has been placed in formalin, procedures have been described to analyze the specimen [5, 12].

Reference laboratories that perform rabies virus variant characterization offer several benefits to the medical story. First, a determination of the virus variant is often only obtainable from postmortem samples. This is typically due to the lack of an animal exposure history from the patient, which subsequently means an absence of animal samples to test. A reference laboratory can aid in the elucidation of the mystery that will often be associated with a rabies case. The identification of the rabies virus variant may result in a clearer understanding of the type of exposure that the patient may have had with a rabid animal [4, 13] or any foreign travel by the patient. In recent years, one of the biggest assets of rabies virus typing has been the discovery that most of the cases for which there is no bite exposure history have been attributed to bat rabies virus variants. A publication by Alan Jackson [14] illustrates the very small puncture wounds associated with a bat bite (see Fig. 14.2). The combination of these factors has led to the enhanced CDC recommendation for bat exposures in that PEP is considered in all situations in which a bat bite or direct contact with a bat may have occurred. Second, a clarification of epizootologic patterns will augment the creation of appropriate public health information and policy for prevention and control of rabies. Third, among the recommendations of the National Working Group on Prevention and Control of Rabies in the United States are expanded resources for regional and national virus typing laboratories. Public health laboratories are facing critical issues, not

Fig. 14.2 Puncture wound of a bite from a silver-haired bat (**a**, *arrow*) and skull of silver-haired bat (**b**) (Reprinted with permission from Elsevier Science (The Lancet, 2001, Vol 357, pp 1714))



the least of which is the problem of accurate surveillance of all the rabies virus variants throughout the USA and other countries. A good model to follow is the successful collaborations between the CDC and the Texas Department of State Health Services (DSHS) rabies laboratory over the last 20 years, which has produced the typing data to identify rabies variants common to animal reservoirs in the southwestern USA and Mexico and mapped their geographic distribution. Appropriately used, this knowledge should allow those who survey rabies to recognize when established reservoirs enlarge or expand into new areas or when different animal species become involved in cycles of rabies virus transmission. Regional laboratories can expand and complement the flow of national surveillance data by increasing their surveillance activities to include antigenic and molecular typing of virus samples from the surrounding states [15–22].

14.3 Methodology

Antigenic (MAb) typing is a simple, inexpensive way to quickly identify rabies virus variants. However, MAb typing has several limitations: it is an indirect FA test, so interpretation can be difficult and subjective at times; very weak specimens generally have to be amplified in mice or cell culture before MAb typing; and it does not differentiate between all variants (e.g., Texas fox and domestic dog-coyote variants have the same MAb pattern but are considered distinct variants). The molecular typing methods described below can be used in cases where MAb typing results are inconclusive.

Table 14.1 Lysis buffer components

Component	Volume
1 M Tris HCL pH 7.5	100 µl
5 M NaCl	333 µl
0.5 M MgCl	33 µl
NP40	65 µl
Molecular grade water	Bring volume to 10 ml

14.4 Protocols

Specimen types: skin biopsy, saliva, brain tissue

14.4.1 Reagents

RNA Extraction Reagents

- Lysis buffer components (see Table 14.1)
- TRIzol
- Chloroform, molecular grade
- Isopropanol, molecular grade
- Ethanol, molecular grade, 75%
- Molecular grade water

Reverse Transcription-Polymerase Chain Reaction Reagents

- RNase inhibitor
- Reverse transcriptase-AMV (RT-AMV) and accompanying 5× RT buffer
- Molecular grade water
- 10 mM PCR nucleotide mix (dNTP)
- Forward and reverse primers
- AmpliTaq
- 1 M Tris-HCl, pH 8.3

Gel Electrophoresis Reagents

- Agarose
- TBE buffer, 10×, molecular biology grade
- Ethidium bromide
- ΦX174 DNA-*HaeIII* digest

Endonuclease Restriction Digest Reagents

- Restriction endonucleases Hinf I and/or Dde I with accompanying buffer H 0.5 M EDTA

Nucleotide Sequencing Reagents

- BigDye® Terminator v1.1 Cycle Sequencing Kit
- DyeEx 2.0 Spin Kit
- Hi-Di™ formamide

Table 14.2 Primers for rabies RT-PCR and nucleotide sequencing

Primer ID	Orientation	Genome position	Sequence	Reference
Lys001	Forward	1–15	ACGCTTAACGAMAAA	[24]
550F ^a	Forward	647–666	ATGTGYGCTAAYTGGAGYAC	^a [24]
1087Sdeg	Forward	1,157–1,173	GAGAARGAACTTCARGA	[25]
1066degB ^a	Reverse	1,136–1,155	TCYCTGAAGAATCTTCTYTC	^a [25]
304	Reverse	1,514–1,533	TTGACGAAGATCTTGCTCAT	[13]

^aReverse complement of a published primer

14.4.2 Procedure

14.4.2.1 RNA Extraction

1. Aliquot 0.1 ml lysis buffer into 1.5-ml PCR tubes (one per sample plus a blank control); place tubes on ice (keep tubes on ice at all times).
2. Remove a small piece of brain material/skin biopsy (~50 mg) from an uncut area of each sample or pipette a sample of saliva (~100–200 µl); place into lysis buffer and homogenize with sterile applicator sticks.
3. Add 1 ml TRIzol to each tube and mix for approximately 30 s by shaking vigorously; vortex and centrifuge briefly.
4. Incubate mixtures at 21°C (room temp) for 5 min.
5. Add 0.2 ml chloroform to each tube and shake vigorously for 30 s.
6. Incubate mixtures at 21°C (room temp) for 2–3 min.
7. Centrifuge samples for 15 min at 4°C at 12,000×G; add 0.5 ml isopropanol to 1.5-ml sterile screw cap conical tubes (one per sample plus one blank).
8. After centrifugation, remove top aqueous layer containing RNA and add to tubes containing isopropanol; shake or vortex briefly.
9. Incubate tubes for 10–15 min at room temperature.
10. Centrifuge tubes for 10 min at 4°C at 12,000×G.
11. Gently pour off supernatant into chemical waste.
12. Add 1 ml cold 75% ethanol to each tube; vortex.
13. Centrifuge samples for 5 min at 4°C at 7,500×G.
14. Remove samples from centrifuge, decant ethanol into chemical waste, and blot tubes on sterile gauze.
15. Rehydrate RNA pellet with 100 µl cold molecular grade water.
16. Vortex tubes on low to medium speed for 2 min.
17. Incubate samples at 56°C for 10 min in dry bath to dissolve RNA pellet.
18. Use immediately (4°C on ice) or store in –70°C freezer.

14.4.2.2 Reverse Transcription-Polymerase Chain Reaction

1. Add 2 µl forward primer (5 µM if specific; 10 µM if degenerate) to 0.2-ml PCR tubes (see Table 14.2).
2. Add 5 µl RNA to each tube and mix with primer.
3. Thaw reverse transcription reaction mix (RTRX; see Table 14.3) and prepare mixture by adding 2 µl of RT-AMV and 2 µl of RNase inhibitor to a 5× RTRX tube (or 1 µl of each if a 2.5× tube is used).
4. Place tubes in thermal cycler; incubate tubes for 1 min at 94°C.

Table 14.3 Reverse transcription reaction mix (RTRX)

RTRX	Amount
Molecular grade water	407 μ l
5X RT buffer	333 μ l
10 mM PCR nucleotide mix (dNTP)	33 μ l
	773 μ l ^a

^aAliquot 66 μ l of mix into eight 0.5-ml tubes for 5X RTRX and 33 μ l into seven 0.5-ml tubes for 2.5X RTRX; store at -20°C

Table 14.4 PCR mix (volumes for 1–5 reactions)

PCR mix	1 (μ l)	2 (μ l)	3 (μ l)	4 (μ l)	5 (μ l)
Forward primer (20 μM^{a})	1.06	2.13	3.2	4.25	5.31
Reverse primer (20 μM^{a})	1.33	2.66	4.0	5.33	6.66
AmpliTaq	0.53	1.07	1.61	2.13	2.66
1M Tris-HCl, pH 8.3	8.55	17.1	25.6	34.2	42.75
Molecular grade water	73.6	147.2	220.8	294.4	368.0

^a40 μM for degenerate primers

- Place tubes on ice for at least 3 min.
- Add 14 μ l of RTRX mixture to each tube; vortex and centrifuge briefly.
- Place tubes in thermal cycler for 90 min at 42°C .
- Prepare PCR mix (see Table 14.4).
- Add 80 μ l PCR mix to each tube; vortex and centrifuge briefly.
- Place tubes in thermal cycler.

14.4.2.3 PCR Program

- 94 $^{\circ}\text{C}$ 1 min
- 94 $^{\circ}\text{C}$ 30 s
- 37 $^{\circ}\text{C}$ 30 s 40 cycles
- 72 $^{\circ}\text{C}$ 90 s
- 72 $^{\circ}\text{C}$ 7 min
- 4 $^{\circ}\text{C}$ hold

14.4.2.4 Gel Electrophoresis

- Make a 4% agarose gel by adding 2 g agarose to 50 ml TBE; microwave the mixture until clear (add additional DI water as needed); add 2.5 μ l ethidium bromide to the gel; allow to cool down sufficiently and pour.
- Place solidified gel in submarine and add TBE buffer.
- Add 5 μ l $\Phi\text{X174 DNA-HaeIII}$ digest to the first and last wells.
- Mix 5 μ l of each sample with 2 μ l loading dye and add to wells.
- Run the gel at ~ 125 mAmp for ~ 30 –45 min or until the markers reach the bottom of the gel.
- Photograph the gel; band strength will be used to determine volume required for sequencing.

Table 14.5 Sequencing reaction mix

Sequencing reaction	Amount (μl)
BigDye Terminator v1.1 Mix	8
Primer (5 μM if specific; 10 μM if degenerate)	2
^a Template	1–8
QS to 20 μl with water	2–9

^aVolume depends on amount of product and genetic analyzer used (e.g., for ABI 310, add 1 μl for very strong bands and 8 μl for very weak bands)

14.4.2.5 Restriction Endonuclease Digestion

This method has been used successfully to differentiate between canine variants in Texas [16]. It may be a suitable tool for identification of variants in other areas; a comparative study would be necessary to determine if this is a viable tool.

1. Heat dry block to 37°C.
2. Label 0.2-ml PCR tubes with sample ID and enzyme used (*Dde I* and/or *Hinf I*).
3. Prepare enzyme mixture(s) for use on all specimens by adding 1 μl of either *Dde I* or *Hinf I* and 2 μl of buffer H to a tube(s). Prepare enough enzyme mixture for the number of samples plus one to ensure there is sufficient mixture for all samples (e.g., if there are three samples, prepare enough mixture for four).
4. Add 3 μl of enzyme mixture to each labeled PCR tube.
5. Add 17 μl of amplicon (PCR product) to each tube; vortex and centrifuge briefly.
6. Place samples in a 37°C dry block for 1.5–2 h.
7. Stop the reactions by placing on ice (or refrigerate for short time period prior to electrophoresis).
8. Use gel electrophoresis and a UV light box to visualize fragment patterns.

14.4.2.6 Nucleotide Sequencing

1. Add the following reagents to 0.2-ml PCR tubes (two tubes for each sample – one for the forward primer and one for the reverse primer) for sequencing reactions (see Table 14.5).
2. Vortex tubes; spin briefly and place in thermal cycler.
3. After cycle sequencing, remove dye terminators from the samples by following the manufacturer's instructions in the DyeEx 2.0 Spin Kit.
4. Place samples in a capillary sequencer (ABI 377, 310, 3730 or other appropriate genomics analyzer).

14.4.2.7 Nucleotide Sequencing Program

- 96°C 1 min
- 96°C 10 s
- 50°C 5 s 25 cycles
- 60°C 4 min
- 4°C hold

14.5 Interpretation of Results

14.5.1 PCR

A sample is considered positive for RV if a band of the appropriate size is present in the lane for that sample (e.g., 377-bp fragment if 1087Sdeg:304 primer set is used). A band should not be present in the blank (negative) control lane; presence of a band is indicative of contamination. The amount of PCR product needed for the sequencing reaction is determined by the band thickness (strength) and genetic analyzer used (see Fig. 14.3).

14.5.2 Restriction Endonuclease Digestion

Following enzyme digestion, compare fragment patterns of samples with characteristic digest patterns (see Fig. 14.4) for specific variants [16]. A restriction digestion should be carefully confirmed with sequence analysis of rabies virus variants due to high mutation rates, especially due to bat rabies virus variants.

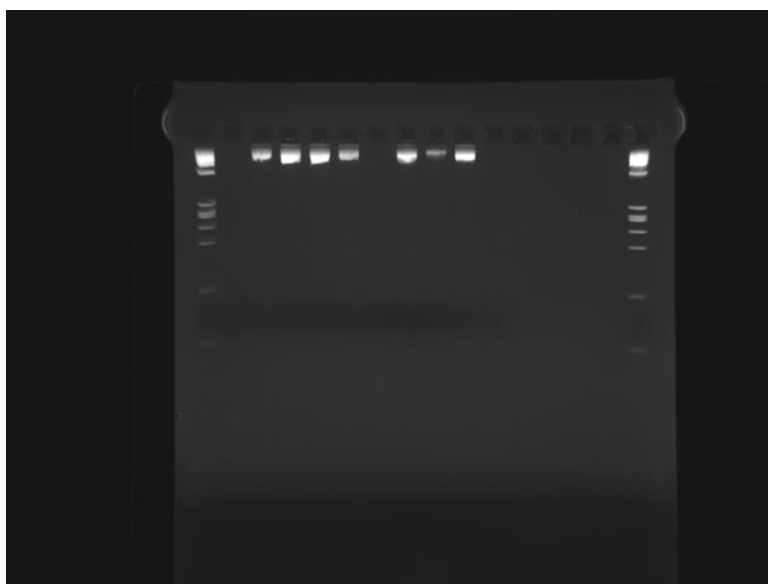


Fig. 14.3 Ethidium bromide-stained agarose gel electrophoresis of RV amplicons. Lanes 1, 16= Φ X174/ *HaeIII* marker fragments; lane 2=blank; lanes 3, 6, 8=strong band, use 2 μ l for sequencing; lanes 4, 5, 10=very strong band, use 1 μ l for sequencing; lane 7=failed amplification; lane 9=weak band, use 8 μ l for sequencing; lanes 11–15=blank. The amount of DNA for sequencing is based on CDC recommendations

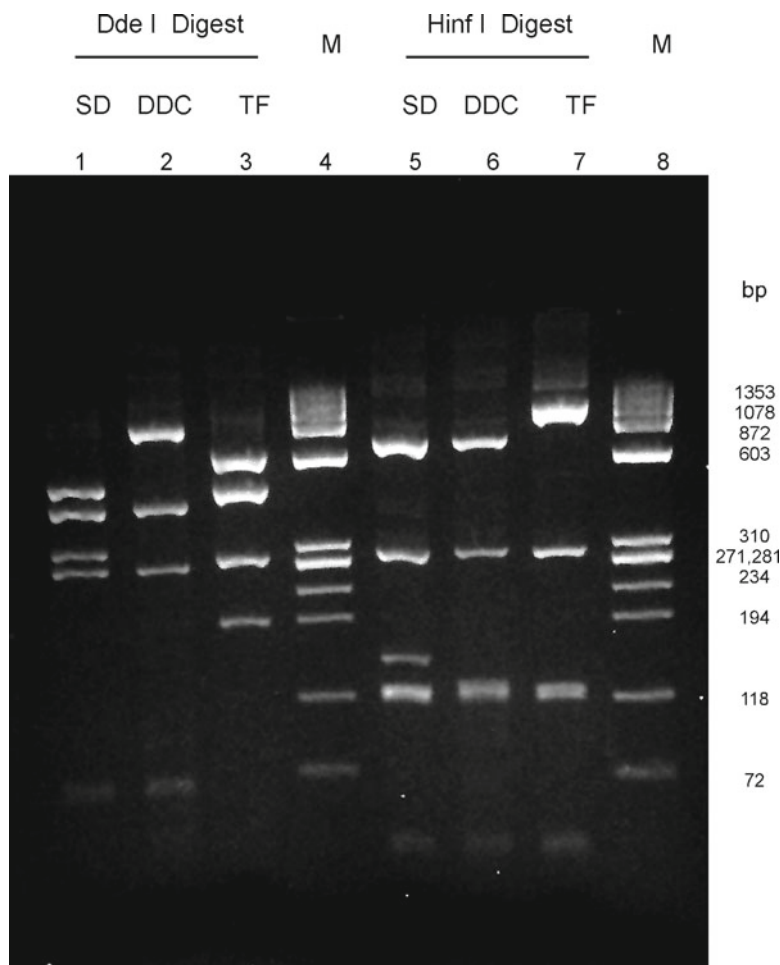


Fig. 14.4 Ethidium bromide–stained agarose gel electrophoresis analysis of cDNA amplified with primers 10g:304 and digested by either *Dde I* (lanes 1–3) or *Hinf I* (lanes 5–7). Lanes 1, 5 (SD variant); lanes 2, 6 (DDC variant); lanes 3, 7 (TF variant). Lanes 4, 8=M (Φ X174/ *HaeIII* marker fragments)

14.5.3 Nucleotide Sequencing

Sequencing data analysis requires software for editing and aligning sequences. BioEdit is a free program that can be downloaded from <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>. The software will align and compare the overlapping sequences (obtained from the forward and reverse primers). Sequencing software, such as DNASTAR Lasergene, can be purchased for performing more comprehensive sequence analysis. After necessary edits are made to a sequence, the sequence needs to be compared to representative RV variant sequences. An inclusive set of such sequences is available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Copy and paste the edited sequence into the search engine Nucleotide BLAST, select “others” for the database, and optimize for “highly similar sequences.” The best matches will generally be the sequences at the top of the table. If the host species is the same for the best matches (e.g., the top ten matches are RV sequence from *Lasiurus cinereus* bats), there is a high probability that the sequence being analyzed represents the variant associated with that host species. Spillover of RV variants does occur, so these results must be interpreted with great care; consider collectively the host species or suspected source of virus, MAb typing results,

BLAST results, and phylogenetic tree analysis [17, 21, 23, 24]. While unnecessary, it is useful to generate a phylogenetic tree from the sample and known RV variants for comparison with geographical host range of RV variants.

14.6 Summary

The DFA assay is the test most frequently used to diagnose rabies in animals. Tissue from the animal is required in suspected rabies cases and can only be performed postmortem. This test has been thoroughly evaluated for over four decades and is recognized as the most rapid and reliable of all the tests available for routine use. All rabies laboratories in the USA perform this test on suspect rabies cases. Other tests for diagnosis and research, such as electron microscopy, histologic examination, immunohistochemistry, RT-PCR, and isolation in cell culture, are useful tools for studying the virus structure, histopathology, molecular typing, and virulence of rabies viruses [24, 25].

Likewise, rabies testing in humans can be performed postmortem on brain tissue by DFA. Antemortem testing should only be performed by highly trained professionals that understand the neurotropic nature of rabies virus in samples such as saliva or hair follicles. The CDC, along with individual state health laboratories and the patient's physician, should always be consulted for specialized molecular testing of rabies samples. For instance, it has become increasingly important to understand human rabies cases where the bite of a bat has gone unnoticed by the physician or individual does not remember an animal exposure. In the USA, most human mortality from rabies over the past decade were the result of unsubstantiated bat bites.

For those laboratories without genetic typing capability, antigenic analysis with MAbs offers a rapid, simple, and inexpensive means of typing RV for epidemiologic surveys. MAb typing can be a useful screening test to differentiate the main terrestrial RV variant from bat rabies viruses [21]. Further, a restriction enzyme analysis can help with more precise differentiation between antigenic strains that are too closely related for MAb typing to separate RV variants. For instance, in Texas, the Texas fox and domestic dog-coyote RV have identical MAb panels, and a restriction digest can usually differentiate between the two. This is typically not the case with bat RV variants [16, 17, 19, 21]. On the other hand, genetic typing, especially nucleotide sequencing, offers the most precise analysis and virus type explanation in regard to diagnosis and epidemiology of the individual case [5, 25].

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Chapter 15

Pyrosequencing for Bacterial Identification

Laura Millecker and Ruth Ann Luna

Keywords Sanger sequencing • Pyrosequencing • Bacterial identification • 16S rRNA • Universal primers • Variable regions • Pyrosequencing platforms

15.1 Introduction

While Sanger sequencing has long been considered the industry standard for microbial sequencing, the introduction of pyrosequencing has provided many potential improvements to the sequencing process. With increased speed and robustness, pyrosequencing has been easily adapted to the clinical molecular laboratory workflow and has been applied in a variety of diagnostic areas including identification of bacterial pathogens from cultured isolates.

15.1.1 Sanger Sequencing

Sanger sequencing was first published in 1977 by Sanger and Coulson and utilized a chain-terminating inhibitor technique for determining the sequences of DNA [1]. This method takes advantage of the effects that randomly incorporated 2,3-dideoxyribonucleotide triphosphates (ddNTPs), which lack a 3'-OH group, have on terminating the elongation of a growing strand of DNA [1]. In order to achieve random incorporation of ddNTPs, each DNA sample was incubated separately with a mixture of one ddNTP (ddATP, ddCTP, ddGTP, ddTTP), the three other respective deoxyribonucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), labeled with ^{32}P , and a sequencing primer [1]. Following incubation, samples were subjected to electrophoresis on denaturing acrylamide gels, with each sample occupying four separate lanes in the gel, one for each ddNTP mixture [1]. An autoradiograph image of the gel then revealed bands of the DNA sample corresponding to the random locations where

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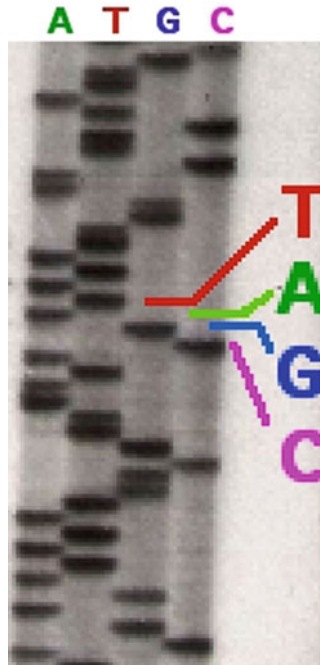


Fig. 15.1 *Autoradiograph of acrylamide gels originally used for DNA sequencing.* Each sample was incubated separately with each of the 4 ddNTPs (shown in the 4 lanes of the gel), and the bands (indicating where ddNTPs were randomly incorporated) were separated on a denaturing acrylamide gel. Sequences of the DNA sample were then determined by “reading” the bands of the gel vertically from *bottom to top* (Image obtained from <http://upload.wikimedia.org/wikipedia/commons/c/cb/Sequencing.jpg> courtesy of John Schmidt)

ddNTPs were incorporated (Fig. 15.1). Corresponding DNA sequences were then deduced by “reading” the bands starting from the bottom of the gel and moving upwards band by band [1].

Over the next 20 years, improvements were made upon this methodology, mainly the replacement of radioactive materials with fluorescently labeled ddNTPs and automation, specifically utilizing capillary electrophoresis for fragment separation and detection [2] (Fig. 15.2). In addition, the average number of bases obtained with Sanger sequencing increased from approximately 200–800 bases per sample [1, 2]. As of 2006, the total average turnaround time, from sample receipt to sequence results, of an 800 base pair region is approximately 24 h [4]. Sanger sequencing technology was instrumental in the generation of the first bacterial and human genomes and has proven to be robust and reliable [3, 4]. Despite the historical success of Sanger sequencing, many other sequencing technologies based on new chemistries or methods have emerged.

15.1.2 Pyrosequencing Technology

In 1996, Nyren et al. developed a technique for DNA sequencing which utilized a substrate-driven (in this case, inorganic pyrophosphate, PPi) enzymatic reaction to detect the emission of light released upon the incorporation of nucleotides onto a growing strand of DNA [5]. This sequencing by synthesis approach, or pyrosequencing, was performed following amplification of DNA in which one of the two primers was labeled with biotin. The biotin-labeled amplicons were then immobilized onto Streptavidin-coated beads and washed with NaOH to capture single-stranded DNA [5]. A complementary sequencing primer was then annealed to the sample. An enzyme mixture consisting of a DNA polymerase, sulfurylase, and luciferase, as well as dNTPs, was then added to the captured single-stranded DNA.

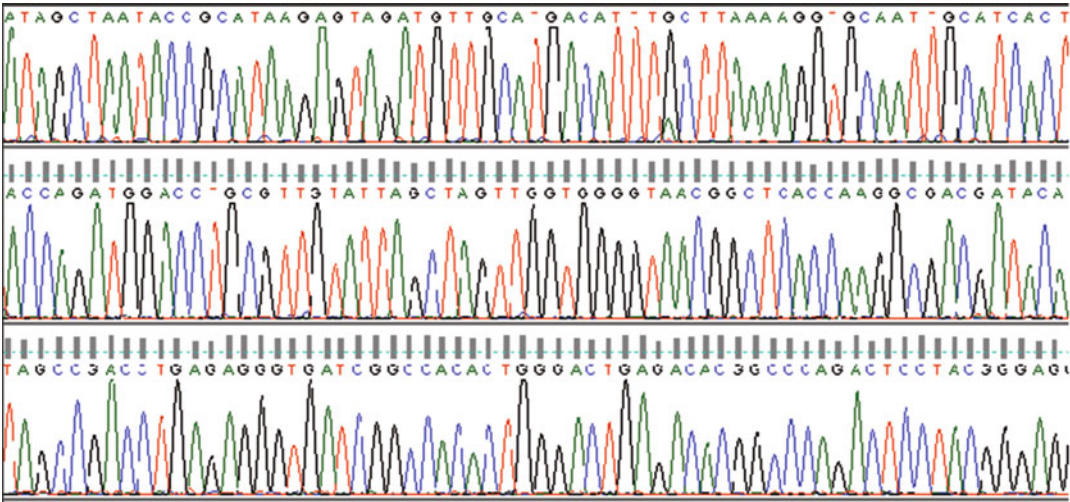


Fig. 15.2 Automated Sanger sequencing chromatogram. Each peak indicates the location of a randomly incorporated dye-labeled ddNTP terminator. The corresponding nucleotide (indicated as A, C, G, or T) is shown above each peak and provides the DNA sequence. The various nucleotides and resulting peaks are also differentiated by color: green for adenine, blue for cytosine, black for guanine, and red for thymine

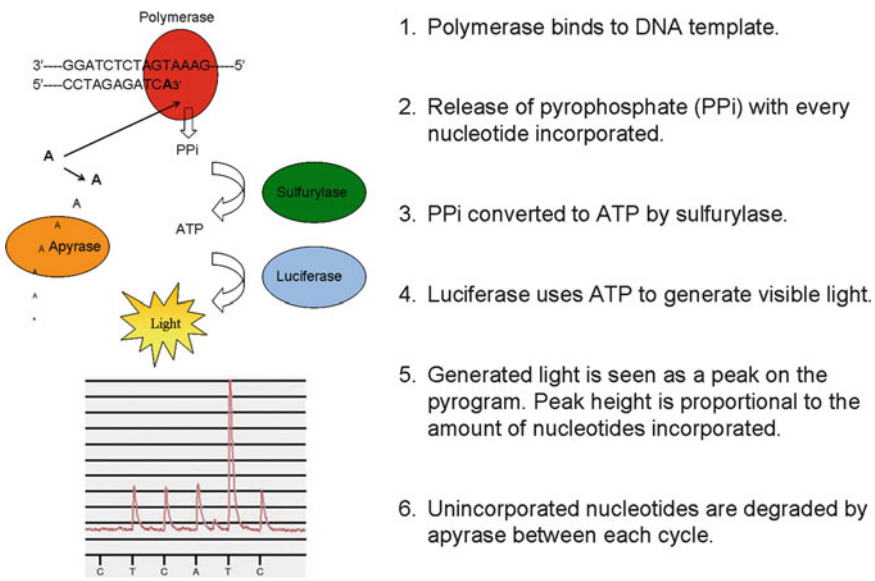


Fig. 15.3 Pyrosequencing methodology

Nucleotide incorporation by the DNA polymerase triggered the release of PPi. The inorganic pyrophosphate was used by sulfurylase to create ATP, which was then used to produce light by luciferase. Light emissions, detected by a luminometer, were directly proportional to the number of nucleotides incorporated. Each sample was then washed with a buffer to remove any unincorporated dNTPs, and the addition of enzyme mixture and each sequential dNTP to the captured DNA strand was repeated over multiple cycles. The resulting DNA sequence could then be determined by analyzing the peaks detected by the luminometer [5] (Fig. 15.3).

Over the next few years, changes were made to the pyrosequencing process which enabled the technology to become a part of the mainstream market. One of the first significant changes included the replacement of dATP with dATP α S [6]. Nyren et al. noted that the structure of dATP is very similar in structure to ATP, causing dATP to be incorrectly converted to light by luciferase, increasing background noise and making sequence analysis difficult; incorporating dATP α S resolved these issues [6]. Another major change was the addition of apyrase to the enzyme mixture (originally a DNA polymerase, sulfurylase, and luciferase) [6]. Apyrase is a nucleotide-degrading enzyme that eliminated the need for washing the samples after each addition of the dNTPs, thus allowing for continual, uninterrupted sequence detection. These changes, along with the addition of a charge coupled device (CCD) camera and the use of microtiter reaction plates, provided the building blocks of pyrosequencing technology that is currently utilized for multiple diagnostic purposes in molecular laboratories.

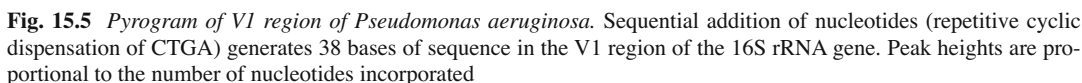
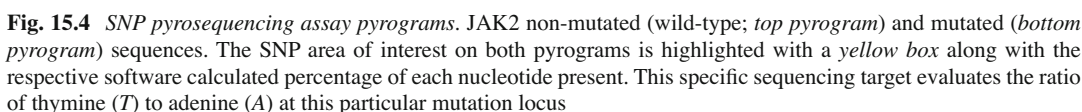
15.1.3 Pyrosequencing Platforms

As of 2011, there were three pyrosequencing platforms available from QIAGEN Inc., the PyroMark Q96 ID (Q96 ID), PyroMark Q96 MD (Q96 MD), and the PyroMark Q24 (Q24) [7]. Both the Q96 ID and Q96 MD are capable of sequencing up to 96 samples simultaneously, while the Q24 has the capacity for a total of 24 samples per sequencing run. Another difference between the three platforms is the presence of 24 individual CCD cameras located on the Q24 in comparison to the single CCD camera on the Q96 ID and Q96 MD platforms. This change in camera format has increased accuracy and detection and quantification of rare mutations on the Q24 platform [8]. All three pyrosequencing instruments are equipped with external software that includes specific analysis algorithms for several genetic applications. The software is also used to customize the nucleotide dispensation order for each pyrosequencing run application. The order in which nucleotides are dispensed can be adapted to user specifications and consist of either a sequence-/mutation-specific dispensation or a repetitive nucleotide cyclic dispensation. Regardless of the number of samples each platform can accommodate, the time required per nucleotide dispensation, incorporation, and detection cycle is approximately 1 min. The approximate sequence read lengths obtained by pyrosequencing range upwards of 35 and 50 bases for the Q96 ID and Q96 MD platforms and the Q24 platform, respectively [7].

15.1.4 Pyrosequencing Applications

Pyrosequencing is most commonly used for SNP quantification and mutation analysis, CpG methylation analysis, and microbial identification and resistance detection [9]. An example of a SNP assay includes the detection of point mutations located in exons 3 and 41 of the beta-catenin gene present in aggressive desmoids tumors [10, 11]. Additional popular SNP assays include mutation detection and quantification of codons 12, 13, and 61 of the KRAS gene; the V617F mutation of the JAK2 gene; and the C677T mutation in the MTHFR gene [12–17] (Fig. 15.4). Detecting CpG methylation in epigenetic studies by pyrosequencing first appeared in 2002 and has provided valuable insight into the relationship between the changes in gene methylation status and tumorigenesis and tumor progression [18]. In particular, the methylation status of the promoter region of the MGMT gene, which plays a role in oncogenesis, has been widely evaluated using pyrosequencing methods [18]. Both SNP and CpG methylation assays typically use nucleotide dispensation orders which are specific to the sequence of interest and mutation prevalent regions.

Bacterial, fungal, and viral identification is commonly achieved through sequencing of well-characterized target genes, such as the 16S rRNA gene for bacterial identification (Fig. 15.5). When utilizing universal primers targeting the conserved areas of the bacterial 16S rRNA gene, it is imperative that



only pure cultured organisms are sequenced to ensure accurate identification based on the resulting DNA sequence. The pyrosequencing technology and software are incapable of separating sequences from a mixture of organisms, with mixed cultures resulting in peaks for most, if not all, dispensations. Following sequence analysis for base calling and base quality, performed automatically by the pyrosequencing software, resulting microbial sequences can be searched against possible matches through user created sequence databases (i.e., QIAGEN's IdentiFire software) or publically available sequence databases (i.e., BLAST, Ribosomal Database Project (RDP)). Stretches of three or more of the same nucleotide (i.e., AAAA), called homopolymers, can be difficult to distinguish by the base calling and quality software and may require manual interpretation to ensure sequence accuracy. In addition to pathogen identification, bacterial and viral resistance detection can also be evaluated using pyrosequencing, as microbial resistance is often acquired through specific and well-characterized point mutations. Examples include pyrosequencing assays for the detection of adamantane resistance in influenza viruses and clarithromycin resistance in *Helicobacter pylori* [9].

While the majority of bacterial pathogens are identified in the microbiology laboratory by conventional morphological and biochemical methods, there are some bacteria which require the additional use of molecular techniques to confirm identification. The 16S rRNA gene is present in all bacteria

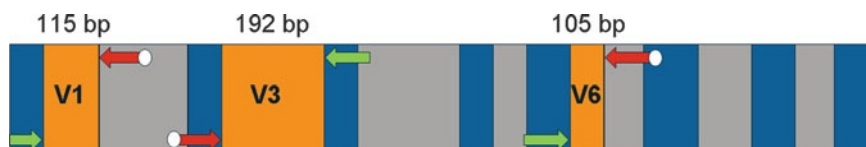


Fig. 15.6 *Bacterial 16S rRNA gene*. Bacterial 16S rRNA gene depicting the eight conserved (blue) and variable regions (grey). The V1, V3, and V6 variable regions, along with their respective amplicon lengths, are highlighted in orange. The red arrows indicate the biotinylated PCR primers and the green arrows indicate the non-biotinylated PCR primers, which are also the sequencing primers

and is approximately 1,500 base pairs in length. It consists of eight conserved and nine variable regions [19, 20]. Universal primers target the conserved regions of the gene, thereby theoretically amplifying any bacterial DNA present. The variable regions are then utilized to differentiate and identify bacteria based on sequence variations [19–22].

In 2007, Luna et al. showed that pyrosequencing the V1 and V3 variable regions was an effective strategy in identifying bacterial pathogens [23]. Future investigations by Luna et al. revealed that the amplification and pyrosequencing primers targeting the V3 region targeted the V6 region, contrary to the original reference describing pyrosequencing for bacterial identification [20, 23]. The amplification primers used in this assay target the conserved regions flanking each respective variable region and generate 115 base pair, 192 base pair, and 105 base pair amplicons for the V1, V3, and V6 variable regions, respectively (Fig. 15.6) [23].

15.3 Procedure

Specimen Types

Genomic DNA extracted from pure microbial isolates (preferred concentration of 10 ng/μL)

Controls

Positive control: genomic DNA extracted from previously identified pure microbial isolate

Negative control: nuclease-free water

Procedural steps

Required reagents

PCR reagents

AmpliTaq Gold LD DNA polymerase (Applied Biosystems)

GeneAmp 10× PCR Buffer (Applied Biosystems)

MgCl₂ (Applied Biosystems)

GeneAmp dNTPs (Applied Biosystems)

10 mM PCR primers:

V1 region

Forward primer: **Bio-pBR5'**: Biotin-GAAGAGTTTGATCATGGCTCAG

Reverse primer: **pBR-V1**: TTACTCACCCGTCGCCACT

V3 region

Forward primer: **V3F-Bio**: Biotin-CTACGGGAGGCAGCAG

Reverse primer: **V3R**: ATTACCGCGGCTGCTGG

V6 region

Forward primer: **Bio-B-V6**: Biotin-ACGACAGCCATGCAGCACCT

Reverse primer: **pJBS.V6**: GCAACGCGAAGAACCTTACC

Pyrosequencing reagents

3-mM sequencing primers: pBR-V1, V3R, pJBS.V6

Streptavidin Sepharose HP (Amersham Biosciences)

High purity water (Millipore or nuclease-free water)

Ethanol (70%)

Binding buffer (QIAGEN)

Denaturation solution (QIAGEN)

Washing buffer (QIAGEN)

Annealing buffer (QIAGEN)

Pyro Gold SQA Reagent Kit (QIAGEN):

- Enzyme mixture
- Substrate mixture
- dATP α S
- dCTP
- dGTP
- dTTP

1. Master mix preparation

- (a) In a nuclease-free 1.5-mL microcentrifuge tube, add the following amounts of reagents for each amplification to be performed. Each sample will be used in three separate PCR reactions. Preparation should be set up on ice or in a PCR cooler tray. Be sure to add extra reagents equivalent to one extra reaction for every 8–12 samples to be run. Taq should be the last reagent added to the master mix and should be kept cold at all times.

Master mix reagent	Volume per sample (μ L)
H ₂ O	32.75
dNTPs	4
MgCl ₂	5
10X Buffer	5
Forward primer (10 mM)	1
Reverse primer (10 mM)	1
AmpliTaq Gold LD	0.25
<i>Total</i>	49

- (b) Aliquot 49 μ L of each master mix into the bottom of the appropriate PCR tube.
- (c) Add 1 μ L sample DNA (concentration of 10 ng/ μ L), positive control, or negative control to the bottom of the corresponding PCR tube.
- (d) Cap the PCR tubes securely. Vortex and quick spin to bring reagents to the bottom.

2. Amplification

- (a) The cycling parameters are the following:

Initial denaturation	95°C	10 min
Repeat cycle	35 cycles	
Denaturation	95°C	40 s
Annealing	55°C	40 s
Extension	72°C	60 s
Final extension	72°C	60 s
Hold	4°C	

3. Pyrosequencing Sample Preparation

- (a) Allow all pyrosequencing reagents to equilibrate to room temperature before starting.
 (b) Shake or vortex the bottle with Streptavidin Sepharose HP beads until a homogenous solution is obtained.
 (c) In a 1.5-mL tube, prepare the bead mix. Per sample (number of PCR products), add:

40 μ L Binding buffer
 3 μ L Streptavidin Sepharose HP beads

- (d) In a 96-well plate, add 40 μ L bead mix and 40 μ L of each PCR product. Be mindful of which samples are V1, V3, and V6.
 (e) Using a mixer/shaker, incubate at room temperature for a minimum of 5 min while agitating constantly at 1,400 rpm to keep the beads dispersed.
 (f) Prepare the sequencing plate (PSQ 96 Low Bottom Plate). Add 40 μ L annealing buffer to each well.

For V1 samples: Add 5 μ L of 3 μ M sequencing primer (pBR-V1) to each well.

For V3 samples: Add 5 μ L of 3 μ M sequencing primer (V3R) to each well.

For V6 samples: Add 5 μ L of 3 μ M sequencing primer (pJBS.V6) to each well.

- (g) Fill pyrosequencing reagent troughs (4 total) with the following: high purity water (180 mL), 70% ethanol (180 mL), washing buffer (180 mL), and denaturation solution (120 mL).
 (h) Prime the probes of the Vacuum Prep Tool by applying vacuum and lowering the tool into the trough with high purity water for approximately 30 s to wash the filter probes.
 (i) Capture the Streptavidin Sepharose beads containing the immobilized templates on the filter probes by slowly lowering the Vacuum Prep Tool into the 96-well plate. Suction as much as possible, but some solution may remain. *NOTE:* Sepharose beads sediment quickly and the capturing of beads must take place within 3 min after termination of the agitation.
 (j) Keeping the PCR plate and the Vacuum Prep Tool together, carefully lift and tilt to check that all beads have been captured onto the probe tips. The surface of the solution should be level with, or lower than, the filter probe tips when they are resting on the bottom of the well.
 (k) Move the Vacuum Prep Tool to the trough with 70% ethanol and let the solution flush through the filters.
 (l) Remove the Vacuum Prep Tool when no more reagent is being flushed through, i.e., only air is being passed through the Vacuum Prep Tool, (*NOTE:* some reagent will always remain at the bottom of the trough) and gently rock the tool back and forth to remove any excess reagent.
 (m) Move to the denaturation solution. Repeat step l.
 (n) Move to the washing buffer. Repeat step l.
 (o) Turn off the vacuum and unplug it.

- (p) Release the beads in the prepared PSQ 96 Low Bottom Plate (pre-filled with sequencing primer and 40-mL annealing buffer) by moving the Vacuum Prep Tool in small circles and rubbing the filter probes against the bottom of the wells for approximately 20 s.
- (q) Shake the Vacuum Prep Tool in high purity water to release any remaining beads.
- (r) Place the PSQ 96 Low Bottom Plate with the samples on a heat block set at 80°C for 2 min.
- (s) Remove the plate from the heat block and place at room temperature for 3–5 min to cool.
- (t) Once the plate has reached room temperature, the plate is ready for pyrosequencing.

4. Pyrosequencing

- (a) Prepare the enzyme and substrate mixtures by adding 620 µL of high purity water to each of the tubes. Mix by swirling the vial gently. *NOTE:* Do not vortex! Leave at room temperature for 5–10 min to ensure complete dissolution. If using a previously prepared mixture, allow to warm to room temperature before loading the cartridge. Only thaw enzyme and substrate mixtures once. Aliquot new mixtures into individual tubes to be frozen.
- (b) Set up pyrosequencing run on the pyrosequencing PC. Open the “SQA 96MA” screen. Under Process Identification, enter the required information (name of the run, lot #s, sample IDs, etc.).
- (c) Under Process Parameters, choose the dispensation order (Cyclic-60 ACGT). Click Run → Info in order to obtain the volumes of reagents that should be placed in the reagent cartridge. Fill the reagent cartridge with the appropriate volumes in the designated areas of the cartridge (refer to QIAGEN PyroMark package insert).
- (d) Load prepared PSQ 96 Low Bottom Plate and reagent cartridge into the pyrosequencer.
- (e) Click Run on the computer screen.
- (f) When run is complete, analyze all wells.
- (g) Note which bases are of “pass” quality. Bases of “uncertain” quality should be verified. The last “pass” quality base should also be noted, and all bases prior to this will be used in the organism identification search.

5. Sequence Analysis

- (a) Access the RDP database on a PC with Internet access <http://rdp.cme.msu.edu> [24].
- (b) Select “Sequence Match.”
- (c) Enter the “pass quality” bases for each region in FASTA format. Separate searches are done for each region.
- (d) Select the following options:
Strain: Type
Source: Isolates
- (e) Click “Submit”
- (f) Choose the organisms with similarity scores of 1.000. If there are no matches at 1.000, then press the “back” button on the internet screen and repeat the search using the “Both” option for Type. If no match with a similarity score of 1.000 is generated, choose the next highest similarity score and note both the organism and similarity score on the pyrosequencing report.
- (g) Confirm the sequences of the positive control, and confirm that there is no recognizable sequence in the negative control.
- (h) ID confirmation is obtained through the correlation of colony morphology, gram stain, biochemical test results, and pyrosequencing data for each sample.

Method Limitations

- 1. This procedure may not produce useful sequence with some microbial samples. Although the primers are located in highly conserved regions of the bacterial genome, it is possible that some

organisms may have sequence differences in or near these regions and will not produce enough sequence information to complete the identification.

2. In cases where there are stretches of repetitive bases or homopolymers (i.e., AAAA), sequences should be checked against the sequence with the highest similarity score. Thorough review of the pyrosequencing data in these homopolymer regions should be done to ensure that the number of repetitive bases is correct.
3. Pure microbial cultures must be used. Mixed or contaminated cultures may result in inconsistent results.
4. Pyrosequencing results should always be evaluated jointly with the clinical microbiology findings. Discrepant findings should be repeated or referred for further identification testing.

15.4 Summary

Advances in sequencing technology have enabled laboratories to utilize these techniques as part of routine molecular diagnostics. The multi-day process of Sanger sequencing was difficult for many clinical laboratories to implement, but with a single-day process paired with robust and reliable instrumentation and reagents, the adoption of pyrosequencing in the clinical laboratory has expanded the potential for sequence-based testing. In targeted sequencing efforts such as pathogen or mutation identification, pyrosequencing can be a valuable resource for providing a wealth of sequence information within a rapid turnaround time. When combined with routine diagnostic testing, pyrosequencing provides an additional effective strategy for clinical testing.

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Chapter 16

Other Pathogens of Significant Public Health Concern

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Keywords Molecular diagnostics • MDx • Sexually transmitted infection • *Chlamydia trachomatis* • *Neisseria gonorrhoeae* • Influenza • Respiratory viruses • *Candida* • FDA • Nucleic acid amplification test • *Staphylococcus aureus* • MRSA • Human papillomavirus • Herpes simplex virus • Herpes • Hepatitis B virus • PCR • Hybridization

16.1 Introduction: Infectious Disease Testing Using Modern Molecular Methods

The intention of this chapter is to educate the diagnostics community on clinical and technical aspects of molecular diagnostics (MDx) with a focus on FDA-approved testing platforms and currently available assays. Relevant topics including assay validation, proficiency testing and special considerations for sample collection, transport, and storage are discussed briefly for implementation of standard operating procedures (SOPs) and ensure reliable and reproducible results from the diagnostic laboratory. Together, this chapter is intended to describe general principles pertaining to the development, validation, and clinical application of molecular diagnostic testing. Prior to commercial availability, the US Food and Drug Administration (FDA; www.fda.gov) plays a definitive role in assessing the performance of molecular diagnostic assays. Therefore, in contrast to other chapters of the compendium, no detailed protocols will be presented herein as all FDA-approved tests rely upon strict adherence to the manufacturer's instructions.

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MDx are now firmly established for clinical detection of microorganisms, and numerous platforms currently exist for clinical detection of nucleic acids. Among the current, FDA-approved MDx for infectious disease testing, all assays can be separated into two general categories: (1) those that utilize “signal amplification” (binding of a nucleic acid probe followed by detection of an amplified fluorescent signal) and (2) those that employ “target amplification” (direct detection of an amplified nucleic acid target, most often via PCR). Between these two categories, several molecular methods are employed for MDx including fluorescence in situ hybridization (FISH), peptide nucleic acid (PNA) probes, the polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), strand displacement amplification (SDA), transcription-mediated amplification (TMA), nucleic acid sequence–based amplification (NASBA), hybrid capture (HC), target capture (TC), and hybridization protection assays (HPA). A more detailed review of these techniques is covered in previous chapters of the compendium.

16.1.1 Specimen Collection and Transport

Validity and proper interpretation of molecular diagnostic test results begin first with optimizing sample collection and then maintaining sample integrity via proper transport and storage. Indeed, training of clinicians, clinical staff, and laboratorians on proper specimen collection, transport, and optimization of workflow are all integral components for successful and accurate MDx. Because the majority of available molecular diagnostic tests target either DNA or RNA, it is essential that handling and transport of collected specimens are consistent with preservation of nucleic acids. Specimens collected for molecular testing should follow the manufacturer’s guidelines as indicated in the package insert in order to prevent degradation of nucleic acids, the target for all MDx. Thus, for each independent test, it is of utmost importance to closely follow the procedures outlined in the package insert as deviations from these guidelines may result in inaccurate results. Even molecular tests from the same manufacturer often employ different transport media, transport and/or storage temperature standards, or acceptable length of time from collection to processing among their tests for optimized preservation of the target nucleic acid.

The off-label utility of molecular MDx is, of course, not endorsed by the manufacturer until proper claims are acquired from the FDA because each specimen type, transport container and medium, extraction method, and assay platform must be validated individually. The FDA independently approves essentially all physical and procedural aspects of a molecular diagnostic test; in turn, deviation from protocol or substitution of an unapproved collection method or container renders the test result invalid. Importantly, prior to approval and marketing of claims, studies of off-label validation are often published in peer-reviewed journals and then endorsed by assay manufacturers as supporting data for their submission of FDA approval. Thus, one can find information on off-label utility by searching databases of peer-reviewed literature, although, again, it is imperative that no unapproved collection containers/devices be employed for clinical diagnostics.

To this end, one important goal for all manufacturers of clinical MDx is to increase the diversity of claims for which an assay is validated. For example, the most versatile test would be validated by the FDA for detection using several specimen collection vessels/containers, transport media, and from all clinically relevant anatomical sites. This scenario provides clinicians and clinical laboratories the opportunity to run different tests, on different platforms, from a single patient specimen. The process of diversifying claims for a particular diagnostic test can be a time-consuming and often a significant financial investment because, depending on the claim, will almost always require comparative clinical trials and subsequent review by the FDA. Currently, cross-platform compatibility of sample collection containers is uncommon, and perhaps future development of universal collection/transport methods will streamline molecular testing.

16.1.2 Sample Preparation and Nucleic Acid Purification

Sample preparation for nucleic acid isolation has evolved from the cumbersome and technically challenging phenol/chloroform methodology to modern kit-based or automated procedures. For FDA-approved assays discussed in this chapter, sample preparation, including purification of nucleic acids, is an included component of molecular diagnostic testing platforms and has been validated extensively prior to approval and implementation for clinical use. As such, standardization has virtually eliminated concerns for suboptimal isolation of nucleic acids, and as a technician performing molecular testing, strict adherence to the manufacturer's protocol will maximize accuracy of the results.

The central dogma of molecular biology, the flow of genetic information from the DNA genome to mRNA that is then utilized for protein synthesis, has been exploited at various steps by manufacturers of molecular assays to develop screening methods for many bacterial, viral, and fungal pathogens. Currently, the majority of molecular diagnostic assays target a conserved region of an organism's DNA genome that is highly specific to the target organism. Assay specificity and classification of pathogen variants or subtypes are determined by comparison of DNA sequences among the target pathogen, other putative pathogens, and, most importantly, DNA from normal or abnormal microbial flora at the anatomical site of sampling. Two central characteristics define the success and clinical utility of a molecular diagnostic assay: (1) specificity, or the ability to detect only the target organism; and (2) sensitivity, the capacity to detect small numbers of organisms from complex specimens. Because MDx can be extremely sensitive and highly specific, newer MDx are now validated with clinical sensitivity and specificity as opposed to analytical specifications. For example, it is well accepted that some molecular methods are highly sensitive (e.g., PCR), but newer molecular platforms not only report a positive/negative result but many also provide quantitative interpretation. Further, some platforms employ optimized processing methods from different sample sources, such as to account for viscosity, volume, or other specimen characteristics. Importantly, not all specimen types are created equal. Modern molecular diagnostic assays have the capacity to tailor the clinical sensitivity/specificity, for example, by applying different reaction conditions, processing conditions, and thresholds for a positive result. Collectively, in collaboration with clinical signs/symptoms, defined clinical specifications allow a molecular assay to better correlate results to a disease state or outcome.

16.1.3 Current FDA-Approved Methods for Molecular Diagnostics

Among target amplification assays that target DNA, the polymerase chain reaction (PCR) has played a central role for MDx. Combined with high sensitivity, PCR can specifically and quickly detect target DNA and is used to amplify that target to quantities capable of detection directly. After denaturation of the double-stranded DNA target, thermal cycling facilitates the binding of specific nucleotide primers, extension of the primers by Taq polymerase, and, ultimately, synthesis of a nascent DNA molecule. This new molecule then serves as a new template for amplification, and, as new PCR products are synthesized, thermal cycling will exponentially increase the amount of amplified DNA. Historically, results of a PCR were determined via end-point analysis (e.g., gel electrophoresis and DNA staining). However, more recent advances in nucleic acid probes and the development of thermocyclers with optical detection modules now allow for real-time monitoring of the PCR, a technology termed real-time PCR.

In contrast, FDA-cleared molecular methods that utilize signal amplification do not rely upon amplification of the target DNA molecule. Instead, using hybrid capture (HC) as an example, an amplified chemiluminescent or fluorescent signal is generated after antibody binding of nascent DNA:RNA hybrid molecules rather than amplified target DNA as with PCR. Several FDA-cleared assays utilize either target amplification or signal amplification for clinical detection of microbial pathogens.

In instances where the molecular test targets an organism's RNA, first RNA must be purified and, in most cases, is then reversed transcribed to generate a complementary DNA strain (termed cDNA) suitable for PCR or other means of target amplification. Two virtually identical examples of RNA-based target amplification methods are TMA and NASBA, both of which rely on RNA as a template for testing. TMA and NASBA are cyclic processes whereby, first, a promoter-primer binds to the RNA target (often ribosomal RNA in the case of bacterial pathogens), followed by reverse transcription of a cDNA molecule. During this process, the RNA template is degraded by the RNase H activity of the reverse transcriptase allowing a second primer to bind the DNA template for creation of a duplex DNA. The nascent DNA molecule then serves as promoter for the RNA polymerase to initiate transcription. The newly synthesized RNA molecules then reenter the TMA process ultimately resulting in exponential amplification of the RNA amplicon that is finally detected using labeled DNA probes. Typically, target amplification methods involve thermal cycling, but TMA assays rapidly amplify the target sequence at a single temperature thereby eliminating the need for thermocycling equipment.

From a clinical laboratory perspective, the RNA amplicon generated during TMA/NASBA amplification is more labile compared to DNA products generated during PCR thereby reducing the potential for laboratory cross-contamination. From a sensitivity perspective, molecular assays that target a pathogen's RNA could have increased sensitivity compared to DNA-targeted tests because numerous copies of the RNA target can be present compared to the single copy of DNA. Additionally, TMA/NASBA allows for direct detection of RNA viruses. It is important to note that the aforementioned methods are not comprehensive but rather a representation of the most commonly employed methods among current FDA-approved MDx.

16.2 Molecular Testing of Clinical Specimens for Bacterial or Fungal Pathogens

As discussed earlier, effective sample collection is the first and most crucial step toward ensuring accuracy for all molecular diagnostic tests. Considering the number of FDA-approved MDx for sexually transmitted diseases, we will exemplify the concerns for specimen collection, handling, and testing by discussing screening of male and female reproductive tract specimens. First and foremost, consider the source. While some bacterial, parasitic, and fungal pathogens of other anatomical sites can be prevalent in acellular samples, sexually transmitted organisms like *C. trachomatis*, and particularly viral pathogens like HPV and HSV, are intracellular or strongly cell-associated. Therefore, sampling efficacy hinges significantly on the technique used by the clinician; ineffective collection of epithelial cells will significantly reduce the assay's sensitivity for intracellular bacterial or viral pathogens. Together, this is why many STI testing methods now utilize cytological brushes or brooms rather than swabs to enhance sampling of the mucosa and ensure adequate cellularity of the specimen. In contrast, detection of some eukaryotic organisms, extracellular bacterial pathogens such as *S. aureus* and *N. gonorrhoeae*, or fungal pathogens like *Candida* spp. does not rely as heavily upon cellularity of the specimen, although cellularity may serve as a proxy for effective sampling regardless of the test.

16.2.1 *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

More than 300 million cases of curable sexually transmitted infections (STIs) are acquired annually throughout the world, making these infections a significant public health concern [1]. STIs can lead to severe reproductive tract morbidity, particularly in women, and are notable causes of urethritis, abnormal vaginal discharge, cervicitis, pelvic inflammatory disease (PID), tubal infertility, and several pregnancy-related sequelae such as preterm birth and spontaneous abortion. More than 25 diseases

Table 16.1 Current FDA-cleared MDx for *Chlamydia trachomatis*

Manufacturer; location	Test name	Detection method
BD Diagnostic Systems; Sparks, MD	ProbeTec™ CT Q ^x Amplified DNA Assay	SDA
BD Diagnostic Systems; Sparks, MD	ProbeTec™ ET CT Amplified DNA Assay	SDA
Gen-Probe, Inc.; San Diego, CA	APTIMA Assay for CT	TC, TMA, DKA
Gen-Probe, Inc.; San Diego, CA	PACE® 2 CT	HPA
Qiagen, Inc.; Germantown, MD	digene HC2® CT ID	HC
Roche Diagnostics; Indianapolis, IN	AMPLICOR® CT/NG Test for CT	PCR
Roche Diagnostics; Indianapolis, IN	COBAS® AMPLICOR® CT/NG Test for CT	PCR

CT *Chlamydia trachomatis*, NG *Neisseria gonorrhoeae*, SDA strand displacement assay, TC target capture, TMA transcription-mediated amplification, DKA dual kinetic assay, HPA hybridization protection assay, HC hybrid capture, PCR polymerase chain reaction

are transmitted through sexual contact, and, other than human immunodeficiency virus (HIV), the most common STIs in the United States are caused by *C. trachomatis* (CT), *Neisseria gonorrhoeae* (NG or GC), *Treponema pallidum* (the causative agent of syphilis), herpes simplex virus (HSV), human papillomavirus (HPV; viral cause of cervical and other mucosal cancers), hepatitis B virus (HBV), *Trichomonas vaginalis*, and bacterial vaginosis (BV). Thus, an extremely large market exists for STI diagnostic screening, and at least one molecular diagnostic assay has been FDA-approved for clinical use for each of these pathogens.

Sexually transmitted pathogens are arguably the most successful group of organisms that cause human disease because of their ability to persist long term in reproductive tract tissues or associated neural tissue despite robust innate and humoral immune responses. Genital chlamydial infections are the most frequently reported infectious disease in the United States [2] and represent a significant public health and economic concern worldwide. *C. trachomatis* is an obligate intracellular pathogen of mucosal epithelial cells and is associated with male urethritis, cervicitis, PID with or without chronic pelvic pain, ectopic pregnancy, and tubal infertility [3]. As *C. trachomatis* is the most prevalent bacterial cause of STI, MDx from several manufacturers, utilizing either DNA- or RNA-based detection methods, are currently FDA-approved and in use worldwide. Due to the volume of STI screening, most *C. trachomatis* assays are currently performed on high-throughput automated platforms such as BD's Viper (BD Diagnostic Systems, Sparks, MD; www.bd.com/ds), the Roche Amplicor (Roche Diagnostics, Indianapolis, IN; www.roche.com), or Gen-Probe's APTIMA (Gen-Probe Inc., San Diego, CA; www.gen-probe.com) (Table 16.1).

N. gonorrhoeae is the etiologic agent of gonorrhea and other inflammatory urogenital syndromes including urethritis, cervicitis, and salpingitis. Although gonorrhea is rare in Canada and much of Western Europe, it remains a relatively common sexually transmitted pathogen in the United States and other developing countries. Classical methodologies for diagnosis of infection with NG include the presence of polymorphonuclear leukocytes and Gram-negative diplococci on Gram stains of male urethral or female endocervical smears. In symptomatic cases, these outcomes are highly predictive of NG infection, but molecular assays achieve far greater sensitivity and allow testing of a wide variety of specimen types including endocervical swabs, vaginal swabs, male urethral swabs, liquid-based cytology containers (commonly used for Pap testing of cervical specimens for signs of premalignant and malignant lesions), and urine from men and women (Table 16.2). Similar to MDx for CT, the majority of NG testing is performed on high-throughput diagnostic platforms due to the significant volume of STD testing, the relatively high prevalence of NG in high- and low-risk populations, and the availability of CT/NG duplex testing.

Indeed, both CT and NG are recognized as independent urogenital pathogens, but the inflammatory syndromes attributed to these organisms are clinically indistinguishable from one another. In addition, it is widely accepted that coinfections with multiple sexually transmitted pathogens are common, and therefore MDx companies have developed multitarget assays able to detect multiple

Table 16.2 MDx assays that target *Neisseria gonorrhoeae*

Manufacturer	Test name	Diagnostic method
BD Diagnostic Systems; Sparks, MD	BD ProbeTec GC Q ^x Amplified DNA Assay	SDA
Gen-Probe, Inc.; San Diego, CA	APTIMA GC Assay	TC, TMA, DKA
Gen-Probe, Inc.; San Diego, CA	PACE [®] 2 GC Probe Competition Assay	HPA
Qiagen, Inc.; Germantown, MD	HC2 [®] GC ID	HC
Roche Molecular Diagnostics; Pleasanton, CA	AMPLICOR [®] CT/NG Test for NG	PCR
Roche Molecular Diagnostics; Pleasanton, CA	COBAS [®] AMPLICOR [®] CT/NG Test for NG	PCR

GC *Neisseria gonorrhoeae*, *CT* *Chlamydia trachomatis*, *NG* *Neisseria gonorrhoeae*, *SDA* strand displacement assay, *TC* target capture, *TMA* transcription-mediated amplification, *DKA* dual kinetic assay, *HPA* hybridization protection assay, *HC* hybrid capture, *PCR* polymerase chain reaction

Table 16.3 Multitarget MDx assays that detect both *C. trachomatis* and *N. gonorrhoeae*

Manufacturer	Test name	Method
Abbott Molecular, Inc.; Des Plaines, IL	Abbott [®] RealTime CT/NG	Real-time PCR
BD Diagnostic Systems; Sparks, MD	BD ProbeTec [™] ET CT and NG Amplified DNA Assay	SDA
Gen-Probe, Inc.; San Diego, CA	APTIMA Combo 2 [®] Assay	TC, TMA, DKA
Gen-Probe, Inc.; San Diego, CA	PACE [®] 2C CT/GC	HPA
Qiagen, Inc.; Germantown, MD	digene HC2 CT/GC DNA Test	HC
Qiagen, Inc.; Germantown, MD	HC	HC
Roche Diagnostics; Indianapolis, IN	AMPLICOR [®] CT/NG Test	PCR
Roche Diagnostics; Indianapolis, IN	COBAS [®] AMPLICOR [®] CT/NG Test	PCR

GC *Neisseria gonorrhoeae*, *CT* *Chlamydia trachomatis*, *NG* *Neisseria gonorrhoeae*, *SDA* strand displacement assay, *TC* target capture, *TMA* transcription-mediated amplification, *DKA* dual kinetic assay, *HPA* hybridization protection assay, *HC* hybrid capture, *PCR* polymerase chain reaction

organisms from a single specimen. This not only reduces cost but also the necessity to collect multiple samples from a patient both at routine STD screenings and also for diagnosis of urogenital complications. Currently, the most widely employed of these multitarget tests are designed to detect both CT and NG as these organisms are commonly detected as coinfections in men and women. Currently approved MDx for simultaneous detection of CT and NG are outlined in Table 16.3.

16.2.2 Staphylococcus aureus and MRSA

Staphylococcus aureus is a significant cause of community-acquired infection (CAI) and healthcare acquired infections (HAIs). Antimicrobial resistance to *S. aureus* has been occurring since penicillin was first introduced in the 1940s and methicillin resistance in the 1960s. HAIs and CAIs due to this microbe complicate patient management. Bacteremia with *S. aureus* increases the risk of death; methicillin-resistant *S. aureus* (MRSA) has shown in most studies to increase mortality in healthcare [4]. Traditional microbiological platforms for testing *S. aureus* usually require at least 24 h for culture and an additional 24 h for antibiotic susceptibility testing. Thus, it is critically important to consider nucleic acid amplification testing to provide same day results for persons needing immediate attention and treatment.

The most common site for MRSA carriage is typically the anterior nares. In turn, lower sensitivities can be expected from nonnasal samples, most likely because of infrequent colonization or competing flora. As discussed earlier, careful consideration of sampling should always be given priority as it typically drives the success of qualitative and quantitative MDx testing platforms.

Rapid, FDA-approved, commercially available diagnostic assays to detect bacteremia caused by *S. aureus* and MRSA have great potential to reduce morbidity and mortality, hospital stay, and costs

Table 16.4 FDA-approved MDx assays for *S. aureus* and MRSA

Manufacturer	Test name	Method
BD Diagnostic Systems; Sparks, MD	IDI-MRSA™ Assay	Real-time PCR
BD Diagnostic Systems; Sparks, MD	GeneOhm™ MRSA ACP Assay	Real-time PCR
Cepheid; Sunnyvale, CA	Xpert™ MRSA	Real-time PCR
Roche Diagnostics; Indianapolis, IN	LightCycler® MRSA Advanced Test	Real-time PCR
Cepheid; Sunnyvale, CA	Xpert™ MRSA/SA Nasal Assay	Real-time PCR
BD Diagnostic Systems; Sparks, MD	GeneOhm™ StaphSR	Real-time PCR
Cepheid; Sunnyvale, CA	Xpert™ MRSA/SA Blood Culture Assay	Real-time PCR
Cepheid; Sunnyvale, CA	Xpert™ MRSA/SA Skin and Soft Tissue Infection test	Real-time PCR
AdvanDx; Woburn, MA	<i>S. aureus</i> PNA FISH	PNA FISH
AdvanDx; Woburn, MA	<i>S. aureus</i> and coagulase-negative Staphylococci PNA FISH	PNA FISH

associated with HAIs [5]. The reduced turnaround time afforded by rapid molecular detection methods leads to earlier identification and isolation of colonized patients, thereby decreasing MRSA transmission. For laboratory throughput, highly sensitive and specific yet user-friendly assays with common platforms are advantageous, and, as outlined in Table 16.4, several FDA-cleared molecular testing platforms and assays are currently available for *S. aureus* and MRSA. MRSA, methicillin-resistant *Staphylococcus aureus*; SA, *Staphylococcus aureus*; PCR, polymerase chain reaction; PNA FISH, peptide nucleic acid fluorescence in situ hybridization.

16.2.3 *Candida albicans* and Other *Candida* spp.

Candida is a genus of yeast that includes pathogenic species of public health concern, most notably *C. albicans*, but also to emerging species like *C. glabrata*. As a component of the normal human flora of the respiratory, gastrointestinal, and female urogenital mucosae, *C. albicans* disease is primarily attributed to fungal overgrowth at these sites but is also associated with rare but severe invasive blood-stream infections, particularly in immunocompromised individuals. Invasive candidiasis, diagnosed primarily from blood culture, is a leading cause of fungus-associated mortality in the United States [6] while vulvovaginal candidiasis is the most common clinical manifestation of mucocutaneous *Candida* disease [7]. Commonly referred to as a “yeast infection,” genitourinary candidiasis in women is a common cause of vaginitis second only to bacterial vaginosis [8]. Vaginitis, bacterial vaginosis, and all inflammatory syndromes associated with vaginal discharge are among the least understood urogenital conditions but are extremely common causes of reproductive tract morbidity of women. Together, *Candida* screening from blood culture bottles and female reproductive tract specimens represent a significant market for MDx testing and, from both a public health and basic science perspective, will ultimately enhance our understanding, prevention, and treatment of *Candida* disease.

Invasive candidiasis can be difficult to diagnose because routine blood cultures are designed primarily for detection of bacterial pathogens and have variable but relatively low sensitivities to fungal pathogens [9]. Considering urogenital disease, the opportunistic nature of *Candida* makes MDx testing a challenge because the presence of the organism is not directly indicative as a cause of disease. Nevertheless, in response to the severity of invasive candidiasis and the prevalence of vulvovaginal candidiasis, MDx assays have been developed and FDA-approved for *Candida* detection from only two sample sources: (1) blood culture bottles and (2) vaginal swabs.

For blood culture detection, AdvanDx (Woburn, MA; www.advandx.com) has three MDx assays based on PNA FISH technology for *C. albicans* or *C. glabrata* detection (see Table 16.5). The Yeast Traffic Light PNA FISH™ test from AdvanDx also detects *C. albicans*, *C. parapsilosis*, *C. tropicalis*,

Table 16.5 *Candida albicans* and other *Candida* spp.

Manufacturer	Test name	Method
AdvanDx; Woburn, MA	<i>C. albicans</i> PNA FISH® for blood cultures	PNA FISH
AdvanDx; Woburn, MA	<i>C. albicans</i> / <i>C. glabrata</i> PNA FISH® for blood cultures	PNA FISH
AdvanDx; Woburn, MA	Yeast Traffic Light PNA FISH™ for blood cultures (<i>C. albicans</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i> , <i>C. glabrata</i> , and <i>C. krusei</i>)	PNA FISH
BD Diagnostic Systems; Sparks, MD	BD Affirm™ VPIII Microbial Identification Test (<i>Gardnerella vaginalis</i> , <i>Trichomonas vaginalis</i> and <i>Candida</i> spp. (<i>C. albicans</i> , <i>C. glabrata</i> , <i>C. kefyr</i> , <i>C. krusei</i> , <i>C. parapsilosis</i> , and <i>C. tropicalis</i>))	Nucleic acid hybridization

PNA FISH peptide nucleic acid fluorescence in situ hybridization

C. glabrata, and *C. krusei*. The current molecular tests for detection of *Candida* from blood culture bottles are indicated only after observing yeast on a Gram stain of a positive blood culture. Therefore, these differential assays provide important information to the health-care team; determining which *Candida* species, or the presence of a non-*Candida* fungus, indicates which antifungal therapy is indicated because drug resistance is common. Although not comprehensive for all fungal pathogens, blood culture identification of *Candida* is an important tool for differential diagnosis of presumed blood-borne or disseminated disease.

Only a single FDA-cleared molecular assay for diagnosis of urogenital *Candida* infections is currently available, the Affirm™ VPIII by BD Diagnostic Systems, which is marketed for the differential identification of the causative agents for vaginitis. In addition to six species of *Candida*, this test also simultaneously detects *Gardnerella vaginalis* and *Trichomonas vaginalis* (Table 16.5). Because *Candida* species are opportunistic pathogens and often reside in the vagina, a threshold of 1×10^4 *Candida* organisms per patient sample was deemed clinically significant. Therefore, any combination of the detected species enumerating to greater than 1×10^4 organisms results in a positive test result. Of course, variability among women requires that this test be carefully interpreted in conjunction with clinical signs.

Similarly, it is now widely accepted that *G. vaginalis* is not the only cause of bacterial vaginosis, and, in fact, many clinical cases show no evidence of infection. Thus, the presence of *G. vaginalis* is suggestive but not diagnostic for bacterial vaginosis. The Affirm™ will give a positive result at concentrations of more than 2×10^5 organisms per sample, but as indicated in the package insert, the diagnostic value of this level is not definitive. The true value of this triplex test is for *T. vaginalis* detection, an independent and well-established cause of vaginitis and other urogenital complications. Collectively, *Candida* detection in female urogenital specimens will provide confirmation for the suspicion of a yeast infection and, because of the other targets in the multiplex assay, provide a more comprehensive testing paradigm for vaginitis/vaginosis.

16.3 Molecular Testing of Clinical Specimens for Viral Pathogens

16.3.1 Respiratory Viruses Including Influenza and Respiratory Syncytial Virus

Respiratory infections are among the most common cause of morbidity worldwide and vary from mild upper respiratory tract complications to lower respiratory tract disease including pneumonia, bronchitis, and bronchiolitis. Viruses associated with respiratory infections include influenza A/B,

respiratory syncytial virus (RSV), parainfluenza virus, adenovirus, rhinovirus, human metapneumovirus (hMPV), and *Enterovirus*. The more common influenza virus is capable of undergoing antigenic shifts and drifts responsible for pandemics and epidemics thru reassortment of the hemagglutinin (H) and neuraminidase (N) genes as was the case in the 2009 H1N1 pandemic [10, 11].

Historically, diagnosis of common respiratory viruses has involved direct examination of specimens using antibodies and inoculation into various cell lines allowing the laboratory professional to observe cells for cytopathic effect (CPE), reflecting viral growth as well as rapid testing methods utilizing antigen/antibody technology. Rapid and accurate diagnosis of respiratory viral infections is critical for proper patient management. Clinically, patients often present with nonspecific febrile-like signs and/or symptoms that are indistinguishable from bacterial infections, ultimately resulting in unnecessary prescription of antibiotics. Therefore, further stratification of potential causative agents associated with respiratory infections can result in expedited treatments reducing costs associated with length of hospital stay [12, 13].

Diagnostic assay performance of point of care influenza testing methodology commonly utilized in acute care settings varies in sensitivity and specificity when compared to viral culture and molecular methods such as RT-PCR [14–16]. Molecular diagnosis of respiratory viruses demonstrates the ability to increase sensitivity allowing detection of viruses difficult to grow in culture as well as the capability to detect multiviral infections on a single platform. Limitation in certain molecular methods to differentiate nonviable from viable virus detection warrants education on the laboratory professional's behalf regarding the viral molecular life cycle and nucleic acid target of detection. A positive result for presence of viral DNA/RNA should be used in conjunction with clinical signs and symptoms as detection of target analytes does not imply that the virus(es) are infectious. Commercial assays have been cleared by the FDA for molecular in vitro diagnostic use to detect various groups of respiratory viruses from symptomatic patients and are included in Table 16.6.

16.3.2 Human Papillomavirus

As a well-recognized and vaccine-preventable cause of sexually transmitted disease, human papillomavirus (HPV) is an independent risk factor for virtually all cases of cervical cancer and is associated with anogenital and orolabial warts. Infection with HPV is a necessary factor in the development of squamous cervical neoplasia despite the fact that most infections will not progress toward significant cervical abnormalities [17–19]. Of the over 100 genotypes of HPV, approximately 40% are transmitted via sexual contact of which several have been associated directly with the enhanced risk of cervical cancer [20]. Current guidelines for cervical cancer screening allow for either cytology alone or cytology in combination with HPV testing; specific identification of HPV genotypes 16 and 18 provides a more informative diagnosis and identifies women at the highest risk for developing cervical cancer. Cytologic evaluation of cervical Pap smear specimens is commonly utilized for cancer screening but can produce variable results due to the subjective nature of interpretation and several potentially confounding scenarios including coinfection with other STI or noninfectious reproductive tract complications. Taken together, considering the high prevalence and pathological consequences of HPV infection in women, it was imperative that MDx be developed for rapid and high-throughput screening/genotyping to complement Pap testing and further stratify a woman's risk for cervical cancer.

Currently, five molecular diagnostic assays are FDA-approved for clinical use in the United States, all of which detect at least two high-risk HPV genotypes (Table 16.7). Among these assays include hybrid capture (Qiagen Sciences, Germantown, MD; www.qiagen.com), invader chemistry (Hologic, Inc. Bedford, MA; www.hologic.com), and real-time PCR (Roche Diagnostics, Indianapolis, IN) technologies capable of detecting HPV DNA, all from liquid-based cytology specimens (Table 16.7). Stratification of HPV genotypes into high-risk and low-risk categories provides

Table 16.6 FDA-cleared MDx assays for respiratory viruses

Test	NucliSENS®		Simpliplexa™		Verigene®		ProFlu™		ProAdeno™		ProParaflu™		Pro hMPV™	
	EasyQ®	bioMeriux	Xpert® EV	xTAG® RVP	FluA/B & RSV	Nanosphere	Gen-Probe®	Gen-Probe®	Gen-Probe®	Gen-Probe®	Gen-Probe®	Gen-Probe®	Gen-Probe®	Gen-Probe®
Manufacturer			Cepheid	Luminex®	Focus		Prodesse®	Prodesse®	Prodesse®	Prodesse®	Prodesse®	Prodesse®	Prodesse®	Prodesse®
Method	Real-time	NASBA	Real-time	RT-PCR, TSPE, Tag sorting	Real-time PCR	Low-density microarray	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR
RSV				✓	✓	✓								
Influenza A														
Nonspecific				✓	✓	✓								
Influenza A														
Influenza A H1 subtype				✓	✓	✓								
Influenza A H3 subtype				✓		✓								
Influenza B				✓	✓	✓								
Parainfluenza 1				✓										
Parainfluenza 2				✓										
Parainfluenza 3				✓										
Metapneumovirus				✓										
Adenovirus				✓										
Rhinovirus	✓		✓	✓										

RSV respiratory syncytial virus, NASBA nucleic acid sequence based amplification, RT-PCR reverse transcription polymerase chain reaction, TSPE target-specific primer extension, PCR polymerase chain reaction

Table 16.7 Current FDA-approved MDx assays for human papillomavirus

Method	<i>digene</i> HC2 High-Risk HPV DNA Test			
	Cervista® HPV HR	Cervista® HPV 16/18	<i>digene</i> HC2 HPV DNA Test	Cobas® 4800 HPV Test
Approved sample sources	Hologic, Inc., Bedford, MA	Hologic, Inc., Bedford, MA	Qiagen, Inc., Germantown, MD	Roche Diagnostics, Indianapolis, IN
Invader chemistry	Invader chemistry	Invader chemistry	Hybrid capture	Real-time PCR
Cervical specimens in liquid-based cytology medium	Cervical specimens in liquid-based cytology medium	Cervical specimens in liquid-based cytology medium	Cervical specimens in liquid-based cytology medium	Cervical specimens in liquid-based cytology medium
HPV subtypes detected	HR: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	HR: 16, 18	HR: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 LR: 6, 11, 42, 43, 44	HR: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68
Individually detect HPV 16 and 18	No	Yes	No	Yes
Internal sample integrity control	Yes	Yes	No	Yes
Detection Technology	Signal amplification	Signal amplification	Signal amplification	Target amplification

HPV human papillomavirus, *HR* high risk, *LR* low risk, *PCR* polymerase chain reaction

important information regarding the potential for progression to cervical intraepithelial neoplasia. The Cervista® tests by Hologic, Inc. can detect either HPV genotypes 16 or 18 individually (Cervista® HPV 16/18 kit) or, on a separate platform, any of 14 high-risk genotypes (Cervista® HPV HR kit). Qiagen's *digene* HC2 HR test detects 13 high-risk HPV genotypes while their *digene* HC2 HPV DNA test detects 18 HPV genotypes and differentiates between several high- and low-risk types. Qiagen's HC2 test does not further discriminate between high-risk types to individually identify HPV types 16 and 18, which are the highest-risk oncogenic genotypes responsible for a significant proportion of cervical cancers. Currently, the only FDA-approved test that individually and simultaneously identifies HPV 16 or 18 from among high-risk genotypes is the Cobas 4800 HPV DNA test (Roche Diagnostics, Indianapolis, IN). This test utilizes the high-throughput Cobas 4800 platform that, after automated sample preparation, employs real-time PCR detection methods to achieve highly specific and sensitive detection of HPV from liquid-based cytology specimens. Clearly, all FDA-approved assays have independent clinical utility.

With exception to Qiagen's HC2 tests, all currently available, FDA-cleared HPV tests utilize an internal sample integrity control to validate the cellularity of the collected specimen (Table 16.7). An internal control for sample integrity is important to prevent false-negative results due to ineffective sample collection or degradation of the specimen during handling/storage. Clearly, HPV testing and further identification of high- and low-risk genotypes is an important component for maintaining reproductive health in women. As described in this section, implementation of high-throughput molecular screening will facilitate rapid identification of oncogenic HPV types and initiation of appropriate treatment paradigms to better manage a significant cause of reproductive tract morbidity in women.

16.3.3 Herpes Simplex Viruses I/II

Herpes simplex virus types I and II are primarily responsible for lifelong orolabial or urogenital infections, respectively. Although both HSV-1 and HSV-2 can cause urogenital infections in men and women, HSV-2 is the most common cause of genital herpes and one of the most prevalent sexually transmitted pathogens worldwide [3]. Importantly, most HSV-1 and HSV-2 infections are subclinical but symptomatic genital infections manifest as recurrent vesicular and/or ulcerative anogenital lesions. HSV-1 is typically associated with recurrent facial or orolabial lesions but appears to be an emerging cause of genital herpes worldwide [21]. Although rare, HSV-1 and HSV-2 infections can cause keratoconjunctivitis leading to blindness and other significant manifestations including meningitis, encephalitis, and disseminated neonatal disease [3]. Currently, there is no cure or commercially available vaccine for HSV, but antiviral drugs can reduce the frequency of herpetic lesions and/or reduce the recurrence of viral shedding. Therefore, considering the lifelong duration of HSV infections, accurate and timely diagnosis is paramount to facilitate appropriate counseling, implement a therapy paradigm, and to monitor the success of treatment.

The gold standard for clinical diagnosis of HSV infections has relied upon inoculation of the virus on susceptible monolayers of eukaryotic cells and, after 2–10 days, looking for cytopathic indications of virus replication and cell lysis. This method is not only insensitive but also lacks standardization, is labor-intensive, and the time-to-results is prohibitive for an accurate diagnosis and quick initiation of therapy. The most significant limitation to the utility of culture-based assays is the necessity to recover live virus particles from the patient specimen at a level that is detectable with culture. As most HSV infections are subclinical, and persons with asymptomatic infection shed virus less frequently [22], only MDx assays offer the enhanced analytical sensitivity and rapid time-to-results needed to screen patients suspected of HSV infection. Despite the worldwide prevalence, lifelong nature and social stigma of HSV infections, only two MDx tests have been FDA-approved, and, at the time of

Table 16.8 MDx assays for detection of herpes simplex virus types 1 and 2

Manufacturer	Test name	Method
Eragen® Biosciences; Madison, WI	Multicode® – RTx HSV 1&2 Kit for vaginal lesions	Multicode chemistry PCR and real-time PCR
BD Diagnostic Systems; Sparks, MD	BD ProbeTec HSV 1 & 2 Q ^x Amplified DNA Assays for male and female external anogenital lesions	SDA

HSV herpes simplex virus, *PCR* polymerase chain reaction, *SDA* strand displacement assay

publication, only the Multicode® – RTx HSV 1&2 Kit for vaginal lesions (Eragen Biosciences, Madison, WI; www.eragen.com) is commercially available for clinical use (see Table 16.8).

The complex life cycle of HSV includes latent localization in neuronal ganglia and trafficking of virus to the epithelium whereby replication and lesion formation occurs. Thus, during infection, viral nucleic acid is not necessarily present in the vagina, cervix, or urethra, and therefore MDx assays require the specimen be collected from an external vaginal or anogenital lesion rather than from swabs or urine. At present, the available test by Eragen Biosciences is approved only for swabs obtained from vaginal lesions. Thus, patients must be symptomatic, and the test serves essentially as a diagnostic aid in addition to clinical signs. Similarly, the BD ProbeTec Q^x test is approved only for external anogenital lesions but is approved for detection of HSV 1 or 2 from males and females. Together, both currently available FDA-cleared tests have clinical utility only in symptomatic individuals and, therefore, are not indicated as a screening assay.

16.3.4 Hepatitis B Virus

Hepatitis B virus (HBV) is a DNA virus associated with liver failure, cirrhosis, and liver cancer. Classification of the virus has been divided into at least eight genotypes (A–H) with further division into subgenotypes identifying distinct viral and epidemiological properties. These genotypes show distinct geographic distributions and are valuable in tracing the origin, evolution, and patterns of HBV transmission. Genotype A is mainly found in North America, Europe, and Africa [23].

Diagnosis of HBV infection commonly utilizes serological methods to detect either specific antigens or antibodies in plasma/serum samples such as hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), hepatitis B core antigen (HBcAg), antibodies to hepatitis B core (anti-HBc), hepatitis B e antigen (HBeAg), and antibody to HBeAg (anti-HBe). These serological patterns are complex and vary with phase of infection (chronic, acute, and immune). HBV genetic material (DNA) detected by molecular techniques can determine both viral presence (qualitative) and viral replication (quantitative). The average time from exposure to detection of HBsAg is from 6 to 60 days [24, 25], but highly sensitive nucleic acid tests can detect HBV DNA in the serum of infected individuals 10–20 days before detection of HBsAg [26]. To this end, FDA-approved HBV nucleic acid tests for in vitro diagnostics aid in the assessment of antiviral therapy response and guide patient management for newly diagnosed HBV infection thru qualitative and quantitative diagnostics (Table 16.9). The CDC recommendations for HBV testing include confirming HBsAg-positive results with IgM anti-HBc, persistence of HBsAg or HBV DNA for at least 6 months [27].

In addition to in vitro diagnostics, HBV is a part of the screening process of blood products and tissue/organ donation. The risk of HBV infection through blood transfusion is between 1 in 200,000 and 1 in 500,000 [28, 29]. To address this, distinct molecular assays have been developed and are marketed for pooled donor testing (Table 16.9) and have increased sensitivity to nonmolecular methods. Single sample nucleic acid tests have been reported to detect potentially infectious HBV before seroconversion where HBV DNA was present although lacking HBsAg and anti-HBc [30, 31].

Table 16.9 Molecular diagnostic tests for hepatitis B virus

Specimen type/utility	Manufacturer	Test name	Method
Blood component screening			
	Roche Diagnostics; Indianapolis, IN	Cobas Ampliscreen HBV Test	Real-time PCR
	Roche Diagnostics; Indianapolis, IN	Cobas TaqScreen MPX	Real-time PCR
	Gen-Probe, Inc. San Diego, CA and Novartis Diagnostics; Cambridge, MA	PROCLEIX® Ultrio	TMA
In vitro diagnostics			
	Abbott Molecular, Inc.; Des Plaines, IL	Abbott® RealTime HBV	Real-time PCR
	Roche Diagnostics; Indianapolis, IN	Cobas Taqman HBV Test v2.0	Real-time PCR

HBV hepatitis B virus, *PCR* polymerase chain reaction, *TMA* transcription mediated amplification

A distinction should be made that commercial molecular assays used for pooled donor screening are not licensed for use as diagnostic assays and serve as a tool to detect HBsAg-negative, anti-HBc-negative blood units donated during early acute infection.

16.4 Quality Control and Postrun Considerations for Molecular Diagnostics

Quality control (QC) of MDx is comparable to other diagnostic assays in that steps must be taken to ascertain the performance of the assay within run. Typically, this involves participation at each step including extraction, amplification or hybridization, and detection. Most kit-based assays include control samples at some level, although these controls are exclusive to the assay being performed. Commercial sources of control and panel materials are available and listed below (Table 16.10). In addition to QC, evaluation of laboratory performance for MDx includes proficiency testing (PT) to further meet accreditation guidelines. Currently, there are limited PT programs available to evaluate laboratory performance. With this in mind, clinical laboratories performing MDx PT should focus on method-based programs rather than specific organism-based programs as a more practical way to address PT programs.

Table 16.10 shows the FDA-cleared positive controls, but, in parallel, most of the above manufacturers also include a certified negative control as well. Considering all of the available assays, overall, there is a lack of FDA-cleared quality controls available for molecular diagnostic testing. However, internal sample integrity controls, positive controls for run validation, and/or negative controls for contamination should always be included on each testing run as required by the manufacturer. Thus, the independent third party quality controls outlined in Table 16.10 should not be substituted for the kit-included controls for any reason. The FDA-approved controls serve as independent validations for assay performance and should be employed as instructed by the manufacturer of the testing platform and/or diagnostic assay.

It is important to note that, despite extensive research and development, and validation through clinical trials for FDA approval, MDx tests have limitations and are just one of many tools available to physicians for accurate diagnosis of disease. Like all diagnostic assays, MDx tests are to be used adjunctively with other clinical and laboratory data available to the physician and, most importantly, it remains imperative for clinicians and clinical laboratorians to adhere strictly to the product insert. Deviations in the sample source, sampling method, collection vessel, transport medium, storage conditions, and any other aspect of the testing paradigm effectively render the results invalid.

In addition, with regard to interpretation of MDx test results, it is essential to bear in mind that these assays detect microbial nucleic acids and, therefore, do not differentiate between viable and dead organisms. This concept would be particularly relevant when monitoring the dynamics of microbial infection or treatment efficacy because even successful antimicrobial therapy inevitably results in

Table 16.10 Summary of currently available, FDA-approved quality controls

Target	Manufacturer	Control test name	Approved claim(s)
CT and NG	Bio-Rad Laboratories; Hercules, CA	Ampliquek CT/GC Controls	Monitor precision of CT or GC detection from urine or endocervical/urethral swabs
HBV	AcroMetrix; Benicia, CA	OptiQual™ HBV DNA Controls	Performance assessment for HBV DNA detection
	AcroMetrix; Benicia, CA		Estimate precision and reproducibility of the Procleix® Ultrio® Assay
HPV	SeraCare Life Sciences	ACCURUN® 378 Series 5000 HPV DNA Positive Control	Estimate precision of HPV DNA detection from cervical samples

FDA Food and Drug Administration, CT *Chlamydia trachomatis*, NG *Neisseria gonorrhoeae*, GC *Neisseria gonorrhoeae*, HBV hepatitis B virus, HPV human Papillomavirus

continued shedding of dead or inactivated organisms that would be detected by MDx testing. Conversely, like all diagnostic tests, negative results can also be difficult to interpret; other considerations of a pathogen's life cycle, cellular tropism, and infection dynamics such as persistence or latency also must be considered.

Understanding the basic pathobiology of the suspected pathogens is essential for accurate interpretation of MDx test results, particularly in conjunction with clinical signs, symptoms and history of disease and/or coinfections. Finally, it is of obvious importance to remember that a positive result does not rule out the presence or absence of other pathogenic organisms. This is of particular importance with MDx for STD screening whereby pathogens are often cotransmitted and contributing to clinical disease.

16.5 Regulation, Accreditation, and Guidelines for Molecular Diagnostic Laboratories

Despite the diversity and breadth of clinical diagnostics, the scope of the current chapter is limited to discussions of current FDA-approved molecular testing for only a select subset of pathogens. Clinical diagnostic laboratories are governed by the federal regulatory standards of the Clinical Laboratory Improvement Amendments (CLIA; founded 1988). However, with the lack of specific guidelines for molecular testing, one must turn to the recommendations of the National Accrediting Agency for Clinical Laboratory Science (NAACLS; www.nacls.org), American College of Medical Genetics (ACMG; www.acmg.net), Clinical and Laboratory Standards Institute (CLSI; www.clsi.org), and College of American Pathology (CAP; www.cap.org) for molecular testing standards and regulatory information. NAACLS describes the programmatic accreditation process for the institution of the diagnostic molecular scientist by providing competencies and requirements for this professional. In contrast, CLIA defines many of the basic quality systems required for laboratories, but lacks specific guidelines pertaining to molecular genetic testing. Not only for infectious disease testing, these standards and guidelines can be applied to many areas of molecular testing regardless of the field of study. Specifically, the ACMG Standards and Guidelines cover cytogenetics, biochemical genetics, and molecular genetics. In addition, the ACMG has developed disease-specific guidelines to address specific technical problems frequently seen in complex assays. Together, these guidelines cover general laboratory practices, assay validation, and technical aspects of molecular methods.

The CLSI, formerly known as National Committee on Clinical Laboratory Standards (NCCLS), used field-specific experts to develop a number of guidelines for MDx, including molecular genetic testing, molecular hematopathology, DNA sequencing, diagnostic microarrays, and proficiency testing.

The CAP is the main accrediting organization for diagnostic laboratories that employ molecular testing. The published inspection checklists are good references for the type of quality systems and procedures that should be maintained in the molecular diagnostic laboratory. Together, guidelines from each of these organizations will continually be updated as new platforms and molecular methodologies are introduced into clinical practice.

16.6 Summary

It is evident that molecular testing in the diagnostic laboratory has significantly advanced our ability to diagnose, treat, and predict outcomes related to infectious disease. Among FDA-cleared diagnostic methods, few testing platforms can compete with the sensitivity, specificity, and high-throughput nature of molecular assays. Therefore, many companies continue to make significant financial investments in the development, clinical testing, and marketing of MDx. Of course, the consumer ultimately absorbs these development costs, and, in general, the heaviest scrutiny of MDx is their cost per test. However, it remains difficult to calculate the true financial characteristics of MDx as they likely reduce other unnecessary diagnostic procedures, incorrect therapies, duration of hospital stay, and nosocomial infections [32]. Further, inconsistency and confusion regarding reimbursement by third party payers remain an impediment to complete acceptance of MDx into the clinical diagnostic laboratory.

Finally, due to space limitations, it was not possible to include and discuss all FDA-approved tests or diagnostic platforms in this chapter. Collectively, however, the most valuable advantage of MDx over other methods is the capacity for early detection, a characteristic directly attributable to the high sensitivity of molecular assays. Compared to culture-based methods, MDx can significantly reduce the time from clinical presentation to accurate diagnosis ultimately improving patient care. Molecular testing for infectious diseases is a burgeoning field with a solid foundation that will continue to serve the diagnostic laboratory for the foreseeable future.

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Part III

Clinical Molecular Oncology Testing

Chapter 17

Gene Rearrangements Testing

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Keywords T-cell monoclonality • TCR gamma gene • Gene rearrangement • T-cell lymphoma • B-cell gene rearrangement • Monoclonality • IgH • IgK • IgL

17.1 Introduction

Stem cells are the progenitor cells that can differentiate into mature B cells or T cells, or into one of the myeloid lineages. The cells that do not go through the maturity process including stem cells possess the Ig and TCR genes in the native, nonrearranged so-called germline alignment. Detection of foreign antigen is the characteristic of the specific adaptive immune response. Two distinct types of molecules are involved in this process – the immunoglobulins and the T-cell antigen receptors. Diversity and heterogeneity are attribute features of these molecules. Extensive somatic gene rearrangements are responsible for generating immunoglobulins and TCRs capable of recognizing many different antigens.

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17.2 Gene Rearrangements Testing

17.2.1 T-Cell Gene Rearrangements

Molecular-based testing of T-cell receptor (TCR) gene rearrangements is a widely used powerful diagnostic tool for detecting monoclonality in suspected T-cell lymphoproliferations. In most suspected lymphoid malignancies, a combination of clinical, histopathological, and immunophenotypical findings are sufficient enough to differentiate between a malignant and reactive lymphoproliferations. However, due to many factors and limitations (both technical and biological), in a minority of case (~5–10%) a clear diagnosis is complicated. In such cases, clonality assessment is often used as an additional tool for a diagnostic confirmation. Detection of a monoclonal expansion of a T-cell population indicates a neoplastic T-cell process. In contrast, reactive T-cell proliferations are polyclonal (heterogeneous/diverse), with no monoclonotypic T cells dominating the T-cell population.

During the development of an individual T cell, the four human TCR genes (α , β , γ , and δ) orderly rearrange and produce heterodimeric cell-surface T-cell receptors, either $\alpha\beta$ (90–95% of T cells) or $\gamma\delta$ (5–10% of T cells). In the ontogeny of T-cell development, TCR $\gamma\delta$ rearranges first, which is expressed in early T cells. TCR $\alpha\beta$ rearranges later which replaces surface $\gamma\delta$ receptor in mature T cells. Hence, TCR γ gene rearrangements can be used as markers of clonality, not only in TCR $\gamma\delta$ positive malignancies (where the rearrangements are functional), but also in TCR $\alpha\beta$ positive malignancies (where nonfunctional TCR γ gene rearrangements are seen as a consequence of the ordered hierarchy of TCR loci gene rearrangements). In addition, with a relatively simple gene structure and restricted germline repertoire, which limits the number of required PCR primers, the TCR γ gene is a highly preferred target for T-cell monoclonality assessment. The human TCR γ gene locus on chromosome 7 (7q14) includes 14 V genes belonging to four subgroups (six are functional; there are three open reading frames and five pseudogenes), 5 J segments, and 2 C genes spanning over >200 kb [1]. Even though many different PCR-based assays have been developed for the analysis TCR γ gene rearrangements, the multiplex PCR assay developed and validated through the European BIOMED-2 collaborative study group is the most widely used test of TCRG gene rearrangements [2–4]. This standardized multiplex PCR assay has been shown to detect the vast majority of clonal TCR γ gene rearrangements utilizing two multiplex master mixes. These two multiplex master mixes target conserved regions within the variable (V) and the joining (J) regions that flank the unique hypervariable antigen-binding region 3 (CDR3). The first mix (Tube A) contains primers that target the V γ 1–8 + V γ 10 genes and all J γ exon segments and the second mix (Tube B) contains primers that target the V γ 9 + V γ 11 genes and all J γ exon segments. When amplified using these multiplex PCR mixes, a clonal population of T cells yields one or two prominent amplified products (amplicons) within the expected size ranges (shown in Table 17.1). Two products are produced in cases when the initial rearrangement was nonproductive

Table 17.1 Expected/valid PCR products with the BIOMED-2 TCRG Gene Clonality Assay [1]

TCRG mix	Target	Valid PCR product size/range (bp)	Peak color
Tube A	V γ 10 + J γ 1.3/2.3	145–175	Green
	V γ 10 + J γ 1.1/2.1	175–195	Blue
	V γ 1–8 + J γ 1.3/2.3	195–230	Green
	V γ 1–8 + J γ 1.1/2.1	230–255	Blue
Tube B	V γ 11 + J γ 1.3/2.3	80–110	Green
	V γ 11 + J γ 1.1/2.1	110–140	Blue ^a
	V γ 9 + J γ 1.3/2.3	160–195	Green ^a
	V γ 9 + J γ 1.1/2.1	195–220	Blue

^aPCR products/peaks are often not produced in this size range due to the highly restricted repertoire of the amplified region

and was followed by rearrangement of the other allele in the homologous chromosome. In contrast, DNA from a normal or polyclonal population produces a bell-shaped curve (or Gaussian distribution) of amplicon products that reflects the heterogeneous population of V-J region rearrangements.

17.2.2 B-Cell Gene Rearrangements

Immunoglobulins are expressed on the surface membrane and secreted by all B cells, where they act as receptors for specific antigens. The prototypical human immunoglobulin receptor consists of two identical heavy (H) chains and two identical light (L) chains, linked together by disulfide bonds. The heavy chain is composed of a variable region (V_H) and a constant region. The light chain is also composed of a variable region (V_L) and a constant region. Diversity of the variable regions is accountable for heterogeneity in antigen recognition. This diversity is created at the DNA level by immunoglobulin (Ig) class switch DNA recombination (CSR). The variable region of the heavy chain is assembled during the rearrangement of variable (V), diversity (D), and joining (J) segments, a process called V-D-J recombination. The light chain variable region is similar but lacks diversity segments and, therefore, undergoes V-J recombination. The IgH and IgL gene rearrangement is, with rare exception, a marker of B-cell development. The H chain rearrangement occurs before the L chain rearrangement and is a useful marker to determine the stage of cell development and to establish the type and stage of the start of the malignancy process in some cases.

Diversity of the variable region is generated at several levels. First, there are many different V, D, and J segments that can be used to form the variable chains. Second, slight variations in the positions at which recombination takes place, as well as nontemplate-dependent insertions and deletions, generate additional diversity called junctional diversity. Third, in immunoglobulin genes, the heavy and light chains undergo structural modification after antigen stimulation in the form of somatic mutation. The result of all of the mechanisms of diversity is that variable regions of immunoglobulins are similar in length but can vary significantly at the DNA sequence level.

17.3 T-Cell Gene Rearrangement Testing

17.3.1 Clinical Utility

- As an adjunct test to clinical, histologic evaluation, immunohistochemistry and flow cytometry in the diagnosis of T-cell lymphomas and leukemias.
- To determine if two samples contain the same T-cell clonal or neoplastic process to help distinguish recurrent or residual disease from reactive/inflammation process or a new lymphoid neoplasm.
- *May be used for the evaluation of residual disease after therapy. However, the sensitivity of this assay (~5%) limits its utility in this regard.*

17.3.2 Methodology

DNA isolated from a variety of sources may be used. This includes genomic DNA extracted from peripheral blood or aspirated bone marrow cells. DNA may also be extracted from solid tissue (fresh or frozen) samples. For frozen tissue, carve away as much of the freezing media as possible prior to continuing the extraction. DNA may also be extracted from FNA samples. In addition, DNA may be extracted from formalin-fixed tissue specimens present in paraffin blocks, and although this DNA is fragmented, it can be successfully used for PCR studies.

17.3.2.1 Handling Conditions

- Reason for the test plus relevant clinical information must be submitted with specimen in order to provide correct interpretation of test results.
- Samples of fresh bone marrow and peripheral blood must arrive within 48 h of collection and be forwarded unprocessed at ambient temperature only.
- Peripheral blood and bone marrow specimens collected in Green- or Tan-top (Heparin) tubes are not suitable for this test.
- Fresh tissue should be frozen within 1 h of collection and the frozen tissue be sent on dry ice or transported immediately at ambient temperature.
- Fine needle aspirate (FNA) sample should be transported in a 15-mL tube containing RPMI or Normosol and forwarded promptly at ambient temperature.

17.3.2.2 Specimen Type

- *Bone marrow*: 1 mL of bone marrow in a lavender-top (EDTA) tube. Invert several times to mix.
- *Peripheral blood*: 1 lavender-top (EDTA) tube (minimum: 1 mL of EDTA peripheral blood). Invert several times to mix.
- *Tissue*: 200-mg tissue.
- *Paraffin-embedded tissue*: In duplicate, five (10 μ m) sections of tissue in a 1.5-mL microcentrifuge tube (two tubes).
- *FNA*: Transport in a 15-mL tube containing RPMI or Normosol.

17.3.2.3 Summary

The TCRG Gene Clonality Assay (available from *InVivoScribe Technologies*, San Diego, CA) utilizes the BIOMED-2 multiplex PCR master mixes targeting the variable (V) and joining (J) regions of the rearranged TCR γ gene locus to assess T- cell clonality. Tube A primers amplify the V γ 1–8 and 10 segments with all J γ exon combinations, while Tube B primers amplify the V γ 9 and 11 segments with all J γ exon combinations. Following PCR amplification, fluorescently labeled PCR products are resolved using capillary electrophoresis on the ABI 3130xl Genetic Analyzer. GeneMapper software (ABI) is then used to visualize and analyze PCR products, allowing detection of clonal cells representing >5% of the cell population.

17.3.3 Protocol Requirements

Equipment

- PCR Set-up Station/Dead Air Box
- Pipettes (10, 20, 200, 1,000 μ L)
- Microcentrifuge
- Centrifuge
- GeneAmp PCR system 9700
- ABI 3130xl Genetic Analyzer

Supplies

- 10, 20, 200, 1,000 μ L pipette tips
- 1.5-mL copolymer microcentrifuge tubes

- PCR tubes, 8-well strips
- Sample racks
- SMX-Septa-3100
- MicroAmp Optical 96-well Reaction Plate (ABI #N801-0560)

Reagents

- Omnipure water
- TCRG Gene Clonality Assay for ABI Fluorescence Detection: (IVS #1-207-0021)
- Positive control DNA: IVS-0021 (IVS #4-088-1210)
- Negative control DNA: IVS-0000 (IVS #4-092-0010)
- TCRG Tube A:V(1-8, 10)J (IVS #2-207-0031)
- TCRG Tube B:V(9,11)J (IVS #2-207-0041)
- AmpliTaq Gold DNA Polymerase (5 U/μL) (ABI #N808-0241)
- Deionized Formamide
- POP-7 polymer (ABI #4363785)
- ROX 50-400HD Fluorescent ladder (ABI #402985)

17.3.4 Procedures

The procedure for the general enzymatic amplification of DNA by PCR should be followed, using the following specific parameters:

1. Follow DNA extraction procedure appropriate for specific sample type.
Use 5 μL of genomic DNA (20–200 ng/μL) extracted from blood, bone marrow, FNA, or tissue (fresh, frozen) per reaction. For paraffin-embedded tissues, limit the total DNA input to 100–150 ng. For all specimen types, set up two PCR reactions for each sample (paraffin-embedded tissues have duplicate extractions, A and B, just do one PCR reaction for each extraction).
2. Label separate amplification tubes for each patient sample to be tested in duplicate, a positive control, normal control, and negative (water) control for both mix A and mix B. Label two 1.5-mL microcentrifuge tubes as A and B for master mix preparation.
3. Prepare master mixes A and B according to the table below:

Master mix (MM)	1× (μL)	_____ X
TCRG tube A or B	45	
AmpliTaQ Gold	0.3	
Total	45.3	

Add 45 μL MM to each labeled tube. Add 5 μL of DNA at a concentration of 20–200 ng/μL or water to the negative control. Total volume = 50 μL.

4. Cover each sample tube and place on thermal cycler, with the following cycle conditions:

95°C for 7 min
 35X 95°C for 45 s/60°C for 45 s/72°C for 1:30 min
 72°C for 10 min
 15°C hold

5. ABI 3130 Sample Preparation:

Number of samples (mix A and mix B) equals N = _____.

ROX Master Mix:

[(0.5 μL ROX 50-400HD ladder) X (N) = _____ μL] +

[(10 μL Formamide) X (N) = _____ μL]

In an ABI 96-well plate, add 10 µL of ROX/formamide mix and 1.0 µL of PCR product to each sample well. **DO NOT combine mix A and mix B products into a single well.**

On a thermal cycler, heat-denature samples at 95°C for 3 min, followed by a 4°C ice bath for 2 min.

6. Capillary gel analysis of parameters for TCRG Gene Clonality Assay.

*Create a sample sheet on the ABI 3130xl with parameters listed below:

Analysis method	Fragment analysis	Application	GeneMapper_LIS_ CLDU_135_S
Injection seconds	3 s	Size standard	GS400HD
Injection kV	15.0	Panel	none
Run kV	15.0	Instrument protocol	Appropriate name
Run °C	60	Results group	TCRG or appropriate
Run time	1,200 s		

*These parameters should be established by each laboratory and is dependent on the type/model of the capillary gel electrophoresis instrument, polymer and data analysis software used, etc.

17.3.4.1 Quality Control

- *Tube A*: Set analysis range from 120 to 280 bp. Expected peaks fall in the following ranges: Green peaks: 145–175 bp/195–230 bp; Blue peaks: 175–195 bp/230–255 bp
- *Tube B*: Set analysis range from 70 to 240 bp. Expected peaks fall in the following ranges: Green peaks: 80–110 bp/160–195 bp; Blue peaks: 110–140 bp/195–220 bp

The following controls should be run with every sample or set of samples:

1. Positive control – 5% IVS-0021, diluted into IVS-0000 or normal patient DNA
2. Normal polyclonal control – IVS-0000
3. Negative “blank” control – substitutes water for DNA template

Expected/Valid Test Control Results

- *IVS-0000 control* – polyclonal bell-shaped curve in all four primer regions of Tube A and two primer regions (160–195 bp and 195–220 bp) of Tube B.
- *5% IVS-0021* – A clonal peak among a polyclonal background in Tube A at 212 bp (ratio >3.0) and in Tube B at 167 bp (ratio >5.0).
- *Negative control* – no amplification in expected size ranges for Tube A and B.
- If the normal control or negative control fails or shows a positive result, the run must be repeated.
- All patient samples are to be tested in duplicate for both mixes, Tube A and Tube B. Samples must show replicating results for interpretation, or be repeated as appropriate.

17.3.5 Troubleshooting

Assay Sensitivity

- Based on the data published by BIOMED-2 consortium, this assay can detect >90% of clonally rearranged T cells.
- Based on in vitro DNA mixing experiments, this assay has been shown to detect a population of clonal T cells that represent at least 5% of the total T cells, at approximately 90% of the time.

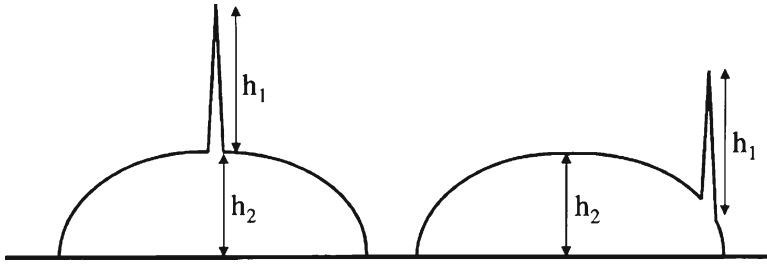


Fig. 17.1 Diagrammatic depiction of the peak height ratio calculation

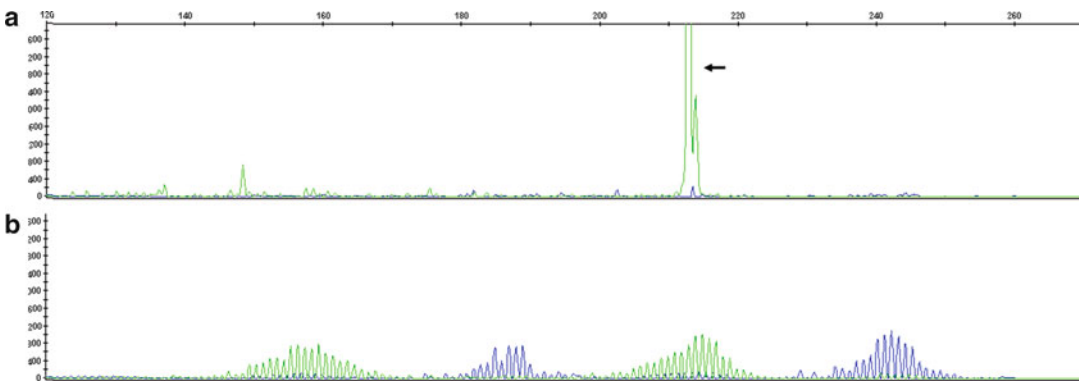


Fig. 17.2 (a) Example of an electropherogram of a 213 bp clonal TCR γ gene rearrangement product/peak detected by the TCRG Gene Clonality Assay Tube A mix. (b) Specimen with a polyclonal/normal test result

However, this ~5% analytical sensitivity is dependent on the number of background normal (polyclonal) T lymphocytes present in the specimen.

Interpretation Guidelines*

To establish a standard/guideline to determine clonality, the method of peak heights in Relative Fluorescent Units (RFU) as compared to the polyclonal T-cell background may be used [5]. The peak height ratio ($h_1:h_2$) can be calculated as $h_1:h_2 = \text{height of the largest clonal peak (h1) above the normally distributed population/the height of polyclonal background (h2)}$. Figure 17.1 is a diagrammatic depiction of the peak height ratio used for calculation.

**The peak height ratio should not be the sole criteria to determine a clonality test result. These criteria are not applicable for evaluation of follow-up specimens or minimal residual diseases. Prominent peaks that are the same base-pair size as previously detected clonal peaks are considered positive, likely related, regardless of the peak height ratio.*

Reporting results (see Figs. 17.2 and 17.3 for examples of electropherograms of clonal gene rearrangement products)

- Clonal population (replicates must match)
 - One or two dominant peaks in appropriate regions without polyclonal background, or
 - One or two dominant peaks in appropriate regions with polyclonal background; **$h_1:h_2 \geq 4.0$**
- Oligoclonal population (replicates must match)
 - Three or more dominant peaks in appropriate regions without polyclonal background, or
 - Three or more dominant peaks in appropriate regions with polyclonal background

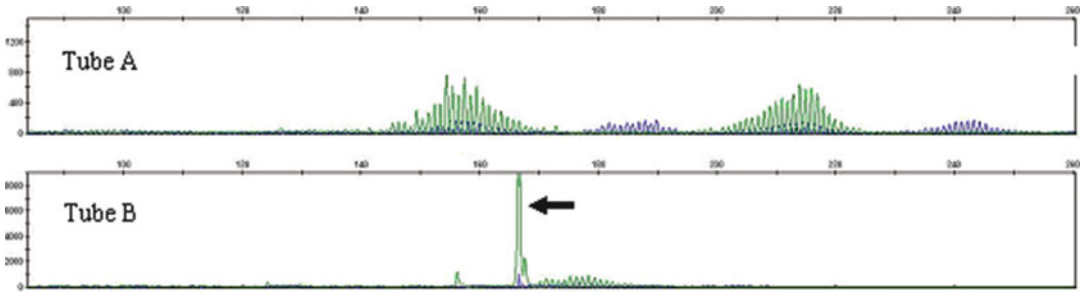


Fig. 17.3 Electropherogram of the polyclonal/normal T-cell test result with the TCRG Gene Clonality Assay *Tube A* mix, but a clonal 167 product/peak with the *Tube B* mix

- Indeterminate for clonality
 - One or two peaks in appropriate regions with polyclonal background, **h1:h2=3.0–3.9**, or
 - One or two small peaks (<500 RFU) in appropriate regions without polyclonal background, or
 - Clonal peak(s) in Mix B only OR Mix A 145–175 bp (green) or 175–195 bp (blue) only and no clonal peaks detected in any other primer regions. *These are called indeterminate, because, per the BIOMED-2 consortium publications, there is a potential risk of false positive results with the variable region primers, targeting Vγ 9, 10, and 11 due to infrequent use and/or restricted heterogeneity of these regions. This risk is even higher in specimens that contain low number of T cells. These cases could be either reflex tested with an alternative methodology such as heteroduplex analysis or by BIOMED-2 TCRB Gene Clonality Assay to further assess for T-cell monoclonality.*
- Polyclonal population (or no clone detected)
 - Polyclonal (bell-shaped) pattern without distinct peaks; same patterns are present in replicates, or
 - One or more small peaks are present in a polyclonal background; **h1:h2<2.9**
- Unable to attain results
 - No amplification, or
 - Small random peaks; consistent with background noise
 - *For (a) and (b), samples should be evaluated by amplification of the specimen control ladder (InVivoScribe Technologies Cat#2–096–002X) to rule out the possibility of PCR inhibition and/or low-quality/degraded specimen DNA*
 - Clonal or oligoclonal peaks showing different patterns in replicates that do not resolve when repeated

Limitations

- This assay detects only the TCRγ chain gene rearrangements.
- A small number of TCRγ rearrangements are not detectable with this assay.
- This assay is not informative for other molecular or chromosomal abnormalities.
- Based on in vitro DNA mixing experiments, this assay is shown to detect a population of clonal T cells that represent at least 5% of the total T cells, at approximately 90% of the time. However, this ~5% analytical sensitivity is dependent on the number of background normal (polyclonal) T lymphocytes present in the specimen.
- For specimen containing a small number of T cells, a pseudoclonal result can occur from the preferential amplification of select DNA sequences among a limited number of amplifiable targets.

- Poor DNA template quality or quantity and the presence of PCR inhibitors can affect the results of this assay. If present, these factors usually lead to an uninterpretable result rather than an inaccurate result.
- Mutations, polymorphisms or rearrangement events in the target DNA primer binding sequences can occur. If present they could lead to a false negative result.
- Occasionally, nonmalignant/reactive processes show clonal gene rearrangements. So, detection of clonality does not always imply the presence of a malignancy.
- Some clinically benign T-cell proliferations have a clonal origin, such as many cases of CD8+ (or sometimes CD4+) T lymphocytosis, benign monoclonal gammopathies, initial phases of EBV driven lymphoproliferations (frequently being oligoclonal) in immunodeficient patients, and benign cutaneous T-cell proliferations, such as lymphomatoid papulosis, etc.
- Due to the complexity of TCRG testing, results should always be interpreted in the context of clinical and pathological findings.

17.4 B-Cell Gene Rearrangement Testing

17.4.1 Clinical Utility

- Differentiation of malignant vs. benign lymphoproliferative disorders
- Lineage determination of leukemias and lymphomas
- Monitoring disease recurrence with same clonality
- Detection of residual disease, as low as 1–10% in certain assay
- Monitoring response of lymphoid neoplasm to monitor therapy

17.4.2 Methodology

All samples should be processed in the laboratory as soon as possible. All types of samples can be tested including: Blood, or bone marrow biopsy, or aspirate. EDTA tube is favored; heparin is a PCR inhibitor and it should be avoided. Fresh tissue should be collected in PBS or culture media or can be frozen with dry ice. Paraffin-embedded tissue can be block or slide. FNA or CSF sample also could be done.

17.4.3 Protocol Requirements

Equipment

- PCR setup station/dead air box
- Centrifuge
- GeneAMP PCR System 9700 (ABI)
- 3130XL Genetic Analyzer (ABI)
- Other general equipment for molecular assays

Reagents

- Qiagen blood DNA Mini Kit, Qiagen Catalog # (51306)
- IGH Framework 1 (FR1) – FAM, IVS Catalog # (2-101-0061)

- IGH Framework 2 (FR2) – FAM, IVS Catalog # (2-101-0091)
- IGH Framework 3 (FR3) – FAM, IVS Catalog # (2-101-0121)
- IGK Tube A, Vk-Jk. IVS Catalog # (2-102-001X)
- IGK Tube B, Vk-Kde. IVS Catalog # (2-102-002X)
- IGL Tube V λ -J λ IVS Catalog # (2-103-001X)
- HotStar Taq Master Mix Kit (ABI, Cat# 203443)
- AmpliTaq Gold DNA Polymerase (ABI, Cat# N808-0241)
- Hi-Di Formamide (ABI, Cat# 4311320)
- Gene Scan-400HD ROX Size Standard (ABI, Cat# 402985)
- POP-6 polymer (ABI, Cat # 4352757)
- Buffer (10 \times) with EDTA (ABI, Cat# 402842)
- Tonsil tissues (polyclonal control) (from Surgical Pathology)
- Other general reagents for molecular assays

17.4.4 Procedures

DNA Extraction

1. Extract DNA by Qiagen blood mini kit (follow different protocol on different sample types).
2. Generally transfer samples into tube, add proteinase K and lysis buffer.
3. Incubate samples at 56 or 70°C for 10 min.
4. Add ethanol to tubes, and then follow steps by transferring all samples into columns, spin and wash, finally elution.
5. Adjust DNA concentration; less than 1 μ g DNA should be in PCR reaction to avoid false negative.

Amplification (6)

1. Aliquot 50 μ L of the master mix/enzyme solutions (0.25 μ L of AmpliTaq Gold or AmpliTaq @ 5 U/ μ L) into individual PCR wells or tubes.
2. Add 5 μ L of sample or control DNA or H₂O to the individual tubes or wells containing the respective master mix reactions and mix well.
3. Amplify the reactions using the following PCR program for FR1, FR2, and FR3.

Standard Program for AmpliTaq Gold (1)	Modified Program for AmpliTaq (1):
Step 1: 95°C for 7 min	Step 1: 95°C for 3 min
Step 2: 94°C for 30 s	Step 2: 94°C for 30 s
Step 3: 55°C for 30 s	Step 3: 55°C for 30 s
Step 4: 72°C for 1 min	Step 4: 72°C for 1 min
Step 5: Go to step 2; 34 more times	Step 5: Go to step 2; 34 more times
Step 6: 72°C for 10 min	Step 6: 72°C for 10 min
Step 7: 4°C forever	Step 7: 4°C forever

4. Amplify the reactions using the following PCR program for IgK and IgL

Standard Program for AmpliTaq Gold (1):

- Step 1: 95°C for 7 min
 Step 2: 95°C for 45 s
 Step 3: 60°C for 45 s
 Step 4: 72°C for 90 s

- Step 5: Go to step 2; 34 more times
- Step 6: 72°C for 10 min
- Step 7: 15 forever

17.4.4.1 Detection

Gel Detection – Polyacrylamide TBE Gels (6)

1. Assemble electrophoresis unit using a 6% nondenaturing polyacrylamide TBE gel made with 1X TBE (Invitrogen Cat# EC62652 Box) and 0.5X TBE running buffer (Invitrogen 5X TBE Cat# LC6675).
2. Add 5 µL of ice-cold nondenaturing bromophenol blue loading buffer to samples.
3. Load 20 µL of mixture into wells of the gel.
4. Run gel at 110 V for 2–3 h or 40–50 V overnight (voltage and electrophoresis time depend on the PCR amplicons size, acrylamide gel thickness, and type of PCR equipment; voltage and run time can be adapted accordingly).
5. Gels are stained in 0.5 µg/mL EtBr (in water or 0.5X TBE buffer) for 5–10 min.
6. Gels are destained 2× in water for 5–10 min.
7. UV illumination is used for visualization.
8. Gel is photographed and data are interpreted.

ABI Fluorescence Detection

1. Add 1 µL of reaction products in separate tubes and add 10 µL of HI-Deionized formamide containing ROX size standards 0.5 µL to each tube, mix well.
2. Reaction products are heated to 95°C for 2 min then snap-chilled on ice for 5 min.
3. A sample sheet and injection list is made up for the samples. As the samples are run on the machine, they are fractionated, detected, and analyzed by the instrument. Runs are 20–40 min in duration. The instrument automatically analyzes and stores data for review or comparison with other test results.

***Note:** Other detection methods that can be used include agarose gel, heteroduplex polyacrylamide gel, etc. Southern blot analysis is still considered a gold standard to detect clonality. The disadvantage is labor and time consumption. The LightCycler is another platform for running gene rearrangement testing to detect B and T clonality. Recently, real-time quantitative polymerase chain reaction (qPCR) of gene rearrangements has been adapted to identify minimal residual disease (MRD) for adults and children with ALL [7, 8]. Sequence analysis of clonal immunoglobulins is also available in some centers.

17.4.4.2 Quality Control

PCR Amplification

To demonstrate that the DNA recovered from the specimen is intact and amplifiable, a control gene beta-globin PCR reaction is also done in parallel. A 324 bp size of the beta-globin gene should be amplified with every experiment. This also ensures that no inhibitors of PCR amplification are present in the reaction as sometimes remain from the extraction process, and there is DNA of adequate quality and quantity to generate a legitimate result. Primer sequences are listed below; program runs same as the FR3 program. All specimens, regardless of cell type, should be applied to this PCR reaction and see this production; otherwise, QNS (or quantity not sufficient report) should be issued.

Forward primer: FAM-GTC TCC TTA AAC CTG TCT TG

Reverse primer: GAA GAG CCA AGG ACA GGT AC

Template Control

Positive, negative, and no template controls always be tested for each of the master mixes with each experiment. Preparation of control is as below:

100 ug/mL 10% Sensitivity Control:

90 ug Tonsil DNA or IVS-0000 Polyclonal Control DNA, Cat # (4-092-0010)

10 ug positive cell line DNA

or

IVS-0010 Clonal Control DNA, Cat # (4-088-0550)

IVS-0029 Clonal Control DNA, Cat # (4-088-1690)

IVS-0007 Clonal Control DNA Cat # (4-088-0370)

H₂O to 1 mL

Contamination Control

Prior to using any new lot of reagent, at least one positive and one negative sample should be run to ensure that reagents work.

To avoid contamination and prevent false positives, the PCR setup is divided into three separate rooms:

- Pre-PCR
- Sample preparation/template addition
- Post-PCR

17.4.5 Troubleshooting

Assay Sensitivity

- Analytical sensitivities are in the range of 1–10% clonal cells in a polyclonal background, even to 1 cell per 10⁴–10⁶ cells by using higher resolution denaturing/sequencing gels.
- Based on the data from most popular FR3 assay, the frequency of clonal detection ranges from approximately 60–80%. By using all assays together, clonality detection rate could be increased to 95% and more.

Interpretation Guidelines

- The expected peak from beta-globin PCR must be higher than 1,000 RFU to continue to interpret patient results.
- The appearance of a peak within the expected size range in the “No DNA” control lane is an indication that contamination has occurred.
- All assay controls must result as expected prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid.
- The expected size ranges for products are listed in Table 17.2.
- The test is used clinically to give support to pathologic diagnosis. The clonal populations of rearranged genes are highly suggestive molecular markers for monoclonality of B cells, confirmation of B-cell lineage, and in some cases to provide tumor-specific markers for follow-up studies.

Reporting Results

- Polyclonal Peaks
 - No dominant peak twofold above background indicates that the B cells are polyclonal. The cells contain rearrangements that vary in size and therefore migrate differently when compared to

Table 17.2 The expected size ranges for each immunoglobulin assay are listed in this table

Master mix	Target	Product size in base pairs
IGH Framework 1	FR1-JH	290–360
IGH Framework 2	FR2-JH	235–295
IGH Framework 3	FR3-JH	69–129
IGK Tube A	Vk-Jk	120–160, 190–210, 260–300
IGK Tube B	Vk-Kde	210–250, 270–300, 350–390
IGL Tube	Vλ-Jλ	135–170

normal tonsil sample in capillary electrophoresis. This type of result is seen in a reactive lymph node or other nonclonal malignant process (shown in Fig. 17.4).

- Two Sharp Peaks
 - A result twofold above background and within the size range of polyclonal peaks is sometimes seen in FR1 and/or other assays including FR2, FR3, IgK, and IgL, which indicates that a particular clone is over-represented in the population of B cells. Because each cell has two alleles that rearrange independently, each rearrangement will have a different sequence that will migrate differently. A monoclonal population of B cells is usually, but not always, indicative of a malignant lymphoid condition. This result should be correlated by the physician with other laboratory or clinical data.
- A Single Sharp Peak
 - A result twofold above background and within the size range of polyclonal peaks seen in FR1 and/or other assays including FR2, FR3, IgK, and IgL indicates that one allele is rearranged, and the other is germline or deleted, and a particular clone is over-represented in the population of B cells. A monoclonal population of B cells is usually, but not always, indicative of a malignant lymphoid condition. This result should be correlated by the physician with other laboratory or clinical data.
- Several Peaks
 - A result three or more peaks, twofold above background, and within the size range of polyclonal peaks. This oligoclonal result may be seen in nonmalignant and premalignant situations. Close clinical follow-up is usually recommended.
- A sample yielding a positive beta-globin reaction but no apparent amplification of IgH locus can occur when there is a lack of the appropriate cell type in the sample, i.e., no lymphoid cells.

Limitations

- False negatives are more frequently seen in follicular, marginal zones and diffuse large B-cell lymphomas.
- Low concentration of DNA or specimen containing a small number of B cells produce pseudoclonal signals.
- Multiplex PCR may potentially yield false-negative results.
- Heparin or fixatives or other inhibitors in samples will inhibit PCR assay.
- Poor DNA template quality can affect the results of this assay and lead to an uninterpretable result.
- Mutations, polymorphisms or rearrangement, or deletion events in the target DNA primer binding sequences can occur. If present, they could lead to a false-negative result.
- Occasionally, nonmalignant/lymphoproliferations disorder show clonal signals. So, clinical correlation is recommended.

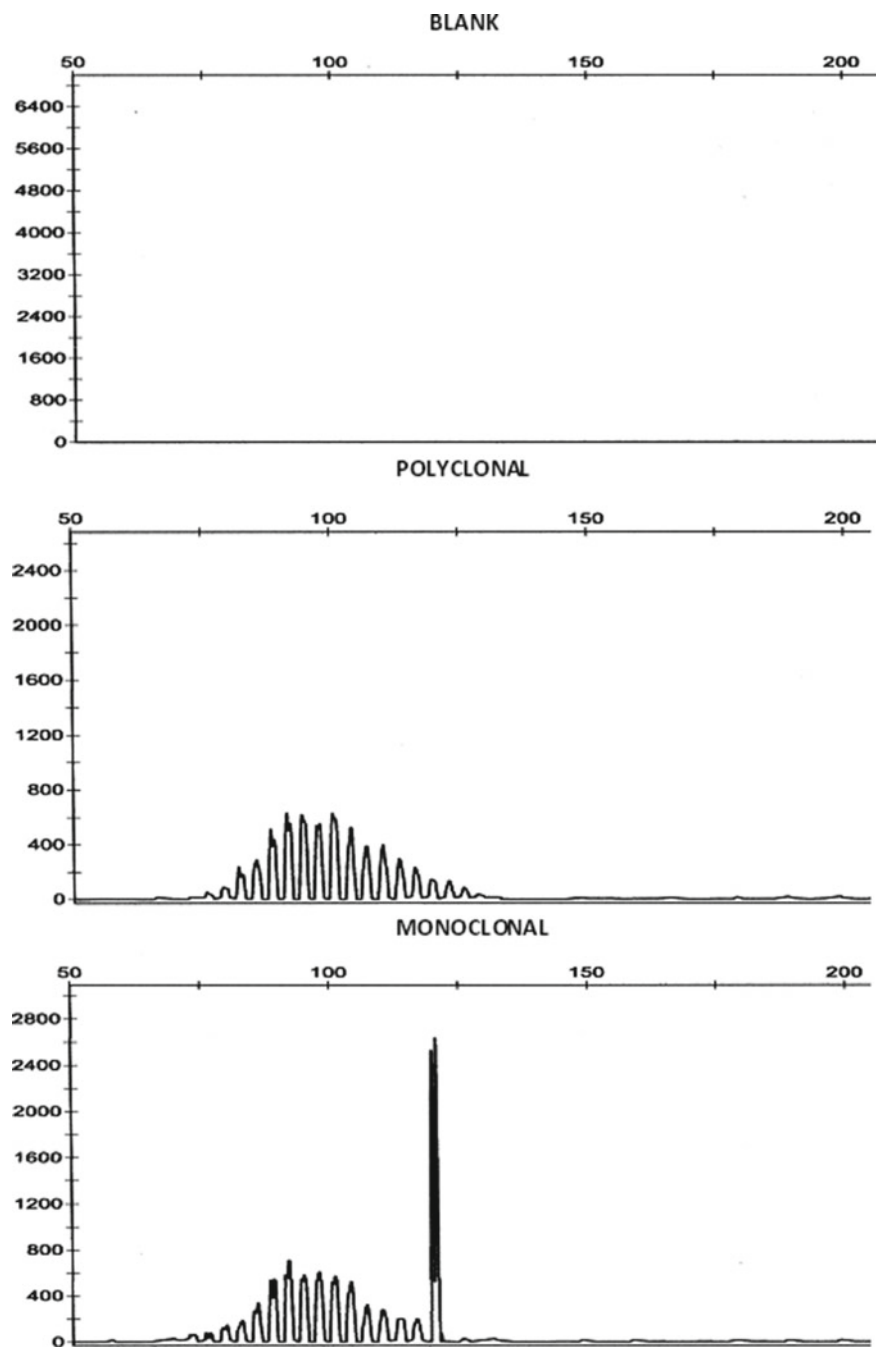


Fig. 17.4 Electropherogram of results from FR3 assay indicating negative, polyclonal, and negative results

17.4.6 Summary

Diagnosis of lymphoid disorders is often intricate due to difficulties in histological differentiations between neoplastic and reactive lymphocytes. Clonality of the lymphocytes within a biopsy specimen can provide an additionally useful deciding factor for the diagnosis of lymphoid neoplasia. The conventional technique of Southern blotting for the detection of gene rearrangements is time-consuming and requires an amount and quality of DNA that is not always available from biopsies. The development of the polymerase chain reaction (PCR) has allowed for the detection of B- or T-cell clonality in a small number of target cells with a relatively quick turnaround time [9]. It also allows for the use of paraffin-embedded samples which, due to fixation, are commonly degraded [10, 11] and, therefore, sometimes unsuitable for Southern blot analysis.

Clonality in B-cell lymphomas is detected by PCR analysis of the immunoglobulin heavy chain (*IGH*) variable region [12]. The advantage of this PCR test over Southern blot is for the most part the ability to analyze DNA extracted from paraffin blocks, as well as the more rapid turnaround time (less than 1 week for PCR vs. 2–3 weeks for Southern blot).

The advantages of this assay (PCR plus capillary electrophoresis) over PCR plus gel electrophoresis is that the assay has higher sensitivity, superior resolution, and less labor-intensive. It also can generate reportable results with less optimal DNA amount.

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Chapter 18

Mutation Analysis in Cancer

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Keywords Mutation testing • Mutation analysis • Gene mutations • Molecular oncology • Personalized cancer care • Sanger sequencing • Pyrosequencing • Targeted therapy • Oncogenic mutations • Polymerase chain reaction (PCR) • Real-time PCR • Allele-specific oligonucleotide PCR • TaqMan probes • Capillary gel electrophoresis

18.1 Introduction

This chapter provides an overview of the impact of molecular genetic testing on cancer treatment and mutation analysis platforms and methodologies used for mutation detection, interpretation of results, and limitations of procedures. Detailed Sanger sequencing and pyrosequencing protocols are provided for *EGFR* and *KRAS* mutation analysis respectively.

18.1.1 Genetic Mutations and Cancer

Identification of mutations in genes that regulate the key cellular processes has contributed greatly to our understanding of cancer biology. On the basis of their role in oncogenesis, oncogenes are classified as either tumor-suppressor genes (mutations that lead to loss of normal function of these genes, including *TP53*, *APC*, and *RBI*, resulting in cancer) or proto-oncogenes (mutations that lead to abnormal

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activation of these genes, including *EGFR*, *KIT*, *PIK3CA*, *NRAS*, and *KRAS*, resulting in cancer) [1]. It is well established that genetic mutations provide cancer cells with survival and/or proliferation advantage over normal cells. Mutations can be hereditary (i.e., germline) or acquired (i.e., somatic). In addition to oncogenic mutations that lead to the development of cancers, cancer cells themselves constantly acquire new mutations, providing them with resistance to therapies. With the increasing availability of next-generation sequencing, clinically significant new genes are being identified at a rapid pace. Improved genomic technologies and informatics tools have enabled the rapid discovery of cellular pathways affected by these mutations.

18.1.2 Impact of Molecular Genetic Testing on Cancer Care

Clinically, genetic mutations are categorized as diagnostic, prognostic, or predictive. Diagnostic mutations are essential to the oncogenesis of a specific tumor, and their presence defines the clinical entity, such as the p.Asp816Val (p.D816V) mutation in *KIT* in mastocytosis and the p.Val617Phe (p.V617F) mutation in *JAK2* in myeloproliferative neoplasms [2].

Prognostic mutations can predict the long-term outcome for patients with a particular cancer, such as in acute myeloid leukemia (AML), where *NPM1* mutations predict a better prognosis [2]. Interestingly, coexisting mutations can change the prognosis; for example, even in the presence of an *NPM1* mutation, a coexisting *FLT3* mutation imparts a poor prognosis [2]. Prognostic mutations in conjunction with other clinicopathologic parameters help in determining overall treatment strategy.

Predictive mutations can predict response to a particular therapy; for example, the *KIT* mutation in gastrointestinal stromal cancer and the *EGFR* mutation in colon cancer predict a good response to their respective inhibitors [3]. Interestingly, a coexisting *RAS* mutation provides chemoresistance to EGFR-inhibitor therapy [4].

Certain mutations can develop during the course of treatment, such as *ABL1* kinase domain mutations in chronic myelogenous leukemia (CML). Testing for *ABL1* mutations is standard practice in patients whose cancers develop resistance to tyrosine kinase inhibitors. Thus, a comprehensive molecular evaluation of a cancer may require testing for multiple genes.

18.1.3 Mutation Analysis Platforms

A wide variety of molecular technologies, based on certain common principles, are available for detecting genetic mutations. Generally speaking, these molecular technologies require polymerase chain reaction (PCR)-based amplification of the target of interest. Depending on the nature and distribution of the mutation within the gene, different detection strategies can be applied. Mutations such as insertions and deletions lead to alteration in the size of a gene segment. Such mutations are easily detected by capillary electrophoresis (CE)-based size separation, which can detect both *NPM1* exon 12 mutation and *FLT3* internal tandem duplications. Mutations that are consistently localized to a specific codon or a limited number of codons within a gene can be detected using a sequence-specific probe-based approach, such as allele-specific oligonucleotide (ASO) PCR, or by a targeted pyrosequencing approach, which can target codon 12, 13, and 61 mutations in *KRAS* and *NRAS* genes. High-resolution melting curve analysis can be used to screen for mutations and to target specific mutations. Direct sequencing of the DNA template using a Sanger-based approach is considered the gold standard in genetic mutation analysis.

The recent development of multiplex genotyping assays using single-nucleotide extension reactions, such as Sequenom MassARRAY (Sequenom, San Diego, CA) and SNaPshot analysis (Applied Biosystems, Foster City, CA), has facilitated the simultaneous detection of multiple mutations using smaller amounts of starting material. Also, the recent availability of desktop next-generation sequencing has made it possible to sequence larger panels of genes in their entirety. Ultimately, the choice of test methodology depends on many factors, including the type and frequency of mutation, anticipated test volume, tumor type, sample type and volume, cost of technology, and preexisting instruments.

18.2 Methodology

Scientifically speaking, a mutation is any aberration from the wild-type gene sequence, which can range from a point mutation to insertions and deletions to chromosomal translocations and large-scale chromosomal abnormalities. In this chapter, the discussion will be limited to mutations at the single-nucleotide level and to smaller genetic alterations such as insertions and deletions. Since there are multiple mutation detection platforms and protocols that a laboratory can choose from, it is beyond the scope of this chapter to provide detailed protocols for all of them. Instead, we provide representative protocols for Sanger sequencing and pyrosequencing, two of the most common and versatile mutation detection platforms. Previously published information on additional mutation detection platforms can be found in Table 18.1. While the principles of Sanger sequencing will be briefly discussed below, principles of pyrosequencing can be found in Chaps. 15 and 20.

18.3 Principles of Sanger Sequencing

Sanger sequencing, widely considered to be the gold standard for DNA sequencing, is an enzymatic method that uses dideoxy terminator nucleotides. The DNA polymerase is able to extend a primer, which is annealed to the template, until a chain-terminating nucleotide is incorporated. Historically,

Table 18.1 Selected mutation detection protocols currently used in the clinical molecular diagnostics laboratory at the University of Texas MD Anderson Cancer Center

Gene	Type of neoplasms	Reference
<i>Sanger sequencing–based assays</i>		
<i>TP53</i>	Hematopoietic, solid	Agell et al. [5]
<i>CEBPA</i>	Hematopoietic	Pabst et al. [6]
<i>EGFR</i>	Solid	Lynch et al. [7]
<i>PIK3CA</i>	Solid	Janku et al. [8]
<i>Pyrosequencing-based assays</i>		
<i>RAS</i>	Hematopoietic, solid	Zuo et al. [9]
<i>JAK2</i>	Hematopoietic	Lin et al. [10]
<i>Fragment-size analysis by capillary gel electrophoresis</i>		
<i>NPM1</i>	Hematopoietic	Patel et al. [11]
<i>FLT3</i>	Hematopoietic	Lin et al. [12]
<i>High-resolution melting curve analysis-based screening followed by Sanger sequencing confirmation</i>		
<i>IDH1, IDH2</i>	Hematopoietic, solid	Patel et al. [13]

radioactive-labeled nucleotides were used during the sequencing, followed by detection of different sizes of fragments on an agarose gel; however, most current protocols use fluorescent-labeled nucleotides followed by capillary gel electrophoresis. The length of the sequence that can be accurately resolved ranges from +1, from the sequencing primer, to approximately +400 positions, depending on the sample quality, sequence composition, and cycling conditions.

Steps in the sequencing reactions are listed below:

1. First-round PCR is used to amplify material from genomic DNA or complementary DNA (cDNA), followed by cleanup of unincorporated nucleotides. PCR products are run out on agarose gels or by Agilent Bioanalyzer to confirm amplification and absence of product in the reagent controls.
2. (Optional step) Second-round nested PCR is used to increase the amount of scant product or to increase specificity of the analyzed product.
3. Cycle sequencing (i.e., asymmetric PCR) is used to generate chain-terminated fragments of various lengths. Generally speaking, bidirectional sequencing is preferred to avoid interpretation pitfalls resulting from sequencing artifacts.
4. CE is used to separate and read terminal base pair (bp) in the sequencing products.
5. Primary analysis of sequencing is used to check for dye incorporation, CE injection, and overall quality of the sequence obtained using sequencing analysis software followed by SeqScape.
6. Secondary analysis is used to identify the sequencing traces, including trimming and assembly of forward and reverse strands, and to compare those with the normal gene template for detection of mutations.

18.4 Protocol

18.4.1 Sanger Sequencing–Based EGFR Mutation Analysis

18.4.1.1 Rationale for EGFR Mutation Analysis

Recently, it has been demonstrated that activation of point mutations and deletions in the kinase domain of the *EGFR* gene occur frequently in lung adenocarcinomas [3, 7]. These mutations occur more frequently in non-smokers and are not seen in tumors that have *KRAS* mutations [14, 15]. The presence of such *EGFR* mutations predicts clinical response to *EGFR* kinase inhibitors such as gefitinib and erlotinib [3, 7, 15]. Detection of mutations in the kinase domain of the *EGFR* receptor in lung tumors may predict response to *EGFR* inhibitors.

18.4.1.2 List of Supplies

Instruments

- ABI 3130 genetic analyzer: 16 capillary array, with POP7 polymer, or
- ABI 3730 genetic analyzer: 48 capillary array, with POP7 polymer

Reagents and Materials

- AmpliTaq Gold Polymerase with buffer II and 25 mM magnesium chloride (ABI product no. N8080247)

Table 18.2 Primers [4] for *EGFR* Sanger sequencing

Step	Exon	Forward	Reverse
PCR ^a	18	M13 CAA GTG CCC TGT CCT GGC ACC CAAG GC	M13 CCA AAC ACT CAG TGA AAC AAA GAG
	19	M13 GTA ACA TCC ACC CAG ATC	M13 GTC TAG AGC AGA GCA GCT G
	20	M13 GAA ACT CAA GAT CGC ATT CAT GC	M13 GCA AAC TCT TGC TAT CCC AGG AG
	21	M13 CAG CCATAA GTC CTC GAC GTG G	M13 CAT CCT CCC CTG GAT GTG TTAAAC
Sequencing ^b		TGTAAAACGACGGCCAGT	CAGGAAACAGCTATGACC

^aM13 sequence tags are attached to the 5' end of all PCR primers. For forward primers the M13 sequence is 5'-TGTAACGACGGCCAGT-3' and for reverse primers the M13 sequence is 5'-CAGGAAACAGCTATGACC-3'

^bM13 primers are used as sequencing primers for all exons

- Sterile water (Sigma W3500)
- 10 mM dNTPs (ABI)
- Agarose, molecular biology grade
- 10× gel loading solution (see Digest section procedure for 10× gel loading solution; dilute 10× gel loading solution 1:4 with sterile water to produce 2.5× concentration)
- Qiagen DyeEx™ 96 Kit

Primers

Primers for Sanger sequencing are listed in Table 18.2 for exons 18, 19, 20, and 21. Primers are diluted to 10 mM for PCR and to 1 mM M13 for sequencing.

Supplies and Equipment

- 0.2-mL PCR tubes or 96-well plate or 0.2-mL strip tubes
- 0.6-mL PCR tubes
- Pipettors and tips
- 8-well caps
- Class II biosafety cabinet
- Gel tray and electrophoresis chamber
- Perkin Elmer Geliance or other gel imaging equipment

18.4.1.3 Sample Requirements, Collection, and Storage

Sanger sequencing requires 5–10 unstained paraffin sections and hematoxylin and eosin (H&E) stained slides with the tumor and normal areas circled. Fresh tissue should be delivered on dry ice. Specimens are microdissected, and DNA is extracted and quantified. DNA is stored in TE buffer at 4°C. The amount of DNA that is required for PCR testing is 2 µg. The optimal amount of DNA is 2.5 µg.

18.4.1.4 Controls

- Negative Control: Tjelerina cell line that is negative for *EGFR* mutation
- Positive control: previously positive patient samples

18.4.1.5 Procedure

PCR Setup and Thermocycling Conditions

1. Prepare the master mix for each exon (18, 19, 20, and 21) for the PCR reactions using the following worksheet:

Reagent	μL per reaction
10× PCR buffer II	5.0
25 mM MgCl ₂	3.0
10 mM dNTP mix	4.0
Water	31.5
<i>EGFR</i> -F- (forward primer)	2.0
<i>EGFR</i> -R- (reverse primer)	2.0
TAQ Gold polymerase	0.5
<i>Total reagent volume</i>	48 μL

2. Label 0.2-μL tubes or strip tubes and number them according to the PCR worksheet.
3. Include a reagent control with no DNA (replace DNA by H₂O only).
4. Add 48 μL of master mix to each tube.
5. Add 2 μL of DNA to be tested, diluted to 0.1 μg/μL, to the appropriate tube.
6. Place the tube in a thermocycler and run Program *EGFR* as follows:

Hold	95°C for 10 min
50 cycles	94°C for 1 min
	60°C for 1 min
	72°C for 1 min
Hold	72°C for 10 min
Hold	4°C for ∞

Post-PCR Evaluation by Gel Electrophoresis

1. Please use standard laboratory protocol for analyzing 8 μL PCR product on a 2% agarose gel.
2. Run the gel at 115 V for 30–45 min or until bands in ladder are completely resolved.
3. After the gel electrophoresis is complete, take a picture and analyze it on the Geliance Gel Doc and obtain a printout.

Post-PCR Purification

The PCR products need to be purified for cycle sequencing. Post-PCR purification is done using the QIAquick PCR purification kit (Qiagen) or the Ampure kit (Agencort).

Cycle Sequencing

1. Arrange and label 0.2-mL PCR strips in two rows aligned from A to H in a 96-well plate for forward and reverse reactions for each PCR product.
2. Prepare a master mix for forward and reverse reactions for exons 18, 19, 20, and 21 as follows:

Reagent	μL per reaction
Water	8.8
5 \times sequence buffer	2.0
<i>EGFR</i> sequence primer (1 μM)	3.2
Big Dye version 1.1	4.0

3. Add 18 μL of master mix to the appropriate tubes.
4. Add 2 μL PCR product to each forward and reverse reaction.
5. Cover the strips and place in a thermocycler and cycle sequence under the following conditions:

25 cycles	96°C for 10 s 58°C for 5 s 60°C for 4 min
Hold	4°C for ∞

Purification of Sequencing Products Using Qiagen DyeEx™ 96 Kit

1. Mix plate by hand to resuspend resin column.
2. Remove foil from wells in an A1-to-H12 orientation corresponding to the PCR tube strips to prevent loss of any of the resin column; remove the bottom foil first.
3. Place the plate on one of the blue plate holders provided in the kit and centrifuge for 3 min at 1,000 RCF, then discard the buffer and blot the bottom of the plate.
4. Place the plate on a 96-well PCR plate with the columns lined up A1 to A1 and carefully transfer the sample from the corresponding PCR tube strips directly to the top of the resin columns. If using a multipipettor, add only four samples at a time to ensure proper control.
5. Centrifuge the plate for another 3 min at 1,000 RCF.
6. Cover the plate with a rubber plate cover and denature in a thermocycler at 95°C for 5 min. After denaturing, place the plate on ice for at least 5 min.
7. If the plate cannot be run for sequencing right away, cover the wells with strip covers and keep at -20°C , protected from light, until ready for sequencing.
8. Alternatively, sequencing purification can be done using Qiagen DyeEx 2.0 Spin Kit.

Sequence Analysis

1. Run plate on 3130/3730 (Following settings are for illustration purpose only. Laboratories should establish their own settings.):
Results group 1: *EGFR-CKit* EX17 Result Group
Instrument protocol 1: Janus_*EGFR*_Kit_Shter
Analysis protocol 1: 3130_BDTv1.-KB-Pop7
3730_BDTv.1-KB-Pop7
2. After the run, evaluate the sequences for mutations using SeqScape software.

18.4.2 Pyrosequencing Assay for *KRAS* and *NRAS*

18.4.2.1 Rationale for RAS Mutation Analysis

The *RAS* family of oncogenes (*KRAS*, *NRAS*, and *HRAS*) plays a central role in the intracellular signaling pathways involved in cell growth. The transforming activity of *RAS* is produced by nonrandom

point mutations at codons 12, 13, and 61 [4]. Mutated *RAS* genes have been found in a wide variety of human cancers, most frequently in lung, colon, and pancreatic cancers. However, AML with monocytic differentiation and chronic myelomonogenous leukemia (*CMML*) show mutated *RAS* at an incidence rate of 30–40% [16]. Recently, it has been documented that the *RAS* mutation yields resistance to anti-EGFR monoclonal antibody therapy [4].

18.4.2.2 List of Supplies

Instruments

- PSQ HS 96 pyrosequencer
- PyroMark Q96 Vacuum Prep Workstation

Reagents and Materials

Pyrosequencing reagents (as recommended by the manufacturer):

- Autoclaved Milli-Q water
- 10× PCR buffer
- MgCl_2 (25 mM)
- dNTP mix (10 mM)
- AmpliTaq Gold (5 U/ μL)
- Autoclaved 0.2 mL PCR reaction tubes (8 tube strips)
- Biomarker Low
- Agarose
- 1× TAE
- Ethidium bromide (10 mg/mL)
- 70% ethanol
- Denaturation solution
- Pyro Gold reagents (Biotage)
- Streptavidin-bound sepharose beads (Amersham Biosciences)
- Washing buffer
- Binding buffer
- Annealing buffer
- Autoclaved Milli-Q water
- Agarose DNA electrophoresis grade
- ABI BigDye™ Terminator Sequencing Kit v1.1
- QIAquick™ Spin Column PCR Purification Kit
- Agencourt Ampure Beads
- DyeEx 2.0 Spin Kit or DyeEx™ 96 Kit

Primers

Adjust primers (Table 18.3) to their respective working concentration and divide into 100 μL aliquots: forward PCR primer (10 μM), reverse PCR primer (10 μM), and pyrosequencing primer (7.5 μM).

Table 18.3 Primers^a for KRAS/NRAS pyrosequencing

Gene	Codon	Step	Forward	Reverse
KRAS	12, 13	PCR	TAT AAA CTT GTG GTA GTT GG	BIOTIN-ATT GTT GGA TCA TAT TCG T
		Sequencing	AAA CTT GTG GTA GTT GGA GC	N/A
	61	PCR	TTG GAT ATT CTC GAC ACA	BIOTIN-CCA CCT ATA ATG GTG AAT ATC T
		Sequencing	TGG ATA TTC TCG ACA CAG C	N/A
		PCR	GTT CTT GCT GGT GTG AAA TGA	BIOTIN-CTC TAT GGT GGG ATC ATA TTC
NRAS	12, 13	PCR	GTT CTT GCT GGT GTG AAA TGA	BIOTIN-CTC TAT GGT GGG ATC ATA TTC
		Sequencing	CAA ACT GGT GGT GGT TGG AGC A	N/A
	61	PCR	GGA CAT ACT GGA TAC AGC T	BIOTIN-CTG TAG AGG TTA ATA TCC GCA
		Sequencing	GGA CAT ACT GGA TAC AGC T	N/A
		Sequencing	GGA CAT ACT GGA TAC AGC T	N/A
		Sequencing	GGA CAT ACT GGA TAC AGC T	N/A

^aAll primers are listed in 5'–3' orientation

Supplies and Equipment

- AmpliTaq Gold (5 U/μL)
- Autoclaved 0.2-mL PCR reaction tubes (8 tube strips)
- 96-well optical PCR plate
- Biohazard bag with holder
- Adjustable pipettors, 1–1,000 (pre-PCR only)
- Adjustable pipettors, 0.5–1,000 (post-PCR only)
- Sterile aerosol filtered pipette tips, 1–1,000
- Nonfiltered pipette tips, 0.5–200
- CDT and RDT tips with cartridge holder (Biotage)
- Pyrosequencing 96-well reaction plate (Biotage)
- Rubber tray seal
- Washing buffer
- Binding buffer
- Annealing buffer
- Vacuum preparation tool
- Thermocycler PE9700 or Hybaid or ABI2720
- Microcentrifuge
- Autoclaved 1.5-mL microcentrifuge tubes
- PCR tray
- Rubber tray seal (septa)
- Adjustable pipettors, 1–1,000 1 range (pre-PCR only)
- Adjustable pipettors, 1–1,000 1 range (post-PCR only)
- Sterile aerosol filtered pipette tips, 10 1–1,000 1 size
- Vortex mixer

18.4.2.3 Sample Requirements, Collection, and Storage

- 1–2 mL of bone marrow (BM) aspirate or 10 mL of peripheral blood (PB) samples should be collected in EDTA (purple top) Vacutainer tubes and delivered to the lab on cold packs as soon as possible and no later than 48 h after collection.
- Specimens shipped in heparinized (green top) containers are not acceptable due to the inhibitory effect of heparin in the PCR reaction.

- DNA is extracted and quantitated. DNA is stored in TE buffer at 4°C. The amount of DNA required for PCR testing is 0.2 µg for each exon. The optimal amount of DNA is 2 µg.

18.4.2.4 Controls

- Negative control: previously analyzed patient samples
- Positive control: previously analyzed patient samples

18.4.2.5 Procedure

PCR Amplification of RAS Sequences

1. Label the 96-well plate according to the PCR worksheet.
2. Mix the DNA sample by vortexing for 5 s. Quick spin the sample to prevent aerosol.
3. Make DNA dilutions of DNA samples to have a final concentration of 0.1 µg/µL (100 ng/µL).
4. Prepare the master mixes for the number of samples being run (plus an additional 2 in case of pipetting error).
5. Aliquot 48 µL of PCR master mix to each reaction well. Close the caps on all the tubes.
6. Add 2 µL water in the reagent control wells. Vortex and spin down sample dilutions and working controls. Opening only one tube at a time, add 2 µL sample DNA (0.1 µg/µL), then close the tube before proceeding to the next sample. If using a 96-well plate, make sure that samples are added to the correct wells and that strip caps are closed tightly. Finally, add 2 µL control DNA (0.1 µg/µL) to the control wells according to the worksheet. Ensure that all wells in use are capped.
7. Briefly vortex the PCR reaction plate to mix, then spin down.
8. Place the PCR reaction tubes in the thermocycler.
9. Start PCR reaction by selecting the correct program. Choose total volume of 50 µL. The thermocycler will perform the following cycles:

Cycle	Conditions
1	95°C for 10 min
50	95°C for 30 s
	55°C for 45 s
	72°C for 30 s
1	72°C for 7 min
1	4°C for ∞

10. After amplification is completed (approximately 2 h), store PCR products at 4°C.

Post-PCR Evaluation by Gel Electrophoresis

1. Please use standard laboratory protocol for analyzing 8 µL PCR product on a 2% agarose gel.
2. Run the gel at 115 V for 30–45 min or until bands in ladder are completely resolved.
3. After the gel electrophoresis is complete, take a picture and analyze it on the Geliance Gel Doc and obtain a printout.
4. Expected results are as follows: reagent control lanes should show no band. Expected band sizes are as follows: KRAS 12, 13 (131 bp); KRAS 61 (125 bp); NRAS 12, 13 (111 bp); and NRAS 61 (110 bp).
5. All PCR products should show single bright bands.
6. If any of the above criteria is not met, the test should not be continued. PCR reaction must be repeated for corresponding exon(s).

Pyrosequencing of the PCR Product

1. On the pyrosequencing workstation computer, open the PSQ 96 HS program.
2. Select “SNP Runs”; select “RAS”; right-click on “RAS 2008”; and select “New SNP Run.”
3. Set instrument parameters to “Capillary Tip Parameters Current Lot #.”
4. Highlight wells to be used on the plate diagram.
5. Select the “Setup” tab.
6. Select the “Entry” tab.
7. For each PCR reaction, select the entry for use in each well from the pull-down menu under the RAS folder.
8. Select the “Sample ID” tab, and enter the information for each sample for each well; also label the “positive” (PosCtrl), “negative” (NegCtrl), and “reagent” (RC) controls.
9. Select the “View” pull-down menu (center of the screen); select “Run” and record the volumes of enzymes, substrates, and highest nucleotide.
10. Save as SNP run.
11. Remove PCR tubes from the thermocycler and take to Pyro setup station (by vacuum tool).
12. Remove the sepharose bead solution and pyrosequencing reagents from the refrigerator and allow them to reach room temperature.
13. Mark a 96-well plate (this is the pyrosequencing setup plate) according to the pyrosequencing worksheet. Add 25 μL of Milli-Q water to each KRAS well and 25 μL Milli-Q water to each NRAS well.
14. Make sepharose bead mixture by combining 38 μL binding buffer with 2 μL bead solution for each sample and mix well.
15. Pipette 40 μL of sepharose bead mixture into each well and cover plate with rubber tray seal.
16. Pipette 15 μL of each KRAS PCR product into each KRAS well and 15 μL of each NRAS PCR product into each NRAS well of the 96-well plate with the water.
17. Place the tray on the thermomixer and mix at 1,000 RPM at room temperature for at least 10 min.
18. Pull apart the springs on the PSQ HS 96 Dispensing Tip Holder and insert the two PSQ HS Reagent Dispensing Tips (RDTs) and the four PSQ HS Nucleotide Dispensing Tips (NDTs).
19. Place the dispensing tip holder with the four CDTs closest to you.
20. Pipette the reagents in alphabetical order from left to right as follows:

RDTs: enzyme (left) and substrate (right)
CDTs: A (left), C, G, and T (right)
21. Place the filled cartridge in the pyrosequencer, again with the CDTs closest to you.
22. Next, perform the dispensation tip test under the Instrument button on the left-hand side of the screen. Use the designated empty plate for the test.
23. Obtain a pyrosequencing 96-well reaction plate (white), and place it on the vacuum prep station.
24. Make a primer master mix according to the table on the PCR worksheet. (Each reaction requires 0.8 μL of sequencing primer at K12, 13 and N61 at 5.0 mM and Kbl and N12, 13 at 7.5 mM and 15 μL of annealing buffer.)
25. Aliquot 15 μL of the primer master mix into the corresponding wells on the pyrosequencing 96-well reaction plate.
26. Fill the plastic tubs with the appropriate reagent: 70% ethanol, denaturation solution, 1 \times washing buffer, and water.
27. Sonicate the vacuum tool before and after each run.
28. When the 96-well pyro setup plate is finished mixing (for a minimum of 10 min), remove it from the thermomixer and place it atop the four probes on the vacuum prep tool workstation.
29. Close the vacuum switch (turn to “Off”) and start the vacuum pump.
30. Apply the vacuum to the vacuum prep tool by opening the vacuum switch (“On”).

31. Place the vacuum tool in the “parking position” and allow to run for 20 s.
32. Capture the beads containing immobilized templates on the filter probes by slowly lowering the vacuum prep tool into the PCR plate.
33. When all liquid is removed from the 96-well plate, place the vacuum tool in 70% ethanol or until liquid is no longer pulled up by tool, denaturation solution until liquid is no longer pulled up by tool, and 1× washing buffer until liquid is no longer pulled up by tool.
34. With the vacuum on, hold the vacuum tool at a vertical angle slightly over 90° to allow the liquid to drain.
35. Close the vacuum switch (“Off”), place the vacuum tool in the pyrosequencing reaction plate, and turn the vacuum pump off.
36. Shake the vacuum tool probes in the reaction plate gently for 10 s and then shake the probes in water before placing the vacuum tool in the holder upside down.
37. Place the pyrosequencing reaction plate on the dry bath at $80^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 min.
38. Remove the pyro plate from the dry bath and allow it to cool to room temperature for 5 min.
39. Place the reaction plate in the pyrosequencer and close the lid. Select “Run.”
40. When the run is finished, select “OK” and then open the run from the “SNP Runs” folder to analyze the results. Analyze selected samples under “SNP Mode.” Remove the cartridge, clean with Milli-Q water, and allow to air dry in the dispensing tip holder box.

18.5 Interpretation of Results

18.5.1 Interpretation of Sanger Sequence Traces

18.5.1.1 Primary Review for Sequence Quality

Results of all tests are first analyzed using the ABI Sequencing Analysis software. The analysis consists of viewing the raw data as well as the electropherogram to determine the signal quality of each reaction and to determine the beginning and end of the target sequence.

18.5.1.2 Sequence Analysis and Reporting

All sequences of the same type on a run (including controls) are imported into the SeqScape software (ABI), and the forward and reverse strands are assembled and aligned with the corresponding template. Instructions for this are included in the gene-specific protocols.

The use of sequencing templates assures that sequences outside the gene (e.g., primer tags) are not analyzed and allows quick localization of changes between the tested sample and the unmutated/reference sequence.

SeqScape has predefined and user-definable analysis settings, which are optimized during assay validation, to allow detection of mutations/base pair changes above the background of the assay. Such changes, as shown by complete replacement of a base (completely mutated and/or homozygous change) or double peaks varying in intensity (mixed mutated and/or heterozygous change), are indicated in SeqScape by a black dot above the base if both F- and R-complement sequences align. Red dots indicate that the nucleotide has been edited in that area. Red dots frequently represent sequencing artifacts but may represent a low-level mutation. A summary of such automatically marked alteration and the effect (if any) on the coding sequence are included in a feature table:

1. Automatically detected mutations that are confirmed by manual review (clear double peaks at the same position on F- and R-complement sequences) are highlighted or underlined in the assembled chromatogram (Fig. 18.1), and a magnified image of that mutation is shown.

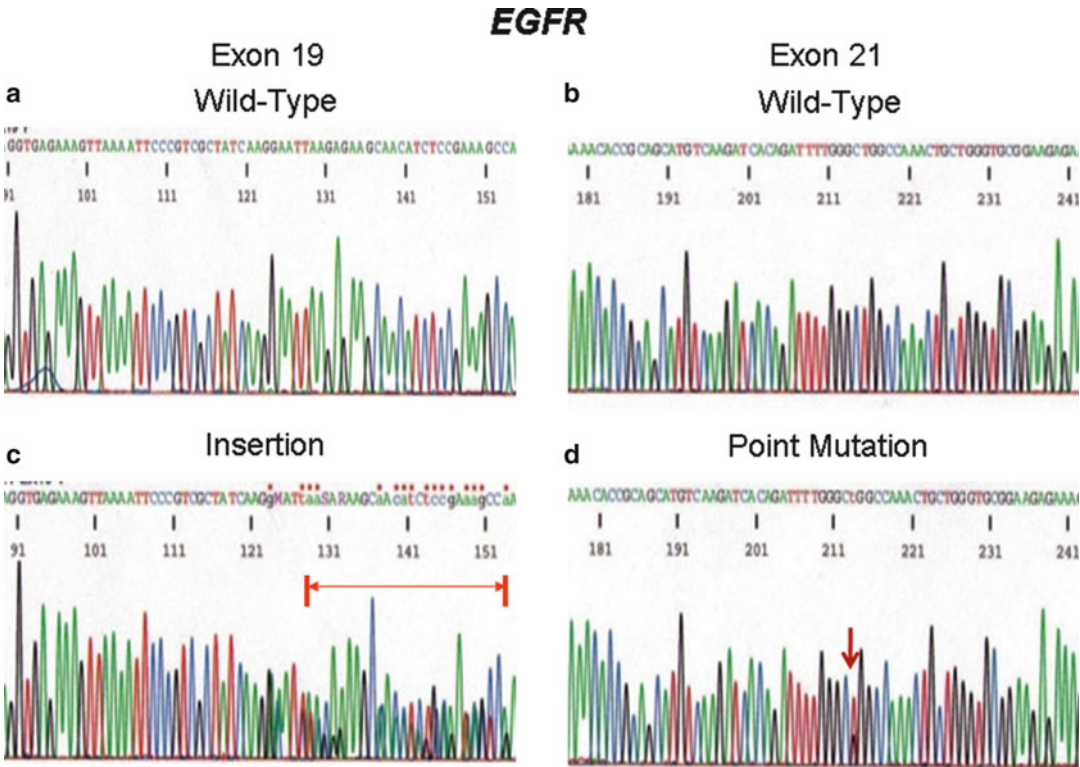


Fig. 18.1 Sanger sequencing for EGFR exons 19 and 21. Wild-type exons 19 (a) and 21 (b) show uniform single peaks with minimal background noise. A 15-base pair insertion in exon 19 is seen as a series of double peaks (c). A point mutation in exon 21 is seen as a double peak at only one base (d). Only the forward strand is shown for each sequence

2. The forward and reverse strands are then scanned by sight looking for insertions or deletions (indels) and low-level point mutations that were not detected by the automated analysis. Indels are characterized by a run of clean sequence on the F strand followed by double peaks and a nonoverlapping similar change on the R-complement sequence (Fig. 18.1). The position on the forward strand where double peaks appear is the front end of the deletions or the limit of the duplicated sequence.

18.5.1.3 Quality Control

Sanger sequencing is a qualitative method for determining the presence of a base pair change or mutation. The frequency and type of controls run is determined by the volume and exon distribution for each assays. In general, controls include the following:

- Negative control is a previously analyzed cell line or patient sample that is negative for the mutations in the codon or exon of interest.
- Reagent control is a PCR reaction containing all components, except the DNA template.
- Sensitivity standards are positive control diluted between 1:2 and 1:5 into a negative control. This can be done using cell lines, or proficiency samples, when appropriate.

18.5.1.4 Analytical Interpretation

The identical insertion or deletion or point mutation must be present in both the forward and reverse sequencing reaction. If there is an equivocal mutation present, the sequencing will be repeated from the PCR product.

18.5.1.5 Examples of Interpretative Comments in Clinical Reports

Wild Type:

EGFR mutation analysis:

No mutation detected in exons 18–21 of the *EGFR* gene.

Point Mutation

EGFR mutation analysis:

Mutation detected in codon 858 (c.2573T>G; CTG to CGG) in exon 21 of the *EGFR* gene that would change the encoding amino acid from leucine to arginine (p.Leu858Arg, p.L858R).

Insertion/Deletion

EGFR mutation analysis:

An alteration is identified in exon 19 of the *EGFR* gene that is consistent with a 15-base pair deletion.

Methodology and Disclaimer (common to all EGFR interpretations)

Methodology: PCR-based DNA sequencing analysis was performed. The analysis was limited to exons 18–21 of the kinase domain of the epidermal growth factor receptor (*EGFR*) gene. The presence of mutations outside the tested exons cannot be excluded. The lower limit of detection is approximately one cell bearing the mutation per five cells (20%).

ASR (Analyte Specific Reagent) Disclaimer: This test was developed and its performance characteristics determined by the Molecular Diagnostic Laboratory (MDL) at M.D. Anderson Cancer Center. It has not been cleared or approved by the U.S. Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary.

18.5.2 Interpretation of Pyrosequencing

18.5.2.1 Interpretation of the Pyrogram

Each test sample pyrogram is compared against the negative control for each sequencing reaction. If no differences in peak heights are observed, the sample is reported as negative for mutation. If there is a difference in a peak height, or an additional peak appears, the mutation is determined and reported.

Results will be in the form of a pyrogram, which is a graph of light intensity versus time. It is from these pyrograms that all mutations can be ascertained. Normal results will be indicated by a pyrogram showing the wild-type sequence with appropriate peaks and peak heights.

Mutations will be indicated as additional peaks to the wild type or alterations in the height of wild-type peaks with relation to a reference peak (Fig. 18.2). Reference peaks are wild-type peaks beside the target codons on each pyrogram.

The sensitivity of detection is approximately 10% mutation-containing cells present in the sample.

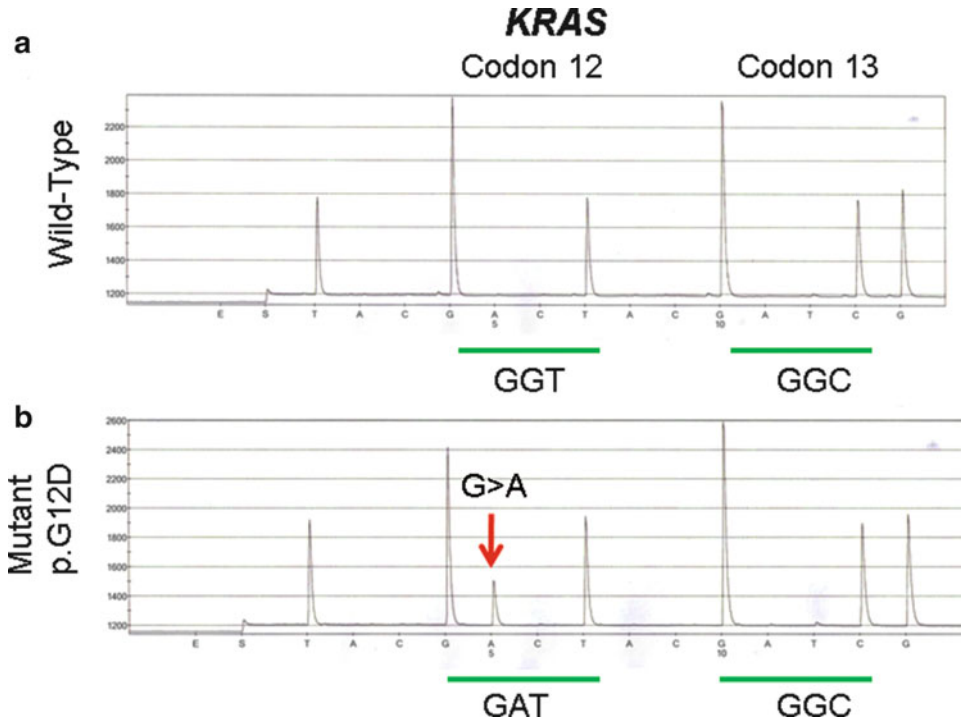


Fig. 18.2 Pyrograms for wild-type (a) and mutant (b) KRAS sequences for codons 12 and 13. Codon 12 in the mutant sample shows an additional peak at A and a reduction in peak height of G (b) corresponding to c.35G>A mutation (p.G12D)

18.5.2.2 Assay Parameters and Reporting Guidelines

Precision: $\pm 10\%$

Analytical sensitivity: Generally, analytical sensitivity is 1:16, based on dilution studies. Mutated peaks comprising at least 10% of the total PCR product are reportable. Lower levels need to be confirmed by other techniques (e.g., ASO-PCR).

Analytical specificity: Analytical specificity is 95–100%, based on comparison with the Sanger sequencing method. Discordant cases when low levels of signal are seen with mutated product, usually owing to high or noisy baseline or delayed or misaligned dispensation of nucleotide.

18.5.2.3 Examples of Interpretative Comments in Clinical Reports

Wild Type

KRAS Mutation Analysis:

No mutation detected in codons 12, 13, or 61 of the *KRAS* gene.

Mutant

KRAS Mutation Analysis:

Mutation detected in codon 12 (c.35G>A; GGT to GAT) of the *KRAS* gene that would change the encoding amino acid from glycine to aspartic acid (p.Gly12Asp, p.G12D).

Methodology and Disclaimer (common to both wild type and mutant)

Methodology: PCR-based DNA sequencing analysis was performed. The analysis was limited to codons 12, 13, and 61 of the *KRAS* gene. The presence of mutations outside the tested codons cannot be excluded. The lower limit of detection of this assay is approximately one cell bearing the mutation per ten cells (10%).

ASR Disclaimer: This test was developed and its performance characteristics determined by the Molecular Diagnostic Laboratory (MDL) at M.D. Anderson Cancer Center. It has not been cleared or approved by the U.S. Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary.

18.5.2.4 Quality Control

- Negative control is a previously analyzed cell line that is negative for the mutation.
- Positive control is a previously analyzed cell line or patient specimen that is positive for the mutation.
- No template control is a PCR reaction containing all components except DNA.
- Sensitivity standards are positive control diluted 1:2, 1:4, 1:8, and 1:16 into a negative control.

18.6 Limitations of the Procedures

18.6.1 Sanger Sequencing

The sensitivity of detection of an insertion, deletion, or point mutation by conventional Sanger sequencing is approximately one mutated cell in five to ten cells without the mutation. Therefore, samples in which the tumor is present in small numbers compared to nonneoplastic cells may give a false-negative result with this assay. Also, the presence of a mutation outside the tested codons cannot be excluded.

18.6.2 Pyrosequencing

During sequence analysis, if the background is too high or the peaks are too low, sequencing is repeated from bead binding and separation. The use of a sterile technique is imperative in the processing of the DNA sample for PCR. The concentrations of the dNTPs in the reaction mix must be kept balanced. If the concentration of any one dNTP is significantly different, the AmpliTaq Gold DNA polymerase will tend to misincorporate them. The reaction will slow down and terminate prematurely. Vortex well.

18.7 Troubleshooting

18.7.1 Sanger Sequencing

Since it is a complex multistep procedure, failure or suboptimal results can occur at many places.

18.7.1.1 When No Readable Sequence Is Obtained

When no readable sequence is obtained, the usual causes are suboptimal or failed injection during CE (remedied by reinjection of product) or absence of PCR product of appropriate size owing to inhibitors in the template, technical PCR failure, loss of product during purification, or, most commonly, lack of adequate template DNA/cDNA.

PCR failures should be handled by repeating the process from the beginning. Reextracting DNA (if >15 µg) to remove inhibitors or redoing cDNA or extracting other pellets for RNA extractions are the preferred methods.

For those cases with limited starting material, rerunning nested PCR with first-round PCR product can be done (where relevant), but this should be used only if no other option exists given the possibility of carryover PCR contamination.

18.7.1.2 When a Suboptimal Sequence Is Obtained

A suboptimal sequence can result from a low amplitude signal (poor injection, low amounts of PCR product template, or poor cycle sequencing), an overlapping sequence, or sequencing artifacts, which are discussed below.

If a single appropriately sized band is seen on the QC gel, the cycle-sequencing reaction should generally be repeated for that particular sample starting from the purified PCR product.

If the QC gel shows a faint band, the amount of PCR product used for cycle sequencing should be increased. Normally, 1.5–3 µL of DNA in the cycle-sequencing reaction is enough when the band is strong. For weak bands, up to 5 µL can be used. If a readable sequence is not obtained, the PCR reaction should be repeated from the original sample.

If multiple differently sized PCR products are seen, there will likely be overlapping sequences present. Repeat purification of the PCR product is recommended if small-molecular-weight PCR products are present. If the problem persists, it may be possible to report portions of the sequence.

18.7.1.3 When Forward and Reverse Strands Cannot Be Assembled

When forward and reverse strands cannot be assembled, it is usually due to poor sequence in one or both strands. Print and review of the chromatograms in CHROMAS to assess cause and manual matching of one strand to exclude mutation.

18.7.2 Pyrosequencing Troubleshooting

18.7.2.1 No Substrate Peak

If there is no substrate peak, one of the reagent tips has an air bubble or is clogged. Remove both tips, and rinse well with water. Allow the tips to soak completely submerged in water for 5 min. Dry the tips and repeat the current run including setup.

18.7.2.2 Out of Phase Peaks

Out of phase peaks are caused by a droplet forming on one of the tips and falling into an adjacent well during robot maneuvering. If this is near a base of interest, repeat the sample; if not, disregard.

18.7.2.3 Reagent Control Shows Peaks for an Individual Nucleotide

If the nucleotide tube, tip, or both are contaminated with PPI, replace the nucleotide tube and tip and repeat the run.

18.7.2.4 Low Signal

A low signal can be caused by many factors. Check the pH and expiration date of all reagents, perform a dispensation tip test (all droplets should be of equal size), make sure the thermomixer was shaking during incubation, check the functionality of the filter probes, and check the QC gel for the product, then repeat the run.

18.7.2.5 Vacuum Prep Tool Not Aspirating

If you know which well(s) is not functioning, replace that filter probe. If not, fill each well in a 96-well plate with water and aspirate for 10 s. Replace the filter probes for each well that still contains liquid.

18.7.2.6 Miscellaneous

If peaks are preceded or followed by a shadow or if degradation of the dye of the same color occurs, it is usually due to polymer problems.

If focal areas are of very high signal, often including all four fluorochromes, it is usually due to “dye blobs” or aggregations of unincorporated nucleotides due to suboptimal purification of cycle sequencing. Other causes include injection problems, failure to fully denature products, or poor formamide, if used.

If misalignment or misidentification of peaks occurs throughout, often a corrupt Matrix file or instrument misalignment is to blame.

18.8 Summary

In summary, mutation analysis in cancer provides a powerful tool for research and clinical diagnostics. The scope and limitation of mutation detection platforms have to be carefully considered before implementation. This chapter provides detailed protocols for *EGFR* and *KRAS/NRAS* mutation analysis and references to selected published protocols for a variety of other genes.

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Chapter 19

Minimal Residual Disease

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Keywords Minimal residual disease • Leukemia • Lymphoma • Real-time quantitative PCR • Acute promyelocytic leukemia

19.1 Introduction

Ongoing advances have tremendously increased our understanding of neoplastic disease at the molecular genetic level. As a result, in addition to clinical and morphologic features, many neoplastic diseases are now also characterized by specific and sometimes unique underlying molecular genetic events that are involved in tumorigenesis [1, 2]. This has led to the development of novel diagnostic approaches using more sensitive immunophenotypic and molecular genetic techniques that are able to detect and characterize exponentially lower numbers of neoplastic cells as compared to the traditional method of routine light microscopy (Table 19.1). In addition, the concept of clonality – that a neoplasm represents an abnormal clonal proliferation of cells – has also formed the basis for the use of these advanced techniques to detect and monitor low levels of disease.

19.2 Rationale for Minimal Residual Disease Monitoring

The term minimal residual disease (MRD) is used to describe the presence of low numbers of tumor cells that are only detected by laboratory techniques more sensitive than routine light microscopy. With the use of more effective therapeutic strategies, a significant number of patients with cancer today may achieve clinical remission after frontline therapy. Yet still, a considerable proportion of these patients sooner or later relapse with disease. Although the cause of eventual clinical relapse is likely to be multifactorial, it is now well established that the presence of MRD plays a significant role in the occurrence of relapsed malignant disease [3]. Furthermore, the presence of MRD has been shown to be predictive of eventual poor clinical outcome in a number of malignancies [4]. Thus, early detection of relapsed or persistent disease may allow for identification and stratification of patients

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Table 19.1 Approximate analytical sensitivities of selected methods used for monitoring MRD

Method	Analytical sensitivity ^a
Light microscopy (morphology)	5% (1 in 20 cells)
Immunohistochemistry	0.01% (1 in 10 ⁴ cells)
Immunophenotyping by flow cytometry	0.001% (1 in 10 ⁵ cells)
Conventional cytogenetics (band karyotyping)	5% (1 in 20 cells)
Cytogenetics by fluorescence in situ hybridization (FISH)	1% (1 in 100 cells)
Southern blot	1% (1 in 100 cells)
Real time qPCR	0.0001% (1 in 10 ⁶ cells)
Dye terminator (Sanger) sequencing	20% (1 in 5 cells)
Nested PCR	0.001% (1 in 10 ⁵ cells)

^aThe sensitivity of any particular method may vary due to the intrinsic idiosyncrasies of that particular tests (e.g., some commercial antibodies used for immunophenotyping are more robust and therefore more sensitive than others) and the quality of the specimen analyzed (e.g., the number of viable cells present)

into appropriate risk groups for which specifically tailored therapeutic strategies can be utilized [5, 6]. Consequently, patients at low risk for relapse (i.e., without detectable MRD at established time intervals) may be spared excessive therapy, thus minimizing the potential for harmful side effects, while on the other hand, early modification of treatment (for example, bone marrow transplant) may significantly increase the chances of improved clinical outcomes in those high risk patients with persistent MRD.

19.3 Molecular Testing for Minimal Residual Disease

Considering the current standard of care in clinical and laboratory practice, a reliable technique to detect MRD should show the following characteristics: (1) analytical sensitivity of at least 10⁴ (one tumor cell in 10,000); (2) specificity, that is, the ability of the test to distinguish between tumor and normal cells; (3) quantifiability across a wide analytic range; (4) stability of the measured target over a prolonged period of time; (5) reproducibility between laboratories; (6) adequate standardization and quality control checks; and (7) rapid availability of results (adapted from Cazzaniga et al. Br J Haematol. 2011) [7]. A number of different modalities may be used to test for MRD, including immunohistochemistry, flow cytometric immunophenotyping, cytogenetic studies (conventional band karyotyping and fluorescence in situ hybridization), and various molecular methods (see Table 19.2). Real-time quantitative polymerase chain reaction (qPCR) is usually the molecular method of choice for monitoring MRD, since it has the greatest analytical sensitivity (i.e., the smallest amount of tumor in a sample that can accurately be measured by an assay) and enables accurate quantitation of PCR products during the exponential phase of amplification. qPCR is also generally less labor intensive than some other methods like band karyotyping and FISH. Additionally, both DNA and mRNA targets are amenable to detection and quantitation by qPCR. For mRNA, cDNA is synthesized by reverse transcription (RT) of extracted RNA, amplified and analyzed (RT-qPCR). Other methods can be used to detect and identify small amounts of DNA/RNA targets after PCR amplification, such as amplicon fragment size analysis using gel or capillary electrophoresis, or DNA sequencing, usually by pyrosequencing or dye-terminator (Sanger) sequencing (see Table 19.1).

19.4 Clinical Applications of Minimal Residual Disease Testing

The concept of minimal residual disease (MRD) was first introduced in the context of low levels of remaining disease after induction chemotherapy for acute leukemia. Over the years, the main focus in this setting has been on monitoring the reduction kinetics of the leukemic clones and early detection of reemerging clones [8]. An ever increasing number of other hematologic neoplasms are now

Table 19.2 Selected list of minimal residual disease testing performed at MD Anderson Cancer Center

Molecular genetic abnormality	Disease	Method
t(8;21)(q22;q22), <i>RUNX1-RUNX1T1</i>	AML	RT-qPCR
t(15;17)(q22;q21), <i>PML-RARA</i>	AML	RT-qPCR
inv(16)(p13.1;q22) or t(16;16)(p13.1;q22), <i>CBFB-MYH11</i>	AML	RT-qPCR
t(9;11)(p22;q23), <i>MLL3-MLL</i>	AML	RT-qPCR
t(6;9)(p23;q34), <i>DEK-NUP214</i>	AML	RT-qPCR
inv(3)(q21q26.2) or t(3;3)(q21;q26.2), <i>RPN1-EVI1</i>	AML	RT-qPCR
t(1;22)(p13;q13), <i>RBM15-MKLI</i>	AML	RT-qPCR
<i>NPM1</i> exon 12 mutations	AML	PCR and capillary electrophoresis
<i>FLT3</i> internal tandem duplication and D835 mutations	AML	PCR and capillary electrophoresis
<i>CEBPA</i> mutations	AML	PCR and Sanger sequencing
<i>IDH1</i> and <i>IDH2</i>	AML	PCR and Sanger sequencing
<i>FIP1L1-PDGFR</i>	Myeloid neoplasms with eosinophilia	Nested RT-PCR and capillary electrophoresis
<i>KIT</i> D816V mutation	Mast cell disease	PCR and Sanger sequencing
<i>JAK2</i> D617F mutation	Myeloproliferative neoplasms:	PCR and pyrosequencing
	– Polycythemia vera	
	– Primary myelofibrosis	
	– Essential thrombocythemia	
t(9;22)(q34;q11.2), <i>BCR-ABL1</i>	– Chronic myeloid leukemia	Multiplex RT-qPCR and capillary electrophoresis
	– Precursor B-lymphoblastic leukemia	
t(14;18)(q32;q21), <i>IGH-BCL2</i>	Follicular lymphoma	RT-qPCR
t(11;14)(q13;q32), <i>IGH-CCND1</i>	Mantle cell lymphoma	RT-qPCR
Consensus immunoglobulin heavy chain gene rearrangement (B-cell clonality)	– Chronic lymphocytic leukemia/small lymphocytic lymphoma	Multiplex PCR and capillary electrophoresis
	– Precursor B-lymphoblastic leukemia	
	– Other mature non-Hodgkin B-cell lymphomas	
<i>T-cell receptor gamma</i> gene rearrangement (T-cell clonality)	– Mature T-cell lymphomas	Multiplex PCR and capillary electrophoresis
	– Precursor T-lymphoblastic leukemia	
<i>T-cell receptor beta</i> gene rearrangement (T-cell clonality)	– Mature T-cell lymphomas	Multiplex PCR and capillary electrophoresis
	– Precursor T-lymphoblastic leukemia	

routinely monitored for MRD using molecular techniques. Table 19.2 provides a list of hematologic neoplasms for which molecular MRD testing is currently performed at MD Anderson Cancer Center. Two basic approaches can be noted: (1) the detection of recurrent molecular genetic abnormalities associated with specific disease entities and (2) demonstration of clonality of lymphoid neoplasms by analyzing T- and B-cell receptor gene rearrangements. Although this is an extensive list, it should be noted that these approaches can potentially be applied to any myeloid or lymphoid neoplasm that harbor similar measurable molecular targets.

19.4.1 Acute Myeloid Leukemia

Three main types of molecular targets are used for MRD monitoring in AML: fusion gene transcripts, small-scale gene mutations (point mutations, insertions, and deletions), and aberrantly overexpressed genes [8]. Fusion gene transcripts that result from disease-specific recurrent chromosomal translocations are the most commonly used targets for monitoring MRD (see Table 19.2). Most protocols are

based on testing of bone marrow samples; however, comparable sensitivities have been shown to be achievable using peripheral blood for some of the most common fusion genes in AML, such as *PML-RARA*, *AML1-ETO*, and *CBFB-MYH11* [9]. Monitoring of *PML-RARA* in AML with t(15;17)(q22;q21), also known as acute promyelocytic leukemia (APL), is the best studied fusion gene target for MRD testing in AML, and unlike other fusion gene markers, its clinical utility has been validated by large prospective trials [6].

Although APL is a relatively rare disease (about 5–8% of all AML), it is the most notorious form of AML because of the potentially dramatic occurrence of sudden death due to disseminated intravascular coagulation (DIC). Paradoxically though, APL has the best survival rate of all AML subtypes (>70%) when treated promptly and appropriately (i.e., a therapeutic regimen that includes all-*trans* retinoic acid) [6]. Molecular remission as measured by RT-qPCR, at completion of therapy is considered a major therapeutic milestone. On the other hand, the presence of persistent MRD at the end of the final course of chemotherapy is highly predictive of relapse, and could potentially be prevented by early regimen adjustments, such as bone marrow transplant [6]. Additionally, because the post-remission disease relapse kinetics of APL can be rapid, frequent monitoring of MRD in a patient with complete therapeutic response could provide the opportunity for successful preemptive therapy that may prevent frank relapse [10, 11]. Current recommendations advocate bone marrow testing every 3 months for 3 years after treatment, which is the period when most relapses occur [6]. Several commercial RT-qPCR-based kits for detection and quantification of *PML-RARA* are available, including, among others, Roche Diagnostics LightCycler® t(15;17) quantification kit and InVivoScribe Technologies PML/RARa t(15;17) translocation assays [12, 13]. The protocol for molecular testing of *PML-RARA* using Ipsogen FusionQuant® kits based on standardized EAC (Europe Against Cancer) RT-qPCR procedures is currently utilized at MD Anderson Cancer Center and is included at the end of this chapter [14]. Other than *PML-RARA*, the kinetics of at least two other fusion gene transcripts, *RUNX1-RUNX1T1* and *CBFB-MYH11*, have also been shown to correlate with risk of relapse and/or to be independently associated with prognosis in AML [15].

Examples of small-scale mutations include, *NPM1* and/or *FLT3* mutations which are present in about 40% of AML with normal karyotype and have emerged as valuable targets for monitoring MRD in these patients [16, 17]. *NPM1* mutation is usually associated with a normal karyotype, but may also occur concurrently with recurrent translocations in about 10% of cases. *NPM1* mutations typically are insertions/deletions in the coding region of the terminal exon (exon 12) at positions 956 through 971, and are usually associated with a good prognosis [18]. Two types of *FLT3* mutations are described in AML – internal tandem duplications (FLT3-ITD) (75–80%) and missense mutations involving the tyrosine kinase domain (TKD) (20–35%) – and are both associated with a poor prognosis [19, 20].

Finally, overexpressed genes in cytogenetically normal AML cases that may serve as targets for MRD monitoring include *WT1* and *EVI*. It has been duly noted, however, that the sensitivity of assays for this class of markers would be no be able to surpass 1:10,000 because of the basic physiological level of expression of the gens found in the background of non-neoplastic cells [8].

19.4.2 Chronic Myeloid Leukemia, *BCR-ABL1* Positive

One of the most well-known diseases on which MRD monitoring has made a significant impact is chronic myeloid leukemia, *BCR-ABL1* positive (CML). In fact, CML serves, in many ways, as a prototype of the ideal setting for MRD monitoring i.e., a disease caused by a single, measurable molecular genetic abnormality (*BCR-ABL1* fusion gene) that is directly targetable by a frontline therapeutic agent (the tyrosine kinase inhibitor, imatinib), which is administered over a prolonged time period (months to years) [21]. Moreover, the fact that a proportion of CML patients invariably develop resistance to imatinib, along with the emergence of second- and third-generation tyrosine kinase inhibitors that can overcome this hindrance, further increases the value of MRD monitoring in these patients

[22]. Long-term follow-up of CML patients involves monitoring response to therapy using clinical, hematologic (morphology), cytogenetic, and molecular criteria [23]. Current guidelines advocate molecular monitoring by RT-qPCR of *BCR-ABL1* transcripts in peripheral blood of CML patients every 3 months indefinitely [23, 24]. Complete molecular remission, defined as undetectable *BCR-ABL1* by RT-qPCR, is the ultimate goal, but is relatively rarely achieved. However, patients who achieve a major molecular response (≥ 3 log reduction of *BCR-ABL1* mRNA transcript) also have a significantly low probability of disease progression [25].

19.4.3 Acute Lymphoblastic Leukemia/Lymphoma

The fact that bone marrow samples in morphologic remission could still contain measurable numbers of leukemic cells (i.e., MRD) was first demonstrated in 1981 by Bradstock et al. [26]. Since then, MRD testing has become an integral part of the management of acute lymphoblastic leukemia (ALL) [27]. Indeed, several studies have shown that the level of MRD at the end of induction and after consolidation therapy is a powerful indicator of prognosis in patients with ALL [28].

Two types of molecular genetic signatures are targeted for monitoring MRD in precursor B- and T-lymphoblastic leukemia (B- and T-ALL): fusion gene transcripts (only applicable in ~40% of ALLs) and *immunoglobulin (Ig)* and *T-cell receptor (TCR)* gene rearrangements (applicable in about 95% of ALL) [7]. Currently, the fusion gene most widely used for qPCR detection of MRD in ALL is *BCR-ABL1* in Philadelphia chromosome–positive (Ph+) B-ALL. Other potential markers yet to be widely established include *MLL-AFF1*, *TCF3-PBX1*, and *ETV6-RUNX1*, and gene fusions involving *CRLF2* (such as *IGH@-CRLF2* and *P2RY8-CRLF2*) [29].

During B-cell development, the V, D, and J segments of the *immunoglobulin (Ig)* genes are rearranged, including deletion and random insertion of nucleotides, eventually generating a unique gene sequence for each cell and its progeny. A similar process occurs in the genes encoding the components of the T-cell receptor (TCR). Normally, the population of B or T cells at any given body site shows a Gaussian distribution of rearranged *Ig* or *TCR* gene sequences respectively. However, since ALL cells purportedly arise from the oncogenic transformation of a single lymphoid precursor, all the cells of the neoplasm harbor virtually the same (clonal) *Ig* and/or *TCR* gene rearrangement sequence. This can be used to distinguish ALL tumor cells from benign lymphoid cells or nonlymphoid cells lacking antigen receptor rearrangements. As expected, *Ig* gene rearrangements are present predominantly in B-ALL as are *TCR* gene rearrangements in T-ALL. However, because ALL cells maintain recombination activity, *TCR* gene rearrangements can also be found in up to 90% of B-lineage ALL cases, and *Ig* gene rearrangements in about 20% of T-ALL due to lineage infidelity. This understanding of the expected clonal *Ig* and *TCR* gene rearrangement profiles of B- and T-ALL cells allows for two basic approaches to detection of MRD in these neoplasms. In the first, *Ig* and *TCR* gene rearrangements can be used as universal PCR targets to detect the presence of a small clonal population of leukemic cells in a background of normal blood cells. In another strategy, once the sequence of the rearranged region is known (by analyzing the first diagnostic specimen), allele-specific oligonucleotide (ASO) primers complementary to and specific for the V-D-J junctional sequence of a given neoplasm can be designed as a target for follow-up monitoring of individual patients.

19.4.4 Lymphoma

As with precursor lymphoid neoplasms (ALL), two basic strategies can be used to monitor MRD in mature non-Hodgkin lymphoid neoplasms: analysis of *Ig* or *TCR* gene rearrangements to demonstrate the presence of clonal neoplastic cells, and detection of a specific disease-associated recurrent molecular genetic aberration. PCR monitoring of MRD is well established for B-cell non-Hodgkin

lymphomas, but is rarely used in T-cell lymphomas [30, 31]. Additionally, unlike the frequent lineage infidelity seen in ALL, *Ig* and *TCR* gene rearrangements are usually restricted to the B-cell and T-cell lineages respectively in mature lymphoid neoplasms, and can therefore be used in a more targeted fashion to detect MRD according to the known lineage of the original tumor [31].

RT-qPCR monitoring of B-cell lymphoma using specific fusion genes is the second strategy frequently used for MRD testing in lymphoma. This approach has been largely restricted to follicular lymphoma (FL) and mantle cell lymphoma (MCL), which are associated with well-recognized chromosomal translocations. The most frequent primary chromosomal aberration in human B-cell lymphomas is the t(14;18)(q32;q21), *IGH-BCL2*, which has been detected by cytogenetics in about 90% of patients with follicular lymphoma and 20% of patients with diffuse large cell lymphoma [32]. By qPCR analysis, t(14;18)-positive cells have not only been identified in the bone marrow of most patients who present with advanced disease, but also in patients in clinical remission after chemotherapy, without any morphological evidence of bone marrow infiltration. Indeed, *IGH-BCL2* RT-qPCR status has been established as a reliable marker of residual disease, and many clinical trials have confirmed the predictive impact of the achievement of complete molecular remission on survival [33].

MCL is characterized by t(11;14)(q13;q32), *IGH-CCND1*, which is present in virtually 100% of cases. Clonal *IgH* gene (*VH-JH*) rearrangements as well as the t(11;14) abnormality are suitable targets for molecular assessment of MRD in MCL. Achievement of molecular remission (MR), defined as achievement of qPCR negativity after allogeneic stem cell transplant, has been shown to be of prognostic significance for progression-free survival and overall survival in patients with MCL [34, 35].

19.4.5 Solid Tumors

Unlike hematologic neoplasms, MRD monitoring in solid tumors (carcinoma, sarcoma, etc.) is still predominantly in its investigational stages. Nonetheless, it is well known that the large majority of delayed cancer-related deaths in patients with solid tumors are due to metastatic disease, and this has been linked to the persistence of low levels of cancer cells after local and/or systemic therapy [36, 37]. These cells may be present locally at the primary tumor site as cancer stem cells for example, in the peripheral blood as circulating tumor cells, or in distant organs as disseminated tumor cells (so-called micrometastases) [38]. Detection of these sub-microscopic residual tumor cells may enable risk stratification as well as optimization and potential individualization of therapeutic strategies that may lead to improved clinical outcomes in these patients [39, 40].

Of the solid tumors, molecular monitoring of MRD is currently well established only for neuroblastoma [41]. The most recent international recommendations by the International Neuroblastoma Risk Group Task Force advocate using RT-qPCR to target tumor-specific or tumor-associated mRNA for analysis of MRD in bone marrow and peripheral blood at the end of induction therapy (before transplantation), before therapy for MRD, and at the end of therapy [41]. The most widely used target is tyrosine hydroxylase. TH is the first hormone in the catecholamine synthesis pathway, and is present in neuroblastoma cells, but is also expressed by normal bone marrow or blood components at lower levels.

Other tumors in which MRD monitoring using RT-qPCR has shown some potential for clinical application include those associated with recurrent cytogenetic translocations such as the Ewing sarcoma family of tumors (translocations involving the *EWS* gene on chromosome 22q12), alveolar rhabdomyosarcoma (involving *PAX3-FKHR* or *PAX7-FKHR* fusion genes), and desmoplastic small round cell tumor (*EWS-WTI*) [42]. Other tumors currently being investigated include lung cancer, breast cancer (HER2 amplification), colon cancer (CEA, cytokeratin 20, guanylyl cyclase), and prostate cancer (androgen receptor, PTEN, EGFR) [41–47].

19.5 Protocol for Detection of t(15;17) – Associated *PML-RARA* Transcripts by RT-QPCR Using Ipsogen FusionQuant Kits

19.5.1 Principle and Methodology

Two predominant types of *PML-RARA* mRNA transcripts are observed in patients with AML with t(15;17), *PML-RARA* (APL) (Fig. 19.1). *RARA* breakpoints on chromosome 17 always occur at intron 2. However, depending on the breakpoint site within *PML* (intron 6, exon 6, or intron 3), three subtypes of *PML-RARA* transcript of varying sizes may be formed – long form (L or bcr1) due to *PML* breakage at intron 6, a variant long form (V or bcr2) due to *PML* breakage at exon 6, and short form (S or bcr3) due to breakage at exon 3 [14]. They represent 55%, 5%, and 40% of cases, respectively. Only bcr1 and bcr3 are assessed in this protocol.

In this assay, extracted RNA is subjected to two separate RT-qPCR procedures to detect the two types of *PML/RARA* fusion transcripts (long and short isoforms) (Fig. 19.2). Additionally, amplification of the *ABL1* gene is performed as a control for sample RNA quality and as a reference for relative quantification. If the *PML/RARA* t(15;17) translocation is detected, the result is considered positive and the ratio of amplified *PML/RARA* fusion transcript to the control *ABL1* transcript is reported.

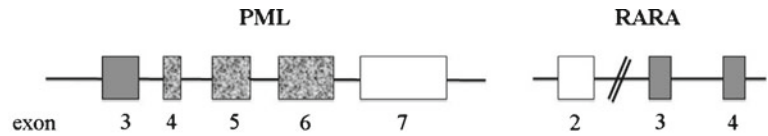


Fig. 19.1 Partial gene maps of *PML* (chromosome 15q24.1, 9 coding exons) and *RARA* (chromosome 17q12, 9 coding exons)

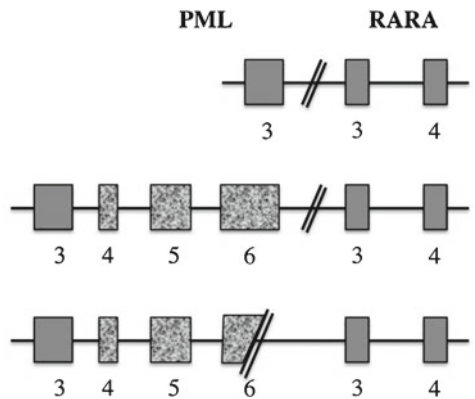
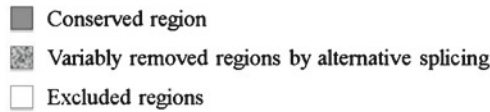


Fig. 19.2 Alternatively spliced long and short forms of *PML-RARA* transcripts. Note that intronic regions (-) are removed during transcription

19.5.2 *Materials*

19.5.2.1 Reagents

- FusionQuant PML-RARA bcr1 kit (Ipsogen, France)
- FusionQuant PML-RARA bcr3 kit (Ipsogen, France)
- Universal Master Mix with UNG
- Patient cDNA
- Positive and sensitivity control cDNA (UB4 and UF-1 cell lines)
- Negative control HL-60 cDNA

19.5.2.2 Primers and Probes

PML-RARA primers and probe

bcr1 forward primer (LF)

5'-TCTTCCTGCCCAACAGCAA-3'

bcr3 forward primer (SF)

5'-CCGATGGCTTCGACGAGTT-3'

PML-RARA reverse primer (common in both kits)

5'-GCTTGTAGATGCGGGGTAGAG-3'

PML-RARA TaqMan probe (common in both kits)

5' FAM-AGTGCCCAGCCCTCCCTCGC-TAMRA 3'

Endogenous ABL1 primers and probe

Forward

5'-TGGAGATAAACACTCTAAGCATAACTAAAGGT-3'

Reverse

5'-GATGTAGTTGCTTGGGACCCA-3'

Endogenous ABL1 TaqMan probe

5' FAM-CCATTTTGGTTTGGGCTTCACACCATT-TAMRA 3'

19.5.2.3 Supplies/Equipment

- 7900 HT Sequence Detection System
- 96-well optical plate
- Sterile 0.2-mL PCR tubes
- Sterile 1.5-mL microcentrifuge tubes
- Pre-PCR black tube rack
- PCR pipettes and filtered pipette tips
- Vortex mixer
- Quickspin microfuge
- PCR hood
- Autoclaved milli-Q water
- Gloves
- Cryocooler

19.5.3 Sample Requirements and Collection

Peripheral blood and/or bone marrow are processed for RNA extraction and purification followed by reverse transcription (RT). After reverse transcription, cDNA is used as a template for PCR using specific primers for t(15;17).

19.5.4 Controls Required for PCR

19.5.4.1 Reagent Control

This control consists of all PCR reagents except the cDNA template. The reagent control is used to assure that there is no contamination with positive or negative templates, which would lead to false results. Any bands seen in the reagent control lane brings into question whether clean PCR technique has been maintained.

19.5.4.2 Negative Control

The t(15;17) negative cell line, HL60, is used. The negative control is used to assure the specificity of the PCR reaction.

19.5.4.3 Positive Control

The positive control is used to assure the specificity of the PCR assay. The cell lines NB4, carrying the long-form fusion transcript, and UF-1, carrying the short-form fusion transcript, are used as the positive controls in this assay.

19.5.4.4 Sensitivity Control

Sensitivity controls comprise of *PML-RAR* α -positive cell line RNA diluted serially into negative cell line HL60 RNA. Thus, a series of ratios of positive cell line to negative cell line is obtained ranging from 1 in 10 to 1 in 100,000. Controls with the ratio of 1 in 10,000 and 1 in 100,000 are run with every assay.

19.5.5 Procedure

1. Remove PCR kits from freezer and allow the reagents to thaw
2. Briefly tap, vortex the thawed PCR reagents to mix
3. Label a 1.5-mL tube as PCR Master Mix for each master mix to be made
4. Prepare the PCR Master Mix according to the master mix worksheet, following the order listed in the worksheet

Reagents	Starting concentration	Working concentration	Vol	Rxns – 36 (μ L)
Universal Master Mix	2 \times	1 \times	12.2	450
Ipsogen primers and probes	25 \times	1 \times	1	36
H ₂ O	–	–	6.5	234

Reagents	Starting concentration	Working concentration	Vol	Rxns – 36 (μL)
Universal Master Mix	2×	1×	12.2	450
ABL Ipsogen primers and probes	25×	1×	1	36
H ₂ O	–	–	6.5	234

5. Briefly vortex and quick spin the master mix after all reagents have been added

Note: Bubbles generated from excessive vortexing will destroy the Taq polymerase. Mix gently.

6. Aliquot 20 μL of the master mix to the appropriate well of a 96-well optical plate
7. Carefully add 5 μL of the corresponding cDNA sample to each well. Total reaction volume at this point should be 25 μL per tube
8. Spin plate briefly and proceed to the 7900 Sequence Detection System
9. Login to computer connected to the 7900 and open Sequence Detection System software
10. Fill in the sample sheet with the accession# of each sample. Do the same for the standards and controls
11. Select the task as UNKNOWN for all the samples including the positive and negative controls. Select the task as STANDARD for all the standards and enter the quantity value represented by each standard. Select NTC for reagent control
12. Select the Thermal Profile tab and enter the following info:
 - 50°C 2 min
 - 95°C 10 min
 - 95°C 15 s
 - 60°C 1 min
 - Repeat the last two steps 45 times.
13. Set volume to 25 μL
14. Save run in designated folder of choice
15. Select Open/Close and place plate in tray
16. Select Open/Close again
17. Select Start
18. When run is finished, immediately remove plate and discard appropriately
19. Analyze data.

19.5.6 Result Reporting/Analytic Interpretation

19.5.6.1 Analyzing and Viewing 7900 Results

1. Select the ANALYSIS menu and select ANALYZE.
2. Click the Results tab to view amplification plot.
3. View the amplification plots of the standards of all three detectors (LF, SF, and ABL) together and adjust the threshold so that it lies at the start of the exponential phase of the curves but is still above the noise.
4. Print CT/Quantity data for each detector, print standard curve plot, print amplification plot for specific standards, print amplification plot for sensitivity controls and amplification plot for negative controls (HL60 and RC).

19.5.6.2 Manual Calculation of Transcript Quantity

The quantity of the sample is determined in this assay by running a set of standards of known quantity and measuring their threshold cycle (CT). Then a graph is plotted using this data with log10 concentration (quantity) of the standards on the X-axis and CT values on the Y-axis. The line of best fit is drawn through the points and slope and intercept calculated. By rearranging the equation of a straight line and substituting for the known variables, the quantity of the unknowns (patient samples) is calculated as follows:

Straight line equation: $Y = -MX + C$

Where $-M$ = slope

C = intercept

Y = CT

X = log quantity

Rearrange equation: $X = (C - Y)/M$

This gives the log quantity, therefore the value of X must be antilogged to obtain the value which is reported.

19.5.7 Limitations of Procedure

19.5.7.1 Minimizing Nonspecific Products

Once pre-PCR is set up, it is necessary to run the samples as soon as possible. If the master mix with added DNA is left at room temperature, it will result in many nonspecific bands.

19.5.7.2 Controlling Contamination

Sterile technique is critical because real-time PCR is a highly sensitive assay. If amplified product is detected in the reagent control at time of analysis, all PCR reagents should be discarded and the run should be repeated with fresh reagents.

19.5.7.3 Sensitivity

Efficiency of the assay is highly dependent on the quality of the sample. The sensitivity indicated by the positive controls represents an ideal sample. It might not represent the tested samples. Therefore, it is mandatory to have a coamplified internal control if the sensitivity control does not reveal the adequate expected sensitivity; the run may be repeated or the results may be reported with a caveat about reduced sensitivity. Samples with fewer than 10,000 copies of ABL amplified should be accompanied by a comment indicating that the low control amplification observed will limit the accuracy and/or sensitivity of PML-RARA fusion transcript detection. Samples with very low *ABL1* control amplification levels are regarded as inadequate for quantitation of a positive test or inadequate for reporting of a negative result.

19.5.7.4 Specificity

Probe breakdown or nonspecific probe binding may occasionally give low signal at a high number of cycles. No "positive" sample with a CT higher than that seen in negative the control should be reported as positive for that particular run.

19.6 Summary

In conclusion, the last seven or eight decades have witnessed extraordinary advances in technology that have resulted in equally significant progress in the understanding of the biology of neoplastic disease at the subcellular or molecular genetic level. The development of sensitive and qualitative molecular techniques to detect disease at levels far lower than what is possible by routine microscopy (i.e., MRD) is an important accomplishment in the management of patients with cancer. In particular, monitoring of MRD by quantitative PCR in hematopoietic neoplasms is now a vital tool for early risk stratification and individualized therapeutic modification in patients with selected diseases. While MRD testing in solid tumors is still largely in its early stages of development, it has the potential to also become a powerful tool that can significantly impact clinical outcomes in affected patients.

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Chapter 20

Pyrosequencing in Cancer

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Keywords Pyrosequencing • Sequencing • Multiplexing • DNA • RNA • Genotyping

20.1 Introduction

In 1970s, the development of DNA sequencing techniques revolutionized the field of molecular biology. Polymerase chain reaction (PCR)–based techniques for DNA sequence determination were invented both by Maxam-Gilbert [1] and Sanger [2]. Of all the conventional sequencing techniques, Sanger sequencing is by far most frequently used. The key principle of the Sanger method is the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. Dideoxy chain termination generates prematurely ended fragments, which are further analyzed with regard to their size by gel electrophoresis. This technique has the ability to determine unknown DNA sequences, i.e., de novo sequencing. The average read length obtained is typically between 500 and 1,000 bases. Thus, it has enabled whole genome sequencing of an increasing number of organisms including human. However, it is labor intensive and is further limited by low throughput and high costs. DNA sequencing technique presented by Maxam and Gilbert is based on sequencing by chemical cleavage and has a read length of up to 500 bp. Though it can also determine unknown DNA sequences, it is slow and involves use of hazardous chemicals which require special handling in the DNA cleavage reactions.

Today, when complete genome sequence references are widely available, analyzing variations among individual genomes is an area of intense investigation. Thus, when using sequence technology for identification of genetic variants, such as SNP genotyping, bacterial or virus typing, detection of specific mutations, gene identification in transcript analysis, etc., shorter read-length DNA sequencing is required. In such instances, faster sequence analysis methods with enhanced sensitivity and throughput

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are of utmost importance. This has led to the development of alternative principles of DNA sequencing. The three methods that hold great promise are sequencing by hybridization (SBH) [3–5], massively parallel signature sequencing (MPSS) based on ligation and cleavage [6], and pyrosequencing [7, 8].

Standard SBH platform consists of sets of oligonucleotides which are hybridized under conditions that allow detection of complementary sequences in the target DNA. These individually detected DNA sequences are then compared to a reference genome with appropriate algorithms to assemble the longest perfect matches. SBH is a sound resequencing approach for identifying sequence variants but can stagger when probing long sequences, tandem DNA repeats, or unexpected sequence insertions. Moreover, SBH has so far failed to sequence even viral genomes because DNA sequence recognition can be error prone when hybridization conditions are identical for all oligonucleotide probes.

MPSS was a bead-based platform that analyzed the level of gene expression in a sample by counting the number of individual mRNA molecules produced by each gene. Tagged PCR products produced from cDNA were amplified. Thus, each corresponding mRNA molecule gave ~100,000 of PCR products with a unique tag. Tags were used to attach the PCR products to microbeads. After several rounds of ligation-based sequence determination using the type II restriction endonuclease *BbvI*, a sequence signature of ~16–20 bp was identified from each bead. This was performed in parallel, and approximately 1,000,000 sequence signatures could be obtained per experiment. However, since this method utilized a complex approach of adapter ligation followed by adapter decoding and sequence read in increments of four nucleotides, it was susceptible to sequence-specific bias or loss of specific sequences. Due to its complex nature, this technology was eventually discontinued.

Due to their significant limitations, technologies like MPSS and SBH were displaced by sequencing-by-synthesis (SBS) based methods. SBS strategy involves use of DNA polymerase or ligase enzymes to synthesize complementary strands of many target DNA sequences in parallel. Nucleotides or short oligonucleotides are provided either one at a time or modified with identifying tags so that the base type of the incorporated nucleotide or oligonucleotide can be determined as extension proceeds. Pyrosequencing is a method of DNA sequencing based on the “sequencing by synthesis” principle. The technique was developed by Pål Nyrén and Mostafa Ronaghi at the Royal Institute of Technology in Stockholm in 1996 [7, 9].

20.2 Pyrosequencing

20.2.1 Methodology

Briefly, the process of pyrosequencing can be separated into different steps, namely, PCR amplification of target DNA, single-stranded DNA preparation, purification of single-stranded DNA template, and nucleotide analysis.

1. *In vitro amplification.* PCR amplification of target DNA sequence, often varying between 50 and 250 nucleotides, is performed in vitro to obtain template DNA for the pyrosequencing reaction. 5–40 μ L PCR products are currently needed depending on the pyrosequencing machine to be used.
2. *DNA template preparation.* Prior to pyrosequencing, PCR product should be purified to obtain DNA template that contains minimal amounts of excess primer, deoxynucleotide triphosphates (dNTPs), and salts, as these can interfere with the pyrosequencing reaction and result in high background signals. The DNA templates can be made both by solid-phase template preparation (streptavidin-coated magnetic beads) and enzymatic template preparation (apyrase+exonuclease) [10, 11]. They can be chosen depending on the individual laboratory requirements, such as speed, automation, and cost. Additionally, alkali denaturation of PCR products is performed to obtain ssDNA templates for primer extension and nucleotide analysis by pyrosequencing.

3. *Nucleotide analysis by pyrosequencing reaction.* The chemical process involved in pyrosequencing is also comprised of multiple separate stages. In summary, a sequencing primer is hybridized to the ssDNA template, which is then incubated with enzymes and substrates in a reaction mixture. DNA sequence is determined by adding individual nucleotides in a predetermined order and detecting subsequent light emission, catalyzed by a series of coupled enzymatic reactions. Data collected are shown as a collection of signal peaks in a pyrogram.

20.2.2 Principles

As mentioned previously, pyrosequencing is based on the principle of “sequencing by synthesis.” In this method, the activity of DNA polymerase is detected with another chemiluminescent enzyme. Essentially, the method allows sequencing of a single strand of DNA by synthesizing the complementary strand along it, one base pair at a time, and detecting which base was actually added at each step. The template DNA is immobile, and solutions of A, C, G, and T nucleotides are sequentially added and removed from the reaction. Light is produced only when the nucleotide complements the unpaired base of the template. The sequence of nucleotides which produce chemiluminescent signals allows the determination of the sequence of the template. These steps are described below in more detail:

- (i) Single-stranded, PCR-amplified DNA template is hybridized to a sequencing primer and incubated with the enzymes (DNA polymerase, ATP sulfurylase, luciferase, apyrase) and the substrates (adenosine 5' phosphosulfate [APS], luciferin).
- (ii) The four nucleotides, deoxynucleotide triphosphates (dNTPs), are added one at a time, iteratively, in a cyclic manner. DNA polymerase catalyzes the incorporation of the dNTP into the DNA strand if it is complementary to the base in the template strand. It should be noted that deoxyadenosine alfa-thio triphosphate (dATP α S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase but is not recognized by the luciferase.
- (iii) Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.
- (iv) ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate.
- (v) This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP.
- (vi) The light produced in the luciferase-catalyzed reaction is detected by a charge-coupled device (CCD) camera and recorded in form of peaks known as pyrogram. Each light signal is proportional to the number of nucleotides incorporated.
- (vii) Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete, another dNTP is added. Addition of dNTPs is performed one at a time.
- (viii) As the process continues, the complementary DNA strand is built up, and the nucleotide sequence is determined from the signal peak in the program.

This four-enzyme process is performed in a closed system in a single well. The reaction is performed in real-time, and the raw data are directly analyzed. The overall reaction from polymerization to light detection takes place within 3–4 s at room temperature.

One pmol of DNA in a pyrosequencing reaction yields 6×10^{11} ATP molecules which, in turn, generate more than 6×10^9 photons at a wavelength of 560 nm. Thus, the processing is simple and relatively fast (~20 min/96-well plate), and the cost of reaction is comparable to other medium-throughput technologies.

The nucleic acid molecule can be either DNA or RNA. However, since DNA polymerases demonstrate higher catalytic activity than RNA polymerases for limited nucleotide extension, efforts have been directed on the use of primed DNA templates for pyrosequencing.

At this point, it is important to note that based on the method of template preparation for pyrosequencing, there are two different pyrosequencing strategies that are currently available:

1. Solid-phase pyrosequencing: This method utilizes immobilized DNA in a three-enzyme system. The four different nucleotides are added stepwise to the immobilized primed DNA template, and the incorporation event is followed using the enzyme ATP sulfurylase and luciferase. However, after each nucleotide addition, a washing step is performed to remove the excess substrate and thus facilitating iterative addition of nucleotide [7].
2. Liquid-phase pyrosequencing: In this system, apyrase, a nucleotide-degrading enzyme, is introduced to make a four-enzyme system. Addition of this enzyme has eliminated the need for solid support and intermediate washing, thus enabling the pyrosequencing reaction to be performed in a single tube [8].

20.2.3 Specimen Types for Pyrosequencing

Pyrosequencing assay is applicable to almost any source of DNA or RNA, e.g., blood, saliva, cell line, plasma, serum, tissue, formalin-fixed and/or paraffin-embedded samples, and whole genome-amplified DNA.

20.2.4 Instrumentation

Two automated commercial versions of multichannel pyrosequencing instruments are currently available (www.pyrosequencing.com). These instruments utilize 24-well and 96-well plate formats. All versions use a disposable inkjet cartridge which contains reagents and each of the four dNTPs. Prior to pyrosequencing, the inkjet cartridge is mounted in the instrument for precise delivery of nucleotides into a temperature-controlled microtiter plate. The microtiter plate contains primed DNA template and is continuously agitated during the pyrosequencing reaction to increase the rate of enzymatic reactions. A lens array converges the light signal generated from each reaction well on the microtiter plate onto a CCD camera. Nucleotides are dispensed into alternating wells with a pulse delay to minimize cross talk of generated light between adjacent wells. A cooled CCD camera images the plate every second to follow the progress of pyrosequencing reactions. Data acquisition modules and an interface for PC connection are used in this instrument. Software running in a Windows 2000 Professional environment enables individual control of the dispensation order for each well. The signals in a pyrogram typically generate high-quality raw sequence data with excellent signal-to-noise ratio with peak height proportional to the number of incorporated nucleotides. These instruments, depending on their well capacity and the sequence to analyze, can analyze up to 96 samples in approximately 30 min, thus facilitating testing of approximately 500–5,000 samples per day. Future formats including array-based pyrosequencing and microfluidics reaction device are currently in development [9, 10].

20.2.5 Software for Program Analysis

Various software programs for SNP genotyping, allele quantification, tag sequencing, DNA methylation, and multiplex analysis are now available (www.pyrosequencing.com). All these software programs are based on pattern recognition. The assignment of quality values is based on a number of different parameters, including difference in match between the best and next best choice of genotypes, agreement between expected and obtained sequence around the analyzed nucleotide, signal-to-noise ratios, variance in peak heights of adjacent nucleotides, and peak width [9, 10].

20.2.6 Applications of Pyrosequencing

Pyrosequencing has been successful in both de novo sequencing and confirmatory DNA sequencing. Since it offers the same accuracy as conventional DNA sequencing for short reads while being more flexible and allowing processing of a large number of samples in parallel, it has rapidly found applications in DNA sequencing, genotyping, single nucleotide polymorphism (SNP) analysis, allele quantification, and whole genome sequencing within the areas of clinical genetics, pharmacogenetics, and microbiology. Pyrosequencing is the only method capable of generating fully quantitative data of allele content. Thus, it is most suitable for quantitative genetic studies, such as CpG methylation analysis, ploidy, pooling, and multisite variation. Recently, several publications have identified pyrosequencing technology as a breakthrough for DNA methylation analysis [12–15]. Additionally, since pyrosequencing makes sequence information rapidly accessible, clinical microbiological labs are using it for routine microbe species identification, epidemiological research, and antibiotic resistance studies. Finally, multiplexing ability of pyrosequencing enables rapid and accurate screening of a large number of samples relatively inexpensively.

20.2.7 Limitations of Pyrosequencing

- (i) De novo sequencing of polymorphic regions in heterozygous DNA material by pyrosequencing becomes a significant challenge when the polymorphism is a deletion or insertion of a kind which is different from the adjacent nucleotide on the DNA template. In such an instance, the sequencing reaction can become out of phase, making the interpretation of the subsequent sequence difficult.
- (ii) At present, pyrosequencing enables only relatively short reads. The lengths of individual reads of DNA sequence are in the neighborhood of 50–100 nucleotides, shorter than the 800–1,000 obtainable with chain termination methods (e.g., Sanger sequencing). This can make the process of genome assembly more difficult, particularly for sequences containing a large amount of repetitive DNA.
- (iii) Pyrosequencing technology also has difficulty in quantifying the homopolymeric repeats, due to nonlinear light response following incorporation of more than 5–6 identical nucleotides. Additionally, it is not able to differentiate nucleotide sequences when different alleles of multiple copy genes exist within the same genome.
- (iv) Finally, pyrosequencing requires specialized equipments and reagents. Although costs are relatively low due to small volume of reagents required, the reagents remain proprietary.

20.3 Pyrosequencing: Applications in Hotspot Mutation Analysis

Pyrosequencing is the fastest method for sequencing a PCR product. As described previously, since pyrosequencing generates an accurate quantification of mutated nucleotides, the resequencing of PCR-amplified disease genes for mutation scanning is one of its most important applications. Programmed dispensing of nucleotides generates a signal for each addition of nucleotide in a pyrogram. Thus, variation in the pyrogram pattern indicates the presence of a mutation. This strategy has been used by Garcia et al. for resequencing p53 tumor suppressor gene where mutations were successfully determined and quantified [16]. In our molecular diagnostic laboratory, we use pyrosequencing for detecting hotspot mutations in various genes such as JAK2, BRAF, MPL, and BCR-ABL. In the following section, we will describe a sequential pyrosequencing assay to assess for JAK2 V617F mutation.

20.3.1 Pyrosequencing Assay for JAK2 V617F Mutation Analysis

Janus kinase 2 (commonly called JAK2) is a human protein that belongs to the family of intracellular, nonreceptor tyrosine kinases, which transduce cytokine-mediated signals via the JAK-STAT pathway. An activating point mutation in JAK2 (V617F) has recently been identified in three different chronic myeloproliferative disorders (CMPD), namely, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). JAK2 V617F has constitutive tyrosine kinase activity and is able to transform hematopoietic cells and activates JAK-STAT signaling when co-expressed with homodimeric type I cytokine receptors. This mutation is present in almost all patients with PV and approximately half of those with ET and PMF. Thus, JAK2 V617F mutation analysis is indicated for diagnosis, exclusion, and follow-up of JAK2-mutated chronic myeloproliferative disorders.

20.3.2 Protocol and Procedure

20.3.2.1 Specimen

1–2 mL of bone marrow aspirate (BM) or 10 mL of peripheral blood (PB) samples should be collected in EDTA (purple top tube). Vacutainer tubes are delivered on cold packs as soon as possible and no later than 48 h after collection.

Specimens shipped in heparinized (green top) containers are not acceptable due to the inhibitory effect of heparin in the PCR reaction.

DNA is extracted by standard methods and quantified. DNA is stored in Tris-EDTA (TE) buffer at 4°C. DNA amount required for PCR testing is 0.2 µg per reaction.

20.3.2.2 Required Reagents

PCR Reagents

10× PCR buffer II (Applied Biosystems)

25 mM MgCl₂

10 mM dNTPs

Ampli Taq DNA polymerase (5 U/µL)

Nuclease-free water

10 µM PCR primers:

JAK2-F: 5'-TCT TTC TTT GAA GCA GCA AGT ATG AT-3'

JAK2-R-BIO: 5'-biotin-ACA AAA ACA GAT GCT CTG AGA AAG G-3'

Pyrosequencing Reagents

7.5 µM sequencing primer

Streptavidin-bound sepharose beads (Amersham Biosciences)

Denaturation solution (QIAGEN)

Washing buffer (QIAGEN)

Binding buffer (QIAGEN)

Annealing buffer (QIAGEN)

70% ethanol

PSQ Pyro Gold Reagents (QIAGEN):

- Enzyme mixture
- Substrate mixture
- dATP α S
- dCTP
- dGTP
- dTTP

20.3.2.3 Setup PCR Reaction, PCR Program

- (a) Stock DNA should be diluted in water to 100 ng/ μ L.
 (b) Prepare the JAK2 PCR master mix using the table below.

Reagent	μ L per reaction
Water	31.75
10 \times PCR buffer II	5.0
25 mM MgCl ₂	4.0
10 mM dNTP's mix	5.0
JAK2F primer 10 μ M	1.0
JAK2R-BIO primer 10 μ M	1.0
AmpliTaq 5 U/ μ L	0.25
Total volume	48 μ L

- (c) Aliquot 48 μ L of master mix to the appropriate PCR reaction tube.
 (d) Add 2 μ L of sample DNA (100 ng/ μ L) or the control DNA to the reaction tube.
 (e) Seal the reaction tube, vortex, and quick spin.
 (f) Amplify the JAK2 amplicon using the following cycling parameters.

Hold	95°C for 2 min
50 cycles	95°C for 30 s
	55°C for 30 s
	72°C for 30 s
Hold	72°C for 10 min
Hold	4°C

- (g) After 3 h, amplification is completed. Store PCR products at 4°C.
 (h) Run a gel of the PCR products using 2% agarose gel at 115 V for 30–40 min. Ensure that the bands of the ladder are completely resolved. The size of the JAK2 product should be around 140 bp using 3.5 μ L of BioMarker Low.

20.3.2.4 Pyrosequencing of PCR Product

<i>Caution:</i>	To avoid contamination, the remaining part of the procedure must be performed in post-PCR areas.
<i>Important:</i>	Turn on instrument PSQ 96 HS and heat block at least 90 min before run.

1. On the pyrosequencing workstation computer, open the PSQ 96 HS program and log in.
2. Select SNP Runs, right-click, and select New SNP Run.
3. Set instrument parameters to “Capillary Tip Parameters.”

4. Highlight wells to be used on plate diagram and select activate.
5. Select the Setup Tab.
6. Select the Entry Tab.
7. Select the JAK2 entry for each well in use from the pull-down menu.
8. Select the Well ID Tab and Enter the MDL number for each sample for each well and label the control wells as follows: Sensitivity Standard 1 (1:4 dilution), sensitivity standard 2 (1:16 dilution) Positive control, Negative control, Reagent control.
9. Select the View pull-down menu (center of the screen); select Run and record volumes of enzyme, substrate, and nucleotides.
10. Save SNP run.
11. Remove PCR tubes from thermocycler or refrigerator.
12. Remove Sepharose bead solution and pyrosequencing reagents from refrigerator and allow them to reach room temperature.
13. In a 96-well plate, add 25 μL of Milli-Q water to each well.
14. Make Sepharose bead mixture by combining 38 μL binding buffer with 2 μL bead solution per reaction and mix well.

<i>Important:</i>	Mix the bottle with Streptavidin Sepharose beads until a homogenous solution is obtained.
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15. Pipette 40 μL of Sepharose bead mixture into each well and cover plate with rubber tray seal.
16. Following the worksheet, pipette 15 μL of each PCR product. Total volume should add up to 80 μL .
17. Place tray on thermomixer and mix at 1,000 RPM at room temperature for at least 10 min.
18. Pull apart the springs on the PSQ HS 96 Capillary Dispensing Tip Holder and insert the two PSQ HS reagent dispensing tips (RDTs) and the four PSQ HS capillary dispensing tips (CDTs).
19. Place the Dispensing Tip Holder with the four CDTs (or NDTs) closest to you.

<i>Important:</i>	When using the CDTs, you must dilute nucleotides by 1:1 with water before use.
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20. Pipette the reagents in alphabetical order from left to right:

RDTs – enzyme (left) and substrate (right)

CDTs – A (left), C, G, T (right)

<i>Caution:</i>	Be sure to point the pipette tip toward the narrowest corner of the dispensing tip to avoid getting air bubbles in the solution.
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21. Place filled cartridge in Pyrosequencer, again with the CDTs closest to you.
22. Next, perform tip dispensing test under the Instrument button on the left-hand side of the screen. Use the designated empty plate for the test.
23. Make a primer master mix using the table on the PCR worksheet. (Each reaction requires 0.8 μL of sequencing primer at 7.5 μM and 15 μL of annealing buffer.)
24. Aliquot 15 μL of the primer master mix into the corresponding wells on the pyrosequencing 96-well reaction plate.
25. Fill plastic tubs with appropriate reagent: 70% ethanol, denaturation solution, 1 \times washing buffer, and water.
26. When the 96-well pyro setup plate is done mixing (at least 20 min), remove from thermomixer and place on top of the four probes on the Vacuum Prep Tool workstation.

<i>Note:</i>	Remember to sonicate Vacuum Prep Tool before and after each use.
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27. Close the vacuum switch (Off) and start the vacuum pump.
28. Apply vacuum to the Vacuum Prep Tool by opening the vacuum switch (On).
29. Place the Vacuum Tool in the “parking position” trough at the top of the workstation and allow it to run for 20 s.
30. Capture the beads containing immobilized templates on the filter probes by slowly lowering the Vacuum Prep Tool into the PCR plate.

Note: Sepharose beads sediment quickly, and capturing of beads must take place within 3 min of the agitation being terminated.

31. When all liquid is removed from the 96-well plate, place the Vacuum Tool in 70% ethanol until liquid is no longer pulled through the Vacuum Prep Tool, then denaturation solution until liquid is no longer pulled through the Vacuum Prep Tool, and then 1× washing buffer until liquid is no longer pulled through the Vacuum Prep Tool.
32. With vacuum on, hold the Vacuum Tool slightly over 90°C vertical to allow liquid to drain.
33. While the probes of the Vacuum Prep Tool are facing the ceiling, close the vacuum switch (Off), place the Vacuum Tool in the pyrosequencing reaction plate, and turn the vacuum pump off.
34. Gently shake the Vacuum Tool probes in the reaction plate for 15 s and then shake the probes in water before placing the Vacuum Tool in the holder upside down.
35. Place the pyrosequencing reaction plate on the dry bath at 80°C ± 2°C for 2 min.
36. Reconnect the vacuum lines to the Vacuum Prep Tool and drain the lines of all liquid by turning on the vacuum. Disconnect the Vacuum Prep Tool and sonicate the tips for at least 1 min before returning the tool to the holder. Place the tool upside down.
37. Remove the iron and allow the plate to cool to room temperature for 5 min.
38. Place the reaction plate in the Pyrosequencer and close the lid. Select run.
39. When the run is finished, select OK and then open the run from the SNP Runs folder to print results.
40. Analyze in the AQ mode and print results. Each patient and control should be printed separately (2–1).
41. Remove cartridge, clean with Milli-Q water, and allow to air-dry in the dispensing tip holder box.

20.4 Interpretation of Results

Interpretation Algorithm: Each patient pyrogram is compared against the negative control for each sequencing reaction. If no differences in peak heights are observed, the patient is reported as negative for mutation. If there is a difference in a peak height, then mutation is determined and reported (Fig. 20.1).

20.5 Quality Control

20.5.1 Controls Included on Each Run

Negative control – A previously analyzed cell line (HL60) that is negative for the mutation

Positive control – A previously analyzed cell line (HEL92.7.1) that is positive for the mutation

No template control – A PCR reaction containing all components except DNA

Sensitivity standards – A positive control diluted 1:4 (Sensitivity standard 1) and 1:16 (Sensitivity standard 2) into a negative control

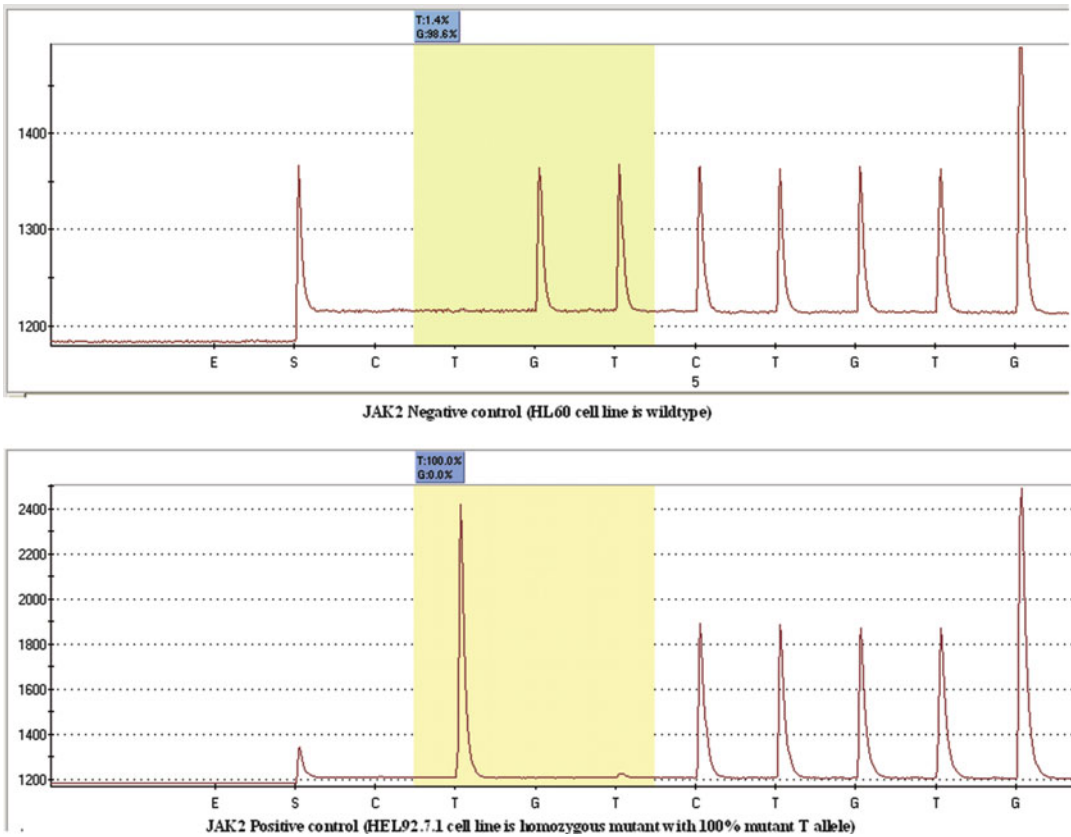


Fig. 20.1 Pyrosequencing assay to detect JAK2 p.V617F mutations. Representative pyrograms for the JAK2 p.V617F mutation assay are shown. The sequence read is (G/T)TCTGTGG. The *highlighted area* indicates the mutation site at base c.G1849 of the JAK2 gene. The dispensation of the enzyme (E) and substrate (S) is also shown. The numbers in the *blue box* show the proportion of each nucleotide at the mutation site. *Top*: HL60 cell line is wild type. *Bottom*: HEL92.7.1 cell line is homozygous mutant with 100% mutant T allele

20.5.2 Assay Parameters

Precision: $\pm 10\%$

Analytic sensitivity: It is generally 1:16 based on dilution studies. Mutated peaks comprising at least 10% of the total PCR product are reportable.

Analytical specificity: It is 95–100%, based on comparison with Sanger sequencing method. The discordant cases have low levels of signal with mutated product, and this usually occurs due to higher/noisy baseline or delayed or misaligned dispensation of nucleotide.

20.6 Troubleshooting

No substrate peak: This usually happens when one of the reagent tips has an air bubble or is clogged. Remove both tips and rinse them well with water. Allow them to soak in water for 5 min, completely submerged. Dry the tips and repeat current run including setup.

Out-of-phase peaks: If a droplet is formed on one of the tips and happens to fall into an adjacent well during robot maneuvering, it results in out-of-phase peaks. If this is near a base of interest, repeat the sample, if not, disregard.

Reagent control shows peaks for an individual nucleotide: In such an instance, the nucleotide tube, tip, or both are contaminated with pyrophosphate. Replace the nucleotide tube and tip and repeat the run.

Low signal: This could be caused by many factors. Check pH and expiration date of all reagents, perform dispensation tip test (all droplets should be of equal size), make sure the thermomixer was shaking during incubation, check functionality of filter probes, check QC gel for product, and repeat the run.

Vacuum Prep Tool not aspirating: If you know which well(s) is not functioning, replace that filter probe. If not, fill each well in a 96-well plate with water and aspirate for 10 s. Replace the filter probes for each well that still contains liquid.

Manual calculation of mutant percent: Manual calculation of mutant percent

$$\% \text{ Mutation} = \frac{\text{Mutant Peak Height}}{\text{Mutant Peak Height} + \text{Wild-type Peak Height}} \times 100$$

20.7 Summary

Pyrosequencing is a DNA sequencing technology based on real-time detection of DNA synthesis monitored by bioluminescence. It is rapid, accurate, reproducible, and automated. Pyrosequencing technology has been used in a broad range of application such as DNA sequencing, de novo mutation detection, genotyping, SNP analysis, allele quantification, and whole genome sequencing, thus useful in the fields of microbiology, clinical genetics, and pharmacogenetics among others. For some applications such as hotspot mutation analysis, this method is an excellent alternative to the traditional Sanger sequencing method. The assay can be multiplexed on a single template, which possesses multiple SNPs, or on multiple templates [16]. Thus, pyrosequencing technology possesses several unique features which make it advantageous compared to other sequencing methods in a number of applications.

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Chapter 21

Clinical Applications of Microarrays in Cancer

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Keywords Cancer microarray • aCGH (microarray-based comparative genomic hybridization) • SNP (single nucleotide polymorphism) • CNV (copy number variation) • LOH (loss of heterozygosity) • CMA (chromosome microarray analysis) • Aberration Detection Method 1 algorithm (ADM-1) • Deletion • Duplication • Amplification

21.1 Introduction

Genetic alterations are important biological markers for cancer diagnosis and prognosis, disease classification, risk stratification, and treatment selection. Microarray-based cytogenetic technology is a superb new method for evaluating cancer genomes. This technology offers high resolution, highly accurate targeted and whole genome evaluation of tumor tissues independent of cell division. Application of microarray-based technology in cancer research has generated a wealth of useful information about copy number variations (CNVs) and their implications in cancer classification, disease progression, therapy response, and patient outcome. There is an increasing interest in the genetic diagnostic community in applying this new technology to cancer diagnosis in CLIA certified laboratories. We anticipate that microarray-based cancer cytogenetics will soon become one of the major diagnostic means for cancer genome analysis, especially for solid tumors and hematological malignancies with prominent genomic imbalances.

21.2 Microarray Technologies

Microarray-based technology has revolutionized modern cytogenetics, allowing high-throughput interrogation of the human genome for copy number variations with unprecedented resolution. The technique has accelerated our capability to discover and characterize critical genomic alterations that

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Table 21.1 Comparison of aCGH and SNP-based arrays

	aCGH	SNP-based arrays
Probes	BAC/PAC, oligos	Oligos
SNP genotyping	No	<i>Yes</i>
SNP desert Coverage	Yes	<i>No</i>
UPD detection	<i>No</i>	<i>Yes</i>

cause and/or promote tumorigenesis. Application of microarray-based technology in cancer research has produced a wealth of useful information about copy number variations (CNVs) and their implications in cancer classification, disease progression, therapy response, and patient outcome [1–5]. The wide spectrum of genomic variations in cancer and broad range of their phenotypic expression uncovered by microarray-based cytogenetics, also known as chromosome microarray analysis (CMA), have raised new questions about our understanding of the complexity of the cancer genome and modified our view of the genomic contribution to human cancer [6]. These discoveries mark the emergence of a new era of genomic medicine and are transforming medical practice in oncology. This chapter provides an introduction to microarray technologies, including different microarray platforms, and a detailed protocol for comparative genomic hybridization-based microarray analysis. We attempt to address issues associated with cancer array data analysis and result interpretation. The application and significance of microarray technology in cancer research and clinical diagnosis are also discussed.

Two major microarray-based platforms are currently used in clinical cytogenetics: microarray-based comparative genomic hybridization (aCGH) and single nucleotide polymorphism (SNP) genotyping-based arrays. aCGH, which directly measures genomic copy number differences between the patient DNA and a normal reference DNA, allows the construction of a high-resolution map of genome-wide copy number alterations. aCGH arrays contain thousands of bacterial artificial chromosome (BAC)/P1-derived artificial chromosome (PAC) clones or in situ-synthesized oligonucleotide probes. These probes may either be enriched for specific disease-associated genes or genomic regions or distributed relatively evenly across the whole genome. SNP-based arrays probe thousands of SNPs and provide data about both copy number and genotype; the latter can be used to study copy number neutral genomic alterations, such as uniparental disomy (UPD) as seen in imprinting disorders and loss of heterozygosity (LOH) as found in cancer. Although SNP-based arrays have the advantage of revealing genotype information, they offer a poor representation of genomic regions with low SNP incidences (SNP deserts) [7]. Additionally, SNP-based platforms do not use an intra-experimental control; rather, they compare patient data to a preestablished laboratory standard. Although the underlying technology and the design of the probes are substantially different among different microarray platforms, studies have shown high level concordance between different platforms [8]. A comparison of the two major microarray platforms is summarized in Table 21.1. As microarray-based technologies continue to improve, many new platforms are being developed. These new platforms provide a combination of high probe density and optimal probe distribution across the genome, including SNP deserts and known chromosome regions, which contain repetitive DNA sequences, and allow detections of both CNVs and absence of heterozygosity (AOH), including UPD and LOH.

21.2.1 aCGH

aCGH evolved from chromosome CGH. In a chromosome CGH study, differentially labeled patient and reference DNA are cohybridized to metaphase chromosomes to generate a map of DNA copy number changes, which allows for screening cells for genomic gains and losses at a chromosomal

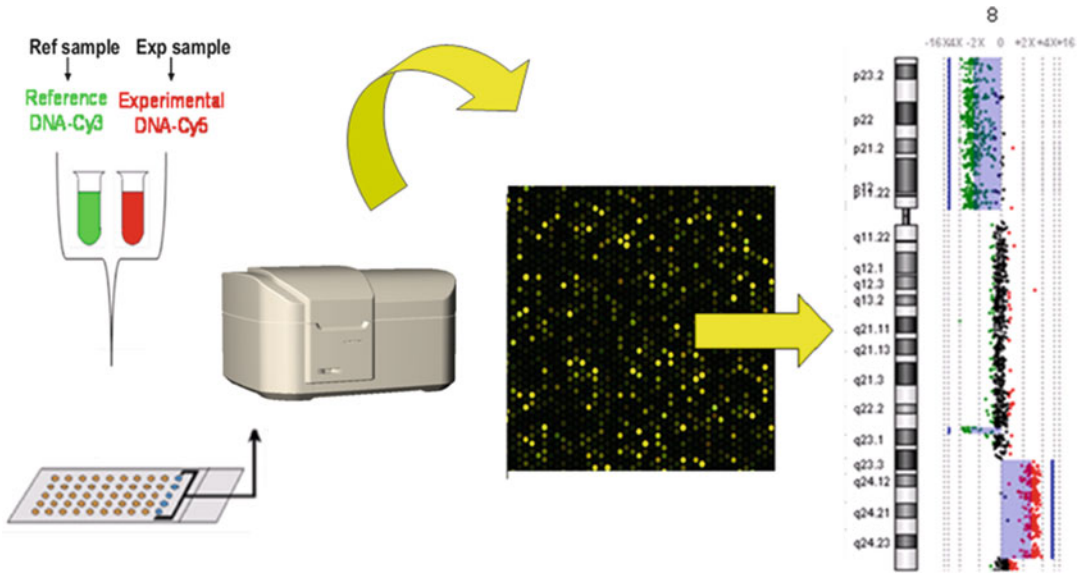


Fig. 21.1 CGH microarray workflow

level. In aCGH, differentially labeled patient and reference DNA are co-hybridized to a microarray chip that contains thousands of DNA sequences representing specific regions (targeted arrays) or the whole genome (whole genome arrays), which allows for identification of genomic gains and losses at the gene or exon level. Recently, a few manufacturers have started to offer CGH arrays with SNP probes to enable the simultaneous detection of both CNVs and AOH. Figure 21.1 illustrates the aCGH workflow. Major companies that offer CGH-based arrays include Agilent Technologies (Santa Clara, CA) and Roche NimbleGen Inc. (Madison, WI).

21.2.2 SNP Arrays

SNP-based arrays utilize SNP genotype information to determine DNA sequence copy number. Each probe is associated with either allele A or allele B. For a given SNP, a person may be AA, AB, or BB. Deletion, duplication, or amplification alters the allelic pattern, which can be used to determine copy number information. There are two major companies that offer SNP-based arrays, Affymetrix and Illumina. Affymetrix arrays are hybridization-based. In Affymetrix arrays, there are two probes for each SNP. Test DNA hybridizes to both probes giving rise to a brighter signal when it is a perfect match or a dimmer signal when it is a mismatch (Fig. 21.2) [9]. Illumina arrays use BeadChip technology. The essential element of BeadChip technology is the attachment of 50-mer oligonucleotides to silica beads. Each 50-mer sequence is complementary to the sequence adjacent to a SNP site. When a DNA sequence binds to a 50-mer, enzymatic single-base extension adds a labeled nucleotide that is complementary to the SNP allele carried by the DNA, resulting in a fluorescent signal (Fig. 21.2) [9]. For both platforms, the raw signal data are converted to inferences concerning the presence or absence of each of the two SNP alleles using different computational algorithms.

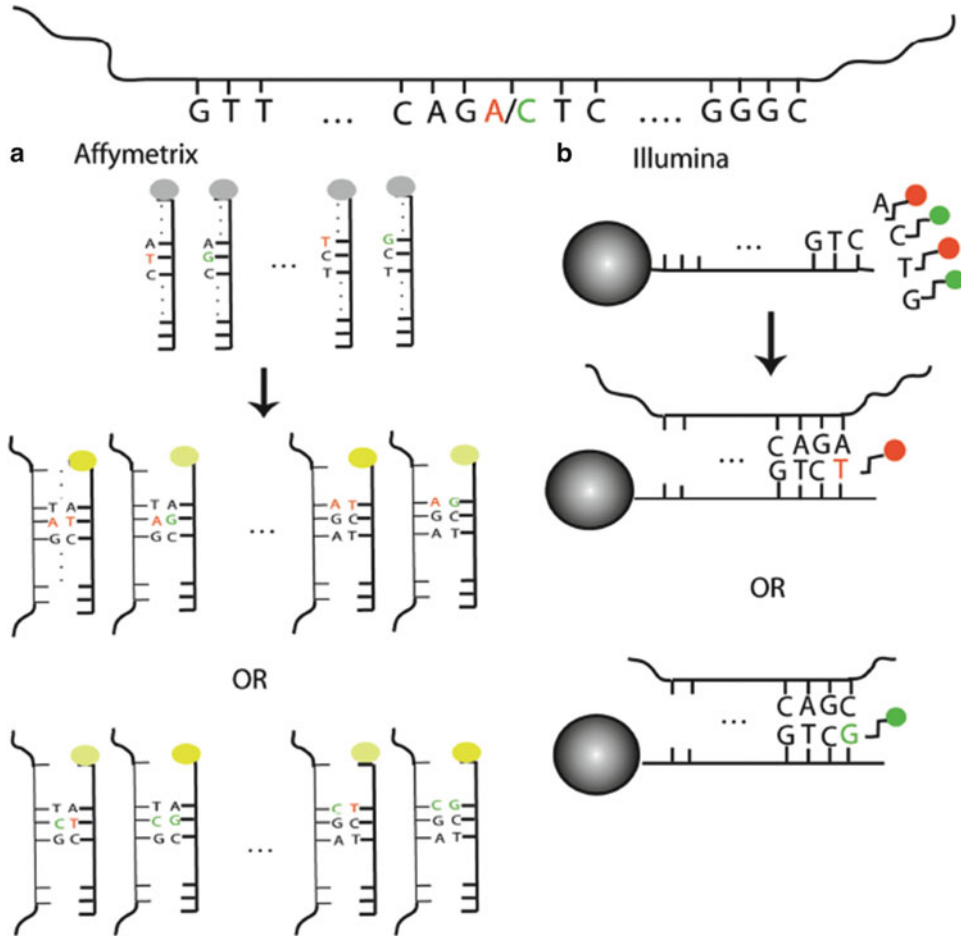


Fig. 21.2 Overview of SNP array technology

21.3 Required Reagents and Materials

Equipment required (based on Agilent 4×180 CGH or 4×180 K CGH+SNP arrays*):

- Agilent Microarray Scanner
- Hybridization chamber
- Hybridization chamber gasket slides
- Hybridization oven, temperature set at 65°C
- Hybridization oven rotator
- Nuclease-free 1.5-mL microcentrifuge tubes (sustainable at 95°C)
- Magnetic stir bar (×2 or ×4)
- Magnetic stir plate (×1 or ×3)
- Magnetic stir plate with heating element
- Eppendorf microcentrifuge
- NanoDrop
- 1.5-L glass dish
- 250-mL-capacity slide-staining dish, with slide rack (×3 or ×5)

- Circulating water baths or heat blocks set to 30°C, 37°C, 65°C, and 95°C
- Clean forceps
- Ice bucket
- Pipetman micropipettors, (P-10, P-20, P-200, and P-1,000)
- Powder-free gloves
- Sterile, nuclease-free aerosol barrier pipette tips
- Timer
- Vacuum dessicator or N₂ purge box for slide storage
- Vortex mixer

Reagents required:

- Genomic DNA Labeling Kit
- Oligo aCGH Hybridization Kit
- Oligo aCGH Wash Buffer 1 and 2 set
- Oligo aCGH Wash Buffer 1 (4 L) and 2 (4 L)
- Agilent Oligo aCGH Spike-in Kit
(optional component to be as an external control for monitoring the microarray workflow)
- Stabilization and drying solution (optional components recommended if wash procedure B is selected)
- Acetonitrile
- DNase/RNase-free distilled water
- Human Cot-1 DNA
- Microcon YM-30 filter units
- 1 × TE (pH 8.0), molecular grade
- Alu I (10 U/μL)
- Rsa I (10 U/μL)
- Human genomic DNA for use as a reference sample
- Ethanol (95–100% molecular biology grade) (optional components recommended if using the amplification method for sample preparation)
(optional components recommended if using the amplification method for sample preparation)
- QIAprep Spin Miniprep Kit
- REPLI-g Mini Kit

21.4 Procedures

21.4.1 Genomic DNA Preparation

High-quality genomic DNA can be obtained from leukemic blood, bone marrow, or tumor tissue by using commercially available kits. Pooled reference DNA can be purchased for aCGH test only. For CGH+SNP arrays, single male or female pregenotyped DNA should be used.

21.4.2 DNA Quality

DNA samples should be free of contaminating proteins, carbohydrates, organic solvents, ethanol, and salts associated with DNA isolation procedures. An effective measure of DNA purity is the ratio of absorbance readings at 260 and 280 nm and the 230 nm reading. The ratio of A₂₆₀ to A₂₈₀ values

should fall in the range of 1.8–2.1, and the A260 to A230 ratio should be above 1.8. These ratios should be determined using a spectrophotometer (e.g., NanoDrop). Table 21.2 is an example of a DNA concentration worksheet. DNA must be suspended in high-quality DNase-free water or TE buffer or the recommended elution buffer. If DNA is contaminated and does not meet the required QC metrics, samples should be repurified using either ETOH/sodium acetate precipitation. Genomic DNA should also be intact with minimal degradation. The quality of the genomic DNA can be checked by gel electrophoresis. Degraded samples can be more challenging to label.

21.4.3 Genomic DNA Digestion

1. gDNA quantitation using NanoDrop

Table 21.2 DNA concentration

Sample	ng/ μ L	260/280 ratio	Sample	ng/ μ L	260/280 ratio

2. gDNA digestion

- (a) Heat water baths to $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $65^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
- (b) Aliquot either **0.5–1 μ g** of test DNA or **0.5–1 μ g** of reference DNA into 1.5-mL nuclease-free microcentrifuge tubes using the worksheet below (see Table 21.3).

Table 21.3 DNA digestion worksheet

Sample	1 μ g DNA (μ L)	H2O (to 20.2 μ L)
1		
2		

Note: We suggest using 0.5 μ g DNA for CGH arrays and 1.0 μ g DNA for CGH + SNP arrays

- (c) Add nuclease-free water to bring to a final volume of 20.2 μ L.
- (d) Prepare digestion master mix (see Table 21.4).

Table 21.4 Digestion master mix

Component	Volume (μ L)/reaction $1 \times$	Volume (μ L)/reactions $(n + 1) \times$
Nuclease-free water	2.0	
10 \times Buffer C	2.6	
Acetylated BSA (10 μ g/ μ L)	0.2	
Alu I (10 U/ μ L)	0.5	
Rsa I (10 U/ μ L)	0.5	
Final volume	5.8	

Note: Alu I – 50 μ L/vial; Rsa I – 100 μ L/vial

- (e) Add 5.8 μL aliquots of the digestion master mix into each reaction tube containing genomic DNA or amplified genomic DNA to make a total volume of 26 μL .
- (f) Incubate the tubes in a water bath at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 h.
- (g) Transfer the samples to a water bath at $65^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 20 min to inactivate the enzymes. Place tubes on ice for at least 5 min. Note: At this point, samples may be covered with aluminum foil and stored at $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for labeling the next day. Samples can also be left at $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ over the weekend.

21.4.4 Fluorescent Labeling and Cleanup of Labeled gDNA

Note: Most commercially available kits use random primers and the exo-Klenow fragment to differentially label test and reference DNA samples. Dye-swap pairs may be used to maximize the accuracy of detection of CNVs, but will double the cost.

1. Fluorescent labeling:

- (a) Set water baths to 95°C , 37°C , and $65^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
- (b) Add 5 μL of random primers to each reaction tube containing 24 μL of digested gDNA to make a total volume of 29 μL (or 31 μL if sample was not run on a gel). Mix well by gently pipetting up and down.
- (c) Transfer the samples to a $95^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (denature) circulating water bath and incubate for 3 min.
- (d) Immediately place the tubes on ice for 5 min and then spin the tubes at maximum speed for 2–10 s. Return tubes to ice.
- (e) Prepare the labeling master mix by mixing the components listed in Table 21.5 on ice in the order indicated.

Table 21.5 Preparation of labeling master mix

Component	Volume (μL)/reaction (1 \times)	Volume (μL)/Cy5 ($n + 1$) \times reactions	Volume (μL)/Cy3 ($n + 1$) \times reactions
5x Buffer	10.0		
10x dNTP	5.0		
Cy3-dUTP (1.0 mM) or Cy5-dUTP (1.0 mM)	3.0		
Exo-Klenow	1.0		
Final volume	19.0		

- (f) Add 19 μL of the **Cy5** labeling and purification master mix to sample tubes 1–12 to make a total volume of 50 μL . Mix by gently pipetting up and down.
 - (g) Add 19 μL of the **Cy3** labeling and purification master mix to sample tubes 1–12 to make a total volume of 50 μL . Mix by gently pipetting up and down.
 - (h) Incubate all tubes in a circulating water bath at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 h.
 - (i) Incubate at $65^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 10 min to inactivate the enzyme and then move the samples to ice. Note: At this point, samples may be covered with aluminum foil and stored at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for cleanup the next day. Samples can also be left over the weekend.
2. Cleanup of labeled genomic DNA – using Microcon YM-30 kit:
- (a) Add 430 μL of 1 \times TE (pH 8.0) to each tube.
 - (b) Place Microcon YM-30 filters in a 1.5-mL collection tube and load each sample onto the filter.

- (c) Centrifuge the column at $12,000 \times g$ for 10 min at room temperature.
- (d) Discard the flow-through and replace the column back into the same collection tube.
- (e) Add 430 μL of $1 \times \text{TE}$ (pH 8.0) to each filter.
- (f) Centrifuge the column at $12,000 \times g$ for 12 min at room temperature.
- (g) Discard the flow-through.
- (h) Invert the column into a fresh 1.5-mL collection tube and centrifuge at $12,000 \times g$ for 1 min to collect the purified sample.
- (i) Measure and record the volume of each elution. If the sample volume is $<20 \mu\text{L}$, then bring the volume up to 20 μL with $1 \times \text{TE}$ (pH 8.0).
- (j) Combine the Cy5 and Cy3 labeled samples for a total mixture volume of 40 μL in a new heat-resistant 1.5-mL microcentrifuge tube. Attempt to match concentrations between Cy5 and Cy3 samples as well as keeping them sex matched.
- (k) Labeled DNA can be stored overnight at $-20^\circ\text{C} \pm 2^\circ\text{C}$ in the dark.

21.4.5 Preparation of Labeled Genomic DNA for Hybridization

Note: This step can differ based on different array formats, as shown in Table 21.6 below.

This protocol is based on the $4 \times 180 \text{ K}$ array.

Table 21.6 Volumes for different Agilent array formats

Component		$2 \times 400 \text{ K}$ array (μL)	$4 \times 180 \text{ K}$ array (μL)	$2 \times 105 \text{ K}$ array (μL)	$8 \times 60 \text{ K}$ array (μL)
1	Cyanine 5-labeled sample	19.5	19.5	19.5	19.5
2	Cyanine 3-labeled sample	19.5	19.5	19.5	19.5
	$1 \times \text{TE}$	40.0	0.0	40.0	0.0
3	Cot-1 DNA (1.0 mg/mL)	25.0	5.0	25.0	2.5
4	Agilent 10x blocking agent	26.0	11.0	26.0	4.5
5	Agilent 2x hybridization buffer	130.0	55.0	130.0	22.5
Final hybridization (sample volume)		260.0	110.0	260.0	68.5

1. Prepare 10x blocking agent:

- (a) Add 1,350 μL of nuclease-free water to the vial containing the lyophilized 10x blocking agent.
- (b) Store at room temperature for 1 h to reconstitute.
- (c) Store at $-20^\circ\text{C} \pm 2^\circ\text{C}$.

2. Prepare hybridization samples:

- (a) Turn on incubation chamber to 65°C and set the rotation speed to **20 rpm**. Equilibrate water baths to 95°C and $37^\circ\text{C} \pm 2^\circ\text{C}$.
- (b) Mix the components listed in Table 21.7 in the order indicated and gently pipette up and down before quickly spinning in a microcentrifuge to drive the contents to the bottom of the reaction tube.

Table 21.7 Hybridization master mix

Component	Volume (μL)/reaction $1\times$	Volume (μL)/reactions $(n+1)\times$
Cy5 and Cy3 labeled gDNA mixture	40	
Human Cot-1 DNA (1.0 mg/ μL)	5	
Agilent 10 \times blocking agent	11	
Agilent 2 \times hybridization buffer	55	
Final hybridization sample volume	111	
Cot-1 – 500 μL /vial		

- (c) Transfer sample tubes to circulating water bath at $95^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and incubate for 3 min.
- (d) Transfer sample tubes to circulating water bath at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and incubate for 30 min.
- (e) Remove samples from water bath. Centrifuge 1 min at **17,900 \times g** (13,000 rpm in Eppendorf 5417 R) in microcentrifuge to collect the sample at the bottom of the tube.

3. Hybridization assembly (see Table 21.8 setup):

Table 21.8 Hybridization setup

Labeling rxn.	Test sample red (Cy5)	Reference sample green (Cy3)	Hybridization description	Barcode	Array
1			Sample 1 vs. normal female		1
2			Sample 2 vs. normal female		2
3			Sample 3 vs. normal female		3
4			Sample 4 vs. normal female		4

- (a) Check and make sure that the hybridization chamber is at $65^{\circ}\text{C} \pm 2^{\circ}\text{C}$ before proceeding.
- (b) Load a clean SureHyb gasket slide into the chamber base with the label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
- (c) Slowly dispense **110 μL** of hybridization mixture onto the gasket well in a “drag and dispense” manner. Avoid touching the sample on the edge of the gasket well.
- (d) Place an array active side down onto the SureHyb gasket, so the numeric barcode side is facing up. Assess that the sandwich pair is properly aligned.
- (e) Place the chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
- (f) Hand-tighten the clamp onto the chamber firmly.
- (g) Vertically rotate the assembled chamber to wet the gasket and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.
- (h) Hybridize at **$65^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 20–40 h.**

21.4.6 Washing the Arrays

Note: (1) Wash slide-staining dishes, slide racks, and stir bars at least five times using copious amounts of Milli-Q water. (2) Prepare oligo CGH Wash Buffer 2 one day in advance of performing the wash procedure. The temperature of CGH Wash Buffer 2 must be at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for optimal performance. Store the solution overnight in an incubator or circulating water bath set to $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$. (3) Put a slide-staining dish into a 1.5-L glass dish three fourths filled with water and warm to $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ by storing overnight in an incubator set to $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

1. Wash conditions (see Table 21.9 setup):

Table 21.9 Washing setup

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	Oligo CGH Wash Buffer 1	RT	
1st wash	2	Oligo CGH Wash Buffer 1	RT	5 min
2nd wash	3	Oligo CGH Wash Buffer 2	37°C ± 2°C	1 min

- (a) Completely fill the first slide-staining dish with aCGH Wash Buffer 1 at room temperature.
- (b) Place a slide rack into slide-staining dish 2. Add magnetic stir bar. Fill the slide-staining dish with enough aCGH Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.
- (c) Remove one hybridization chamber from incubator and record time. Record whether there are bubbles formed during hybridization and if all bubbles are rotating freely.
- (d) Disassemble the hybridization chamber and place the array-gasket sandwich in the first slide-staining dish filled with aCGH Wash Buffer 1 at room temperature. With the sandwich completely submerged in aCGH Wash Buffer 1, pry the sandwich open with blunt-ended tweezers. Remove slide and place into slide rack in the second slide-staining dish containing aCGH Wash Buffer 1 at room temperature. Minimize exposure of the slide to air.
- (e) Repeat steps for up to **5 hybridization chambers**.
- (f) When all slides in the group are placed into slide rack in staining dish, stir using 200 rpm for 5 min.
- (g) During Wash 1 incubation described, put the prewarmed 0.5-L dish filled with water and containing a slide-staining dish on the magnetic stirrer with heating element. Fill the slide-staining dish approximately three fourths full with aCGH Wash Buffer 2 (prewarmed to 40°C ± 2°C). Add magnetic stir bar. Maintain temperature of Wash 2 at 40°C ± 2°C.
- (h) Transfer slide rack to slide-staining dish filled with aCGH Wash Buffer 2 at 40°C ± 2°C and stir using 200 rpm for 1 min.
- (i) Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5–10 s to remove the slide rack.
- (j) Repeat steps for the next group of five slides using fresh aCGH Wash Buffer 1 and aCGH Wash Buffer 2.

21.4.7 Scanning

1. Verify default scan settings:

- (a) Scan region is set to Agilent HD (61 × 21.6 mm).
- (b) Scan resolution (3 μm) is set to 180 K microarrays.
- (c) Dye channel is set to red and green.
- (d) Green PMT is set to 100%; red PMT is set to 100%.

2. Settings for the automatic file naming:

- (a) Prefix 1 is set to instrument serial number.
- (b) Prefix 2 is set to array barcode.
- (c) Verify that the scanner status in the main window says **Scanner Ready**.

- (d) Click **Scan Slot m-n** on the Scan Control main window where the letter **m** represents the Start slot where the first slide is located and the letter **n** represents the End slot where the last slide is located.

21.5 CGH or CGH+SNP Array Data Analysis

Scanned image data are extracted using Agilent Feature Extraction software. Since Feature Extraction software is revised periodically to accommodate continuously improved arrays, use of the correct and updated software protocol is critical. Extracted array CGH data are analyzed with Agilent Genomic Workbench. It is important to use the right version of the software for the arrays in use and to check the software updates periodically (see Agilent Genomic Workbench Software User Guide, <http://www.chem.agilent.com/en-US/Pages/Homepage.aspx>). For cancer data analysis, we suggest using the Aberration Detection Method 1 algorithm (ADM-1) with threshold set at 6.0. Data may be further filtered with two different stringency settings: the higher stringency filter sets fuzzy zero correction on and uses 5–19 consecutive probe alterations at \log_2 ratio ± 0.20 to make a call, and the lower stringency filter sets fuzzy zero correction off and uses 20 or more consecutive probe alterations at \log_2 ratio ± 0.20 to make a call. The purpose of using the lower stringency filter is to catch lower level mosaic aberrations. With this setting, CGH arrays can detect 20% mosaic deletions and duplications. Each laboratory should determine its own cutoffs for array data analysis as part of the test validation process. The SNP data can be analyzed using the newest version of Genomic Workbench. However, for a highly complicated genome, such as a mixture of diploid/triploid/tetraploid genome, currently released software may not be sophisticated enough to make correct SNP calls. Better software that can accurately analyze cancer CGH+SNP array data is in an urgent need. We expect such software will be released in a near future.

*For SNP-based arrays, protocols can be downloaded at <http://www.affymetrix.com/estore/> for Affymetrix arrays and <http://www.illumina.com/> for Illumina arrays. Protocols for NimbleGen CGH arrays can be downloaded at <http://www.nimblegen.com/>.

21.6 General Consideration of Microarray Data Analysis

There are many algorithms used in different analytical software to determine the presence of a significant gain or loss of a chromosomal region. Laboratories may decide to use software of their preference instead of manufacturer-suggested ones. Most software manufacturers provide standard algorithms to set the cutoff points. However, these rules and cutoffs are largely based on an uncomplicated diploid genome. Laboratories should thoroughly test the rules or filters as part of the validation of the assay and establish laboratory criteria, such as the minimum number of consecutive probes deleted or amplified and the \log_2 ratio, based on the density of probes and the laboratory experiences. It may be necessary to set different criteria for different chromosome regions. The laboratory personnel should be familiar with the principles of the software program being applied. In cases of mosaicism, which will typically show ratio values less than that expected for a nonmosaic case, a less stringent set of analytical criteria may be used as suggested earlier. Each laboratory should determine the sensitivity of the microarray for detection of mosaicism and indicate this limitation in the final report. Laboratories should never depend solely on software analysis. Visual examination of the whole genome will help in identification of large copy number changes, especially important in identification of mosaicism.

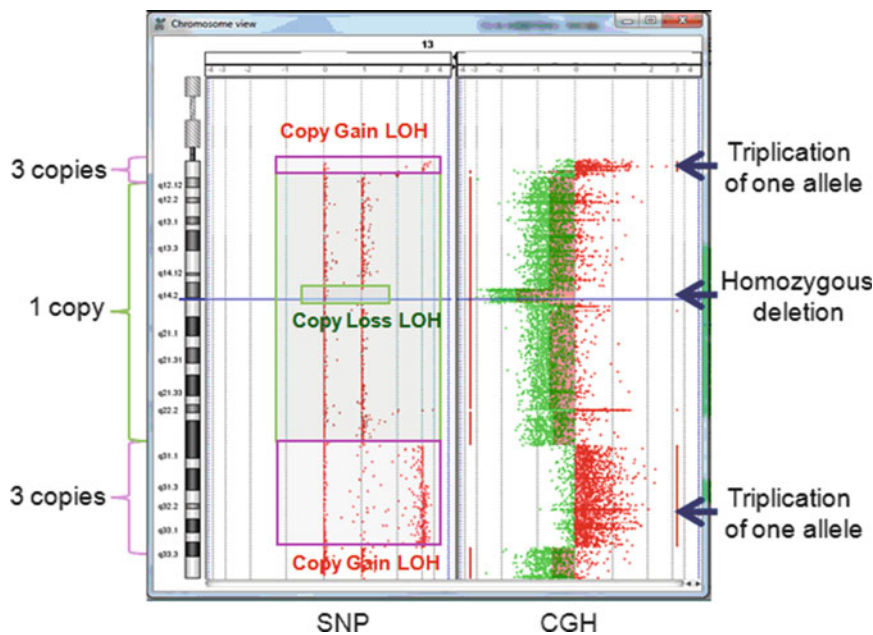


Fig. 21.3 CNVs and LOH on chromosome 13 from a patient with myelodysplastic syndrome using BCM 400 K CGH+SNP array

Table 21.10 Pathogenic CNVs and LOH on chromosome 13 in a patient with MDS

Column	Gain/loss/LOH	Chr	Band	Start (bp)	Stop (bp)	Size (Mb)	# Copy
1	Gain/LOH	chr13	q12.11	19590141	21620651	2	3
2	Loss/LOH	chr13	q12.11–q22.2	22995810	76220277	53	1
3	Loss	chr13	q14.2–q14.3	48703733	51933894	3.2	0
4	Loss/LOH	chr13	q22.2–q31.1	76746976	84458300	7.7	1
5	Gain/LOH	chr13	q31.1–q33.3	84541850	107785824	23	3
6	Loss/LOH	chr13	q33.3–q34	107886452	115083342	7.2	1

21.6.1 Interpretation of Cancer Array Results

It is expected that microarray test may reveal a large amount of CNVs in certain cancers. These CNVs may include somatic mutations as well as constitutional normal variants. We suggest categorizing cancer CNVs into different groups, such as “known cancer-associated,” “likely cancer-associated,” “CNVs of unknown significance,” “likely benign,” and “benign” CNVs. All known cancer-associated genomic alterations detected by microarray analysis should be reported. The report should include the nature (deletion/amplification/LOH), the size, the copy number, and the clinical significance of the aberration. Figure 21.3 shows the genomic changes of chromosome 13 from a patient with myelodysplastic syndrome. Table 21.10 is an example of reporting genomic alterations showed in Fig. 21.3. Since absence of heterozygosity can be the result of identity by descent (IBD), laboratories should determine the size of the LOH to be reported and clearly indicate the cutoff in the report. Deletion or duplication at or near-recurrent cancer-associated translocation breakpoints may indicate the presence of a cytogenetically balanced translocation (Fig. 21.4). CNVs encompassing known oncogenes or tumor suppressor genes may be clinically significant even though the implication of the CNVs to the

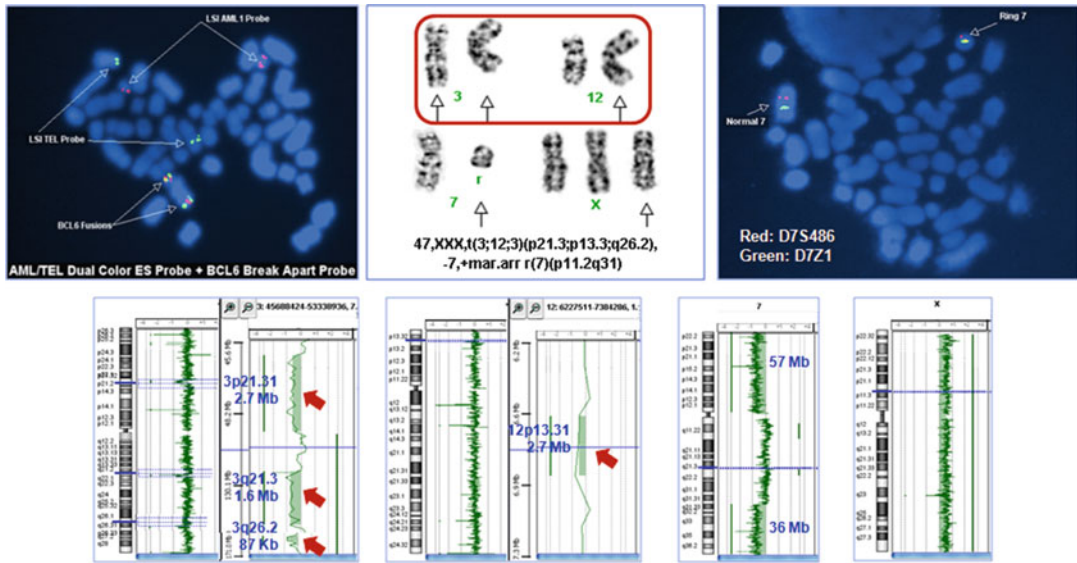


Fig. 21.4 CNVs associated with cytogenetically “balanced translocations”

patient or the disease may not be clear at present time. The interpretation of cancer microarray results will be inevitably complicated by common as well as private constitutional benign CNVs. The laboratory director and staff should be familiar with common constitutional benign CNVs on the arrays used in the laboratory. The laboratory director and staff should also be updated with current literature and databases on the common benign CNVs and should interpret the microarray results in this context. Nevertheless, it should be recognized that not all common CNVs are benign. For example, a cancer-associated abnormality can occur in known variant regions. In order to accurately interpret the cancer array results, laboratory directors should possess solid knowledge of common genetic disorders caused by genomic CNVs, as these CNVs could be incidental findings of cancer microarray study. Appropriate genetic counseling should be provided to the patient in this situation. Commonly used databases that help in determining the significance of CNVs include the Database of Genomic Variants (<http://projects.tcag.ca/variation/>), Online Mendelian Inheritance in Man (www.ncbi.nlm.nih.gov/omim/), and DECIPHER (<http://www.sanger.ac.uk/PostGenomics/decipher/>). Two new NCBI databases are now available: dbVar (database of Structural Variation (www.ncbi.nlm.nih.gov/dbvar/)) and dbGaP (database of Genotypes and Phenotypes (www.ncbi.nlm.nih.gov/gap/)). Other incidental findings may include nonpaternity and incest when SNP probes are used in microarray analysis. The laboratory director should treat each case carefully in order to protect patient privacy. When the results suggest that the minors in the household may be at risk of abuse, child protection service or other social service agencies should be notified. Interpretation of microarray result should also correlate with histopathology, immunophenotype, flow cytometry, and clinical-pathological information.

21.7 Clinical Application of Cancer Microarray Analysis

Genetic alterations in cancer cells are important biological markers for cancer diagnosis and prognosis, disease classification, risk stratification, and treatment selection. Metaphase chromosome analysis (MCA) has been used to detect genomic aberrations in cancer since the identification of the

Philadelphia chromosome in chronic myelogenous leukemia (CML) in 1960 [10]. However, MCA relies on cell division and has a limited resolution of 5–10 Mb or larger for cancer tissues, resulting in underestimation of the degree of genomic aberration. Fluorescent in situ hybridization (FISH) technology, which laid the foundation for molecular cytogenetics [11], is widely applied as a reliable diagnostic tool in the search for specific balanced or unbalanced genetic aberrations in cancer due to its straightforward application, fast turnaround time, high sensitivity, and independence of cell division [12–15]. Nevertheless, FISH can only target specific sequences which are known and suspected to be associated with specific cancers. Hence, a comprehensive screening for chromosomal aberrations cannot be achieved using this technique. Microarray-based cytogenetic techniques rely on the difference in the copy number between differentially labeled patient and reference DNA samples or SNP genotype information; therefore, cell division is not required. The high-density probe coverage allows for detection of small genomic alterations and for delineation of boundaries of deleted/duplicated regions [16, 17]. Arrays can be customized for different density and coverage, which enables even, targeted, or combined targeted/whole genome probe distribution. This is especially useful for cancer applications since the array coverage can be tailored to target known cancer genes and cancer-associated genomic regions [18].

Microarray-based cytogenetics in cancer research has produced a wealth of useful information about CNVs and their implications with regard to cancer classification, disease progression, therapy response, and patient outcome. For example, in breast cancer, CNVs in certain genomic regions are linked to specific clinical and pathological characteristics, including tumor grade, estrogen receptor status, *p53* mutation status, and overall survival [19]. In renal tumors, CNVs are extremely helpful in tumor subtype classification, such as deletion of 3p in clear cell renal cell carcinoma [20], deletion or loss of chromosome 1 and Y/X in renal oncocytoma [21], and loss of chromosomes 1, 2, 6, 10, 13, 17, 21 in chromophobe renal carcinoma [22]. CNV profiles can also be used to define distinct therapeutic response subgroups for a given type of tumor. Clustering of aCGH data was able to distinguish multiple myeloma cases into subclasses with different clinical outcomes [23]. Certain CNVs, such as deletions at 17q11.2 or 5q31.1, are associated with high genomic instability and confer an unfavorable outcome upon patients with acute myeloid leukemia (AML), even those otherwise classified in the favorable or intermediate cytogenetic prognostic groups [24].

The application of microarray-based cytogenetics in cancer has also led to the identification of putative oncogenes and tumor suppressor genes. aCGH analysis of mantle cell lymphoma (MCL) identified three recurrent homozygous deletions which led to the identification of the proapoptotic gene *BIM* as a novel candidate tumor suppressor gene in MCL [25]. Analysis of CNVs in ovarian cancer resulted in the discovery of the *FGF-1* gene that was amplified and overexpressed in ovarian cancer cells as a putative oncogene [26]. The discovery of cancer-specific CNVs and the genes involved provides the basis for a better understanding of cancer initiation and progression. More importantly, these putative genes and their associated pathways could be potential therapeutic targets. Internal tandem duplication of *FLT3* gene (*FLT/ITD*) occurs in more than 15% of patients with pediatric AML [27]. *FLT/ITD* constitutively activates the FLT3 receptor tyrosine kinase and causes autonomous, cytokine-independent proliferation in vitro. A few FLT3-inhibitors, such as PKC412 [28], are currently being tested in clinical trials in adults and show great potential for treatment of pediatric AML. Another recent discovery is the identification of a deletion of the *IKZF1* gene and its association with acute lymphoblastic leukemia (ALL) prognoses [29]. ALL patients with the *IKZF1* deletion or mutation commonly have mutations resulting in activation of kinase signaling cascades, including activating mutations of Janus kinases (*JAK1* and *JAK2*), and may benefit from JAK inhibitor therapy [30].

Genomic alterations have also been found to confer increased cancer susceptibility. Using aCGH that targeted six known cancer-associated genes and their flanking sequences, Staaf et al. [31] revealed germ line deletions or duplications in *BRCA1*, *BRCA2*, *MSH2*, and *MLH1* genes in patients with breast cancer or hereditary nonpolyposis colorectal cancer (HNPCC), indicating a cancer predisposition. Using SNP-based microarrays in two independent studies, analysis of patients from nonfamilial

adenomatous polyposis (FAP)/non-HNPCC colon rectal cancer (CRC) families uncovered a novel CRC susceptibility locus on chromosome 3q [32]. These findings will potentially aid in the early identification of at-risk individuals and allow earlier detection of cancer, optimizing prognosis and the chance for cure.

21.8 Summary

Chromosome microarray analysis is a superb new method for evaluating cancer genomes. The technology offers high-resolution, highly accurate, whole genome evaluation of tumor tissues independent of cell division. CMA can not only confirm, clarify, and further characterize cytogenetic and FISH results but also identify many submicroscopic genomic aberrations which may be of clinical significance. At the present time, CMA should be an adjunct test to current standard care in hematological malignancies. However, it should be the first tier test for many solid tumors and some hematological disorders, such as CLL.

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Chapter 22

Basic Theories and Clinical Applications of Molecular Flow Cytometry

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Keywords Flow cytometry • Antigen • Monoclonal antibody • Cluster of designation marker • Immunophenotyping • Immunostaining • Deoxyribonucleic acid content/ploidy

22.1 Introduction

Flow cytometry is a dispensable molecular diagnostic technology being capable of detecting and enumerating normal and abnormal cellular components of various tissues. By using various fluorescent probes targeting to moleculars of interest with non-overlapping emission spectra and optical sensing system, this technology has proven particularly useful for simultaneous multiparametric analysis of physical characteristics, surface/cytoplasm immunophenotypes and DNA content of up to thousands of single-cell(s) suspended in fluid per second. Not only Flow cytometry is routinely utilized in providing data relevant to both diagnosis/prognosis and monitoring of patients with a variety of health diseases, especially hematologic neoplasms, but also has many other applications in both clinical practice and preclinical studies. However, there are many technical obstacles involved in clinical flow cytometry testing. This chapter presents a comprehensively standardized flow cytometry protocol, predicated on analyzing polychromatic parameter of individual cells and facilitating identification of different cell types within heterogeneous populations, to enable a correct detection of specific cells of interesting in a variety of human samples types for routine assay in clinical applications.

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22.1.1 Clinical Importance of Flow Cytometry

For any flow cytometric application, monodispersed cell ought to be prepared so that they are single and suspended in an apposite medium. Once the single-cell suspension is ready, the cells could be then labeled with a monoclonal antibody conjugated with a fluorochrome or fluorescent dye. The immunolabeling procedure could be affected by a lot of factors, including antigen density and antibody specificity being studied in and/or on the cells of interest. Herein, we appropriately cover technical aspects of flow cytometry with providing a step by step, optimized immunostaining procedure with procedural variation for specific situations, a daily quality control checkpoint, and troubleshooting checklist for ensuring both accuracy and precision of flow cytometry testing. Although still important in preclinical studies, many flow cytometric applications have become routine clinical tests required for diagnosis, estimating of prognosis, and monitoring of treatment. Subsequently, there is a detailed description that comprehensively focus on the most common flow cytometry diagnostic testing, their interpretations including monitoring of CD4+ T-lymphocyte counts in HIV patients, diagnostic immunophenotyping of leukemia/lymphoma, evaluation of CD34+ stem cells for transplantation, and DNA content/ploidy analysis for prognosis of malignant diseases.

22.2 Features of Flow Cytometry

Fluorescent molecular-based flow cytometry is a powerful, rapid, and cost-effective technology that identifies, enumerates, and monitors cellular markers in various tissues from patients with hematopoietic neoplasms as well as other diseases [1]. Together with the availability of specific monoclonal antibodies and fluorochrome reagents, flow cytometers allow laboratory professionals to quantitate cellular/intracellular antigens from the surface, cytoplasm, and DNA content of thousands of cells suspended in fluid to determine their lineage and estimate their potential biologic and pathologic behaviors.

Flow cytometry has the advantage of being more efficient, sensitive, accurate, and reproducible than manual techniques for cellular immunology. The ability to perform more than a four-color multiparametric analysis on an individual cellular basis at flow rates of up to 25,000 cells per second is a unique feature of this technique and offers distinct advantages over immunohistochemical and immunocytologic analysis. It is typical for leukemia, lymphoma, and other tumor cells from fresh biopsy tissues to be identified by a search for an abnormal gain or a change from normal antigen expression patterns, i.e., immunophenotypic analysis [2]. With a modern flow cytometer, multiple specimens can be simultaneously processed with a panel of four or more monoclonal antibodies, and these tests can be completed within several hours. The ease of operation of this automated instrument makes its use feasible in routine laboratory procedures not only in large medical centers but also in smaller hospitals and professional reference laboratories.

22.3 General Principles of Flow Cytometry

22.3.1 Basic Concepts of Flow Cytometry

Flow cytometry is defined as the simultaneous detection and enumeration of multiple cellular physiognomies (characteristics) from a single cell as cells flow in a stream through a measurement device. Flow cytometric testing passes thousands of cells per second through a laser beam and captures the light that emerges from each cell as it passes through. The data gathered can be analyzed statistically

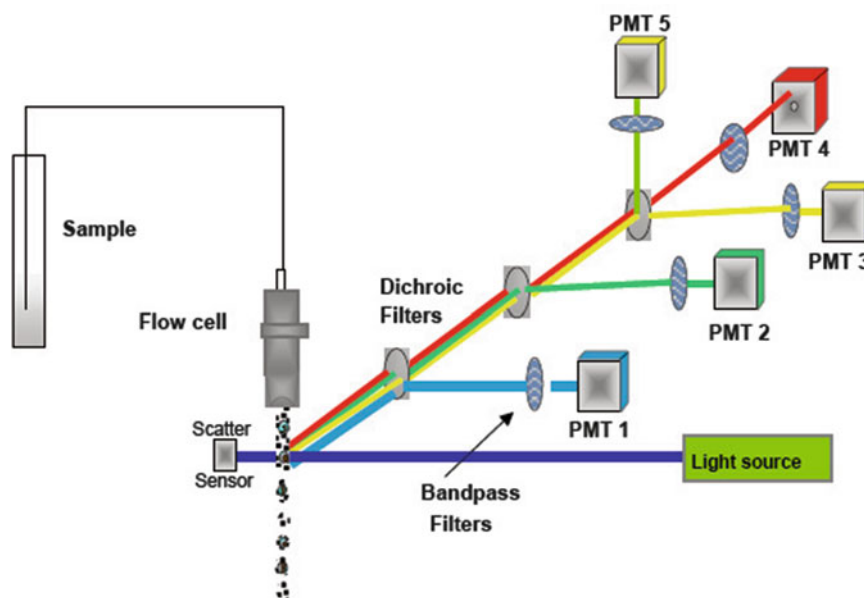


Fig. 22.1 Simplified overview of a typical flow cytometry setup (Courtesy of Dr. S.J. Ochatt, Flow cytometry in Medicago Truncatula Handbook, 2006; and Dr. R.F. Murphy, Basic Flow Cytometry Theory, 2006. Accessible at <http://www.cyto.purdue.edu/flowcyt/educate/pptslide.htm>)

by flow cytometry software, which can report cellular characteristics such as size, complexity, phenotype, and viability [3].

Most clinical flow cytometers have the capacity to make simultaneous measurements on every cell using four or more colors; however, some specialized research instruments are equipped with much greater power and with six lasers can analyze up to 18 parameters [4]. A typical flow cytometer consists of three functional units: (1) the fluidic system, which controls the delivery and focus of cells at the intersection of the laser beams; (2) the sensing system, which comprises the generation and collection of light signals from the sample/flow chamber and the optical assembly; and (3) the electronics system, which collects data and performs routine analysis of measurable electronic signals that are then converted from light signals from the sensing system (Fig. 22.1).

Fluidic System. The fluidic system is comprised of the flow cell, a pressurized sample, and a pressurized isotonic buffer in a sheath tank (Fig. 22.2). Two important processes used to deliver cells in suspension to the point of laser interrogation are laminar flow and hydrodynamic focusing; these ensure that only one cell is interrogated at a time. Laminar flow is the process by which the sample fluid flows into the central core of the sheath fluid without mixing. This is accomplished by creating a difference in pressure between the sample tube and the sheath tank, the sample tube being at a higher pressure than the sheath tank. When the sample is injected into the sheath fluid, the difference in pressure creates a “core” or space for the sample without mixing it with the sheath fluid. In the hydrodynamic focusing process, cells are focused into the center of the laser beam one cell at a time. The pressured sheath fluid forces the cells in the sample to flow in single file as they pass through the laser beam (Fig. 22.3).

Sensing System. After hydrodynamic focusing, each cell passes through one or more laser beams. The optical system is composed of light excitation sources, usually lasers of a specific wavelength, and a series of optical filters and mirrors that direct specific wavelengths of light to their appropriate detector. The most commonly used laser wavelengths in flow cytometry are 488 nm (blue), 633–637 nm

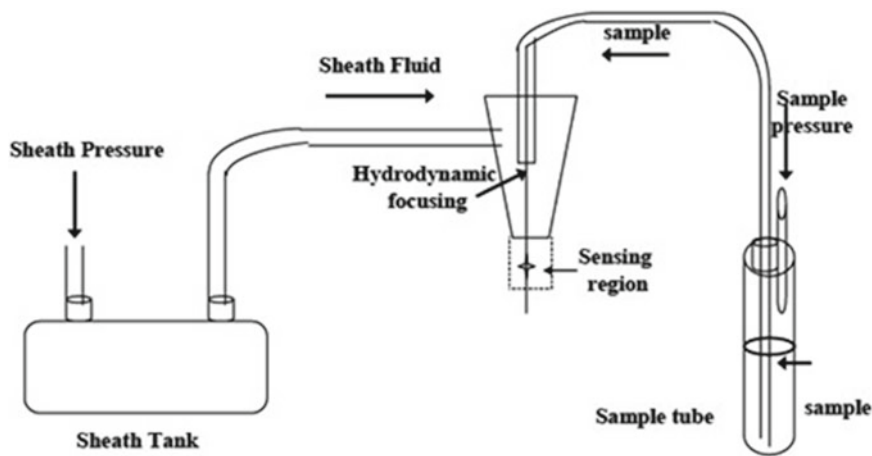


Fig. 22.2 Basics of a fluidic system in flow cytometry

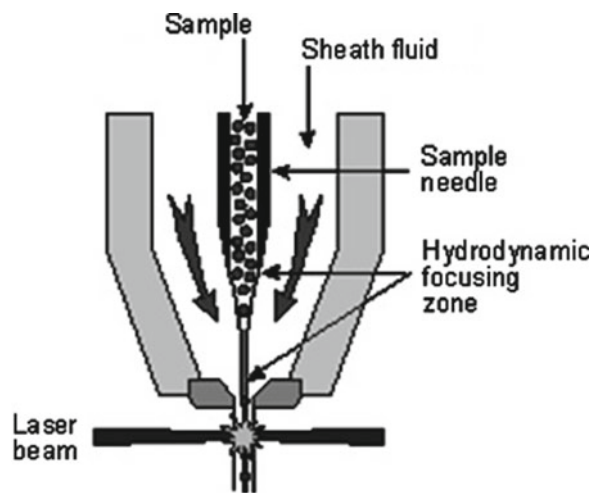


Fig. 22.3 The shape of the flow cell causes hydrodynamic focusing, which pushes cells to line up single file along their long axis (Courtesy of Dr. SJ Ochatt, Flow cytometry in Medicago Truncatula Handbook, 2006; and Dr. RF Murphy, Basic Flow Cytometry Theory, 2006. Accessible at <http://www.cyto.purdue.edu/flowcyt/educate/pptslide.htm>)

Table 22.1 Types of filters or mirrors used in flow cytometry

Filter/mirror type	Definition
Long-pass filter	Transmits wavelengths of light \geq the spectral band of the filter
Short-pass filter	Transmits wavelengths of light \leq the spectral band of the filter
Band-pass filter	Transmits wavelengths of light within a specific range of wavelengths
Dichroic mirror	Either a short-pass or long-pass filter placed at a 45-degree angle, which splits wavelengths of light so that they travel in two directions

(red), 405–407 nm (violet), and 514 nm (green). Fluorochromes have a specific spectrum of wavelengths with which they are maximally excited, so the more lasers an instrument has, the more fluorescent colors can be measured.

A flow cytometer uses a combination of long-pass, short-pass, and band-pass filters and dichroic mirrors to direct specific wavelengths of light to a particular detector (Table 22.1). Light emission

from fluorescence provides information about each cell's properties. Light is scattered forward, normally up to 20-degree offset from the laser beam's line, and is collected by a lens known as the forward scatter channel (FSC). The FSC intensity approximately correlates with the cell's size. Light measured at an approximate 90-degree angle to the excitation axis is called the side scatter channel (SSC). The SSC provides information about complexity within a cell. Both FSC and SSC have unique parameters for every cell type, and the combination of FSC and SSC is routinely used to differentiate cell types in a heterogeneous sample (Fig. 22.4).

Electronics System. When light reaches a photodetector, a small current is generated. Its associated voltage has amplitude proportional to the total number of light photons received by the detector. The voltage is then amplified by a series of linear or logarithmic amplifiers and by analog-to-digital converters into electronic signals large enough to be plotted graphically. To capture details, the computer system controls the function of (1) converting an optical signal (photons) to a proportional and measurable electronic signal (photoelectrons) with photomultiplier tubes that take in photons and release several thousand photoelectrons; (2) measuring the amplitude, area, and width of the photoelectron pulse; (3) compensating for spectral overlap of fluorochromes; (4) amplifying the pulse through the use of linear or logarithmic amplification pulse boards; (5) transforming the output from the amplification boards to a digitized value; and (6) communicating the digitized data to the computer.

22.3.2 *Fluorochrome Measurements and Antibodies*

Fluorochromes are a group of unique fluorescent molecular probes capable of absorbing light energy and then emitting light at a longer wavelength. This energy is released in the form of dissipated heat and photons. Because some energy is lost through heat, the photons that are released are of a lower energy (longer wavelength) than the excitation wavelength, which is referred to as the "Stokes shift" (Fig. 22.5). The fluorochrome molecule, which can be excited by a range of wavelengths, will then emit a range of wavelengths of light; these two ranges are referred to as the excitation and emission spectra. A fluorochrome molecule will have an excitation (or absorption) maximum (the wavelength at which the fluorochrome is optimally excited) as well as an emission maximum (the strongest emitted wavelength). A fluorochrome may have several different ranges of excitation, but it will always emit over the same range of wavelengths, although the extent of emission will vary (Fig. 22.6).

Over the years, the list of available monoclonal antibodies used to define human cluster of designation (CD) markers has expanded immensely and has been accompanied by a steadily growing list of fluorochromes for monoclonal antibody conjugation [5]. Like other molecular probes, monoclonal antibodies specifically bind to antigens used to define immunophenotypic characteristics of the cells (Table 22.2). A major benefit of monoclonal antibodies is their reliably reproducible binding strength, which ensures the same clone is selected. Flow cytometric analysis relies on monoclonal antibodies coupled to fluorescent molecular probes to facilitate identification of cell lineage, maturation, activation state, diploidy/cell cycle status, and viability, which has resulted in widespread use of those reagents for cellular marker detection methods in clinical laboratories. CD is a standardized nomenclature for monoclonal antibodies. Anti-CD antibodies are monoclonal antibodies used for the identification and investigation of cell surface molecules present on white blood cells (WBCs) and other types of cells, such as those in bone marrow or biopsy specimens. These CD molecules often present as cellular receptors or their ligands existing on the cell surface, in the transmembrane, and/or in the cytoplasm of the cell being studied. As of 2009, human CD markers numbered up to 350 [6]; the most commonly used human CD markers in clinical phenotypic analysis are summarized in Table 22.3.

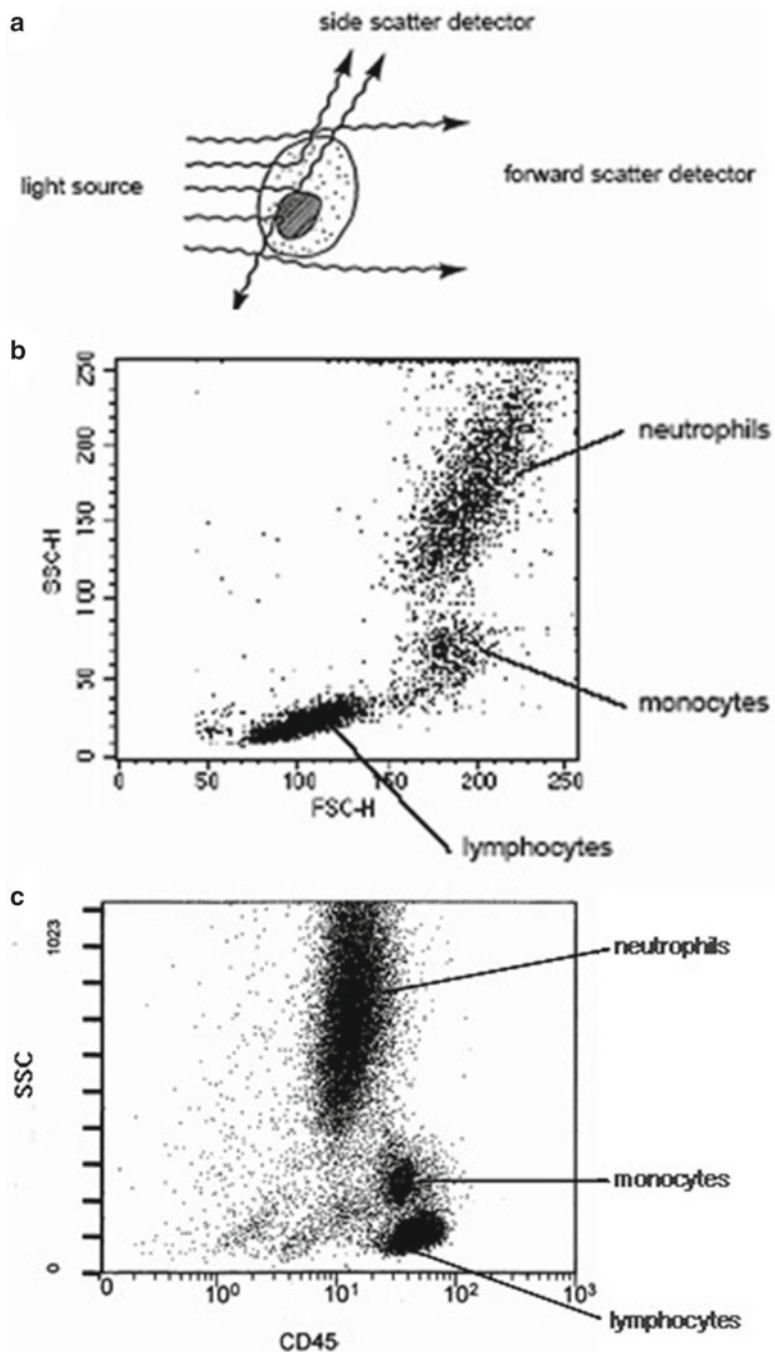


Fig. 22.4 (a) A schematic illustration showing transitive relationship of fluorescent light and cell size as well as complexity. (b) Histogram with forward scatter channel (*FSC*) and side scatter channel (*SSC*), showing clusters of neutrophils (granulocytes), monocytes, and lymphocytes in lysed normal peripheral blood. (c) Leukocyte population is ascertainable in lysed peripheral blood with CD45 gating (CD45 vs. SSC_{linear})

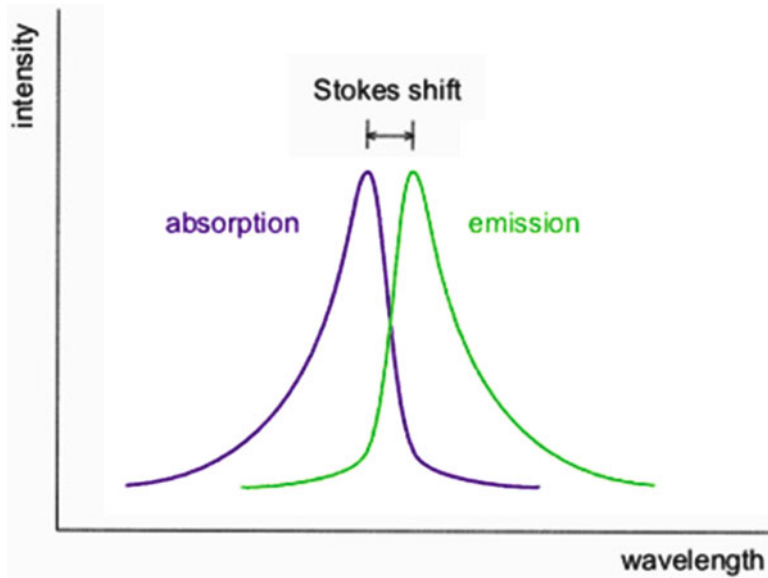


Fig. 22.5 Stokes shift. The distance (wavelength) between distinct positions of band maxima of the fluorescent absorption and emission spectra is known as Stokes shift ([wikipedia.org](http://en.wikipedia.org/wiki/Stokes_shift); http://en.wikipedia.org/wiki/Stokes_shift)

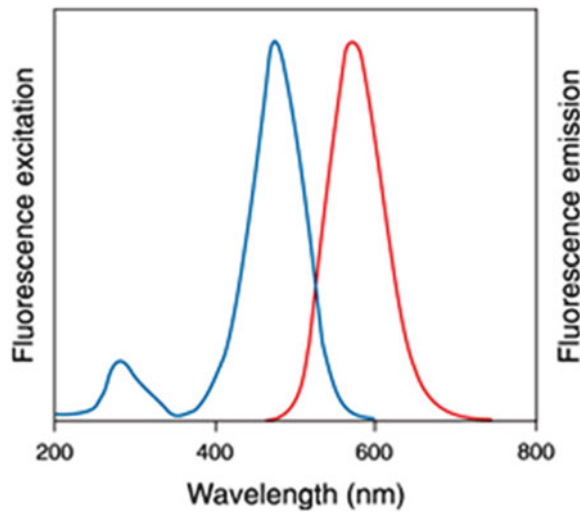


Fig. 22.6 Light absorption and emission. A fluorochrome absorbs energy of a specific wavelength (excitation) and reemits energy as fluorescence at a different wavelength (emission). The light emitted as fluorescence is a longer wavelength than the absorbed light

22.3.3 Spectral Overlap and Compensation

The optical filters in a flow cytometer are designed to capture ranges of wavelengths, with the center of each range near the maximum emission of a specific fluorochrome. Because fluorochromes emit over a range of wavelengths, some of the wavelengths may be identified by multiple detectors (Fig. 22.7).

Table 22.2 Fluorochrome commonly used in a clinical flow cytometry laboratory

Fluorochrome molecular probe	Excitation wavelength (nm)	Emission wavelength (nm)	Molecular weight (kDa)
4',6-Diamidino-2-phenylindole (DAPI)	345	455	227
Pacific blue	403	455	406
R-Phycoerythrin (PE)	480, 565	578	240
PE-Cy5 conjugates	480, 565, 650	670	N/A
PE-Cy7 conjugates	480, 565, 743	767	N/A
Peridinin chlorophyll protein (PerCP)	490	675	N/A
Alexa Fluor 488	499	519	643
Fluorescein isothiocyanate (FITC)	495	519	389
Propidium iodide (PI)	536	617	668
LDS 751 (C ₂₅ H ₃₀ ClN ₃ O ₄) [§]	543, 590	712, 607	472
Actinomycin D	546	647	1,270
SYTOX Orange ^{§§}	547	570	~500
Texas Red (or sulforhodamine 101 acid chloride)	589	615	625
Allophycocyanin (APC)	650	660	104
APC-Cy7 conjugates	650,755	767	N/A

[§]LDS 751 (C₂₅H₃₀ClN₃O₄) is a cell-permeant nucleic acid stain which has definition as Quinolinium, 6-(dimethylamino)-2-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl, perchlorate

^{§§}SYTOX Orange is a DNA/RNA dye for cell-impermeant nucleic acid SYTOX dead cell staining which is available from Molecular Probes (provided by Invitrogen)

Table 22.3 Immunophenotypic cellular markers commonly used in diagnostic flow cytometric analysis

Category of phenotypes	Antibody reactivity with extracellular and intracellular antigens
Pan-hematopoietic cell antigens	CD45 (common WBC antigen), CD45RA (naïve T-cells), and CD45RO (activated and memory T-cells)
T lymphocyte antigens	CD2, CD3, CD4/CD8, CD5, CD7, TCR _{αβ} and TCR _{γδ}
B lymphocyte antigens	CD10, CD19, CD20, CD22, CD23, CD79, CD138, Igκ and Igλ
Natural killer cell antigens	CD16, CD56, and CD57
Myeloid/monocyte antigens	CD11b, CD11c, CD13, CD14, CD33, CD64, CD123, and CD163
Erythrocyte antigens	CD235a (glycophorin A)
Platelet antigens	CD41, CD42b, and CD61
Stem cell/progenitor antigens	CD10, CD34, and TdT
Activation (non-lineage) antigens	CD25, CD30, CD38, CD54, CD71, and HLA-DR

CD cluster of designation, *WBC* white blood cell, *TdT* anti-terminal deoxynucleotidyl transferase, *HLA-DR* Human leukocyte antigen DR, *TCR* T-cell receptor, *Ig* Immunoglobulin

To ensure measurements in each of the fluorochrome-specific detectors come only from that specific fluorochrome, one must compensate for the spectral overlap from the other fluorochromes [7]. Compensation is an electronic process by which spillover fluorescence is removed from secondary parameters so that the fluorescence value for a parameter reflects only the primary fluorescence. This process is also referred to as spectral compensation and can be completed either by manipulating the electronics (digital signals) of the flow cytometer itself or by applying a software algorithm.

22.3.4 Data Analysis

Before analyzing flow cytometric data, patient’s age, sex, specimen type, and past medical history, along with other relevant clinical laboratory results (cytology, cytogenetics, molecular testing) and morphologic evaluations should be reviewed. Analysis of flow cytometric data is generally performed

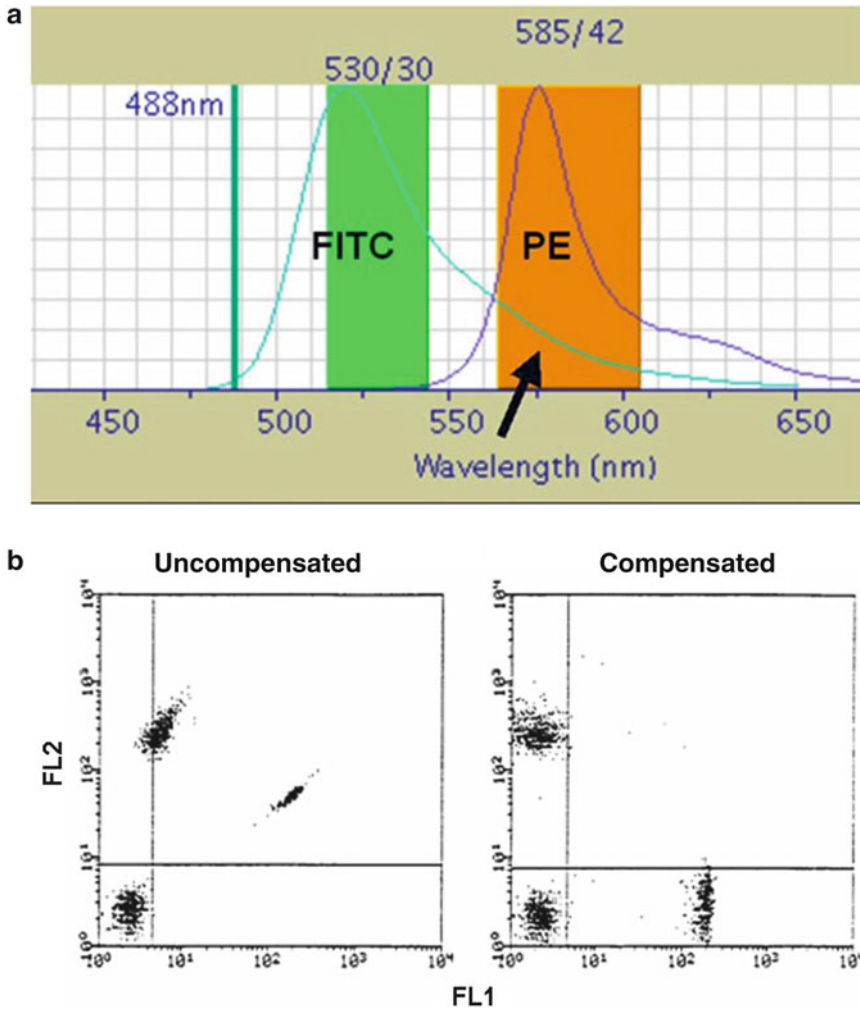


Fig. 22.7 Spectral overlap and compensation. (a) Two fluorochromes with distinct emission spectra. Although only the peak portion of the spectrum is distinct, there is a small overlap that requires compensation. (b) Fluorescence signal acquired from two immunostaining fluorochromes (FL1, FL2) with overlapping emission spectra before and after compensation

to ascertain subpopulation frequencies and relative antigen values. Once a signal has been digitized, this digitized value is assigned a channel number (usually 125 or 1,024) to which it will be plotted on a graph. This plotting is done for each cell for each parameter collected (i.e., FSC, SSC, all FL channels) and is displayed in the form of a histogram. These data are stored in a listmode file for each cell as it comes through the cytometer. Flow cytometry can be used to analyze any of the parameters as a single parameter or as a combination of correlated parameters from the listmode results. Multiple options also exist for the way the data can be displayed (see Fig. 22.8):

Histograms. Histograms are used for displaying single-parameter data where the x-axis represents scatter or fluorescence intensity and the y-axis represents the number of events. Histograms represent a single dimension and as a result have limited usefulness. Therefore, histograms are best suited for well-resolved homogeneous populations or for comparing intensities of multiple samples for a single parameter.

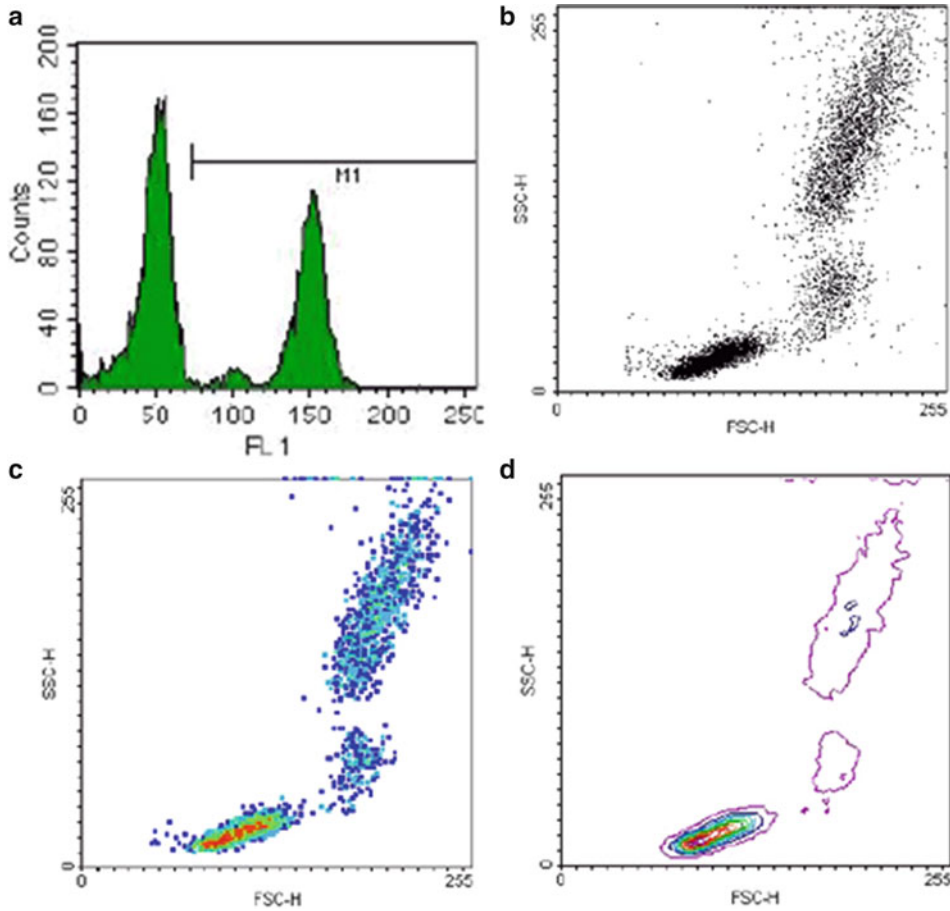


Fig. 22.8 Commonly used displays of flow cytometric data analysis from lysed peripheral blood. (a) Histogram. (b) Dot plot. (c) Density plot. (d) Contour diagram

Dot Plots. Dot plots can plot data values for each cell for any two acquired parameters (X and Y), where one dot on the display is related to the amounts of these parameters for each cell that passes through the flow cytometer. Dot plots are good for detecting small numbers of events that are well separated from the large subpopulations of the cells present, but give little or no indication of the relative density of the numbers of events in those populations.

Density Plots. Density plots simulate a three-dimensional display of events, with the third parameter being the number of events. Density plots can dramatically emphasize, or hide altogether, subpopulations of cells.

Contour Plots. Contour plots are another two-dimensional display of relative X and Y amounts of two parameters, with contour lines being drawn to form x- and y-axis coordinates that have similar numbers of cells.

Gating. Gating is establishing a graphical or numerical region (boundary) in the flow cytometric data that defines a subpopulation of cells to be further analyzed. Gates can be single or multidimensional, and they often include rectilinear, ellipsoid, and linear shapes for univariate histograms and freehand shapes for other areas of interest. Advanced software allows gate setup to be done before or during acquisition to selectively acquire data (live gate) or specify the subpopulation of interest to sort, or gate setup can be done after data acquisition so that data for a specific subpopulation can be analyzed [7, 8].

22.4 Procedures

22.4.1 Specimen Types and Handling

In general, the following specimen types are suitable for flow cytometric analysis: whole blood, bone marrow, body fluid, lymph nodes, fresh biopsy tissues, and adherent/nonadherent cells from culture. For precise antigen detection and immunophenotyping, samples must be collected and submitted to the flow cytometry laboratory as soon as possible. For example, all blood/bone marrow and fresh biopsy tissue samples collected for hematology neoplasm analysis should be tested for viability if the time since the sample was collected exceeds 48 h.

Note: Recommendations for hematologic samples: (1) Collect venous blood specimen aseptically by venipuncture into a blood collection tube using the recommended anticoagulant ethylenediaminetetraacetic acid (EDTA). Whole blood specimens obtained in tubes with heparin or acid-citrate dextrose as an anticoagulant are also acceptable. Heparin is the preferred anticoagulant for bone marrow. (2) Perform a total WBC count using an established procedure [9, 10]. (3) Dilute or adjust the concentration of the single-cell suspension for specimens with WBC counts outside the normal range $3\text{--}10 \times 10^3$ cells/ μl .

22.4.2 Reagents and Materials

22.4.2.1 Sample Type

Sample materials such as bone marrow, fresh biopsy tissues, and cultured cells may include single-cell suspensions.

22.4.2.2 Instrumentation and Materials

Equipment

- Multiparameter flow cytometer equipped with two lasers, four- to six-color in vitro diagnostics (IVD) flow cytometer, e.g., Coulter FC-500 (Beckman Coulter, Brea, CA), BD FACSCaliber, BD FACSCanto (BD Biosciences, San Jose, CA), or equivalent
- IVD automated sample-preparation workstation, e.g., Coulter TQ-Prep Plus 2 (Beckman Coulter), BD FACS Sample Prep Assistant III (SPA-III) (BD Biosciences), equivalent
- Cell counter or hematology analyzer

Materials (required but not supplied)

- Disposable 12- \times 75-mm polypropylene tubes
- Blood collection tubes with anticoagulant (EDTA)
- Transfer pipettes
- Pasteur pipettes
- Micropipettes
- Vortex
- Centrifuge
- Additional appliances (e.g., surgery knife, scissor, tweezers, and mesh) for dissociating cells from solid tissues

Note: IVD refers to an in vitro diagnostics medical device, reagent product, or relevant kit that has been approved or cleared by the Food and Drug Administration for clinical laboratory use.

22.4.2.3 Required Reagents

Reagents (required but not supplied)

- Phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) staining buffer (PBS, pH 7.4, and 1% BSA)
- PBS/1% paraformaldehyde (PFA) fixing buffer (PBS, pH 7.4, and 1% PFA)
- Permeabilization/staining buffer (PBS, pH 7.4; 1% BSA; and 0.5% saponins)
- Monoclonal antibodies labeled with fluorochrome to human leukocyte antigens and other cellular markers
- 1× ammonium chloride lysing solution
- Coulter lysing/fix kit or equivalent
- Coulter Flow-Count fluorospheres or equivalent
- Human FcR blockage reagent (Miltenyi Biotec Inc., Auburn, CA)

22.4.3 Preparation of Single-Cell Suspension

A monodispersed (single) cell suspension must have a density of $0.5\text{--}1.0 \times 10^7$ cells/ml to prevent the narrow bores of the flow cytometer and its tubing from clogging up. A common suspension buffer is PBS/1% BSA staining buffer, and the most straightforward samples for flow cytometry include non-adherent cells from culture, whole blood, bone marrow, and solid tissues. Whole blood is the most common type of specimen for flow cytometric testing, and it is easy to use unless RBCs must be removed by a simple lysis/no-wash or lysis/then-wash step [9, 10]. The resulting blood sample without RBCs allows for quick identification of lymphocytes, granulocytes, and monocytes by their FSC/SSC characteristics [11, 12].

22.4.3.1 Preparation from Fresh Biopsy Tissues

1. Place tissue on a sterile Petri dish. Remove cells by gently perfusing the tissue using a syringe and needle containing approximately 15 ml of PBS/1% BSA staining buffer.
2. Transfer the cell suspension from the Petri dish into a 15-ml conical centrifuge tube.
3. Wash the cells with PBS/1% BSA staining buffer by centrifugation at $500 \times g$ for 5 min.
4. Dispose of the supernatant and resuspend the pellet in the proper amount of PBS/1% BSA staining buffer.
5. Add 10 ml of 1× ammonium chloride lysing buffer.
6. Gently mix and incubate the solution for exactly 10 min.
7. Collect the cells by centrifugation at $500 \times g$ for 5 min.
8. Add 10 ml of PBS/1% BSA staining buffer and mix gently.
9. Wash the cells again with PBS/1% BSA buffer by centrifugation at $500 \times g$ for 5 min.
10. Dispose of the supernatant and resuspend the pellet to a final volume of 10 ml with PBS/1% BSA staining buffer.
11. Count the number of cells using a hematology analyzer or cell counter.

12. Adjust the single-cell suspension to 1×10^7 cells/ml as appropriate.
13. Keep the cells on ice or at 4°C until immunofluorescence staining is performed.

22.4.3.2 Preparation from Suspension-Culture Cell Lines

1. Using 15-ml conical centrifuge tube(s), collect the cells from the tissue culture flask.
2. Wash the cells with PBS/1% BSA staining buffer by centrifugation at $500 \times g$ for 5 min.
3. Dispose of the supernatant and resuspend the pellet in 10 ml of PBS/1% BSA staining buffer.
4. Wash the cells with PBS/1% BSA staining buffer by centrifugation at $500 \times g$ for 5 min.
5. Dispose of the supernatant and resuspend the cell pellet in PBS/1% BSA staining buffer to a concentration of 1×10^7 cells/ml.
6. Keep the cells on ice or at 4°C until immunofluorescence staining is performed.

22.4.3.3 Preparation from Adherent-Culture Cell Lines

1. Rinse the cells using 2 ml of PBS/1% BSA staining buffer and harvest the cells by gentle scraping.
2. Transfer the harvested cells to a 15-ml conical tube and add buffer until the volume in the tube is 10 ml.
3. Wash the cells with PBS/1% BSA staining buffer by centrifugation at $500 \times g$ for 5 min.
4. Dispose of the supernatant and resuspend the cell pellet in 10 ml of PBS/1% BSA staining buffer.
5. Wash the cells with PBS/1% BSA staining buffer by centrifugation at $500 \times g$ for 5 min.
6. Dispose of the supernatant and resuspend the cell pellet in PBS/1% BSA staining buffer to a concentration of 1×10^7 cells/ml.
7. Keep the cells on ice or at 4°C until immunofluorescence staining is performed.

22.4.3.4 Preparation from Frozen Cells

1. Carefully remove the frozen cells from liquid nitrogen or -80°C deep freezer storage.
2. Rapidly thaw the cells at 37°C and collect them in a 15-ml conical centrifuge tube containing 10 ml of PBS/1% BSA staining buffer.
3. Wash the cells with PBS/1% BSA staining buffer by centrifugation at $500 \times g$ for 5 min.
4. Repeat the wash described in step 3 one time.
5. Dispose of the supernatant and resuspend the cell pellet in PBS/1% BSA staining buffer to a concentration of 1×10^7 cells/ml.
6. Keep the cells on ice or at 4°C until immunofluorescence staining is performed.

22.5 Immunostaining Procedure

22.5.1 *Direct Immunofluorescence Staining of Lyse/No-Wash Whole Blood Samples*

1. For each sample, label two 12- \times 15-mm test tubes: one for the ordered antibody reagent (clinical panel) and one for the isotype antibody reagent control.
2. Pipet 100 μl of the sample with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant into the correspondingly labeled tubes.

3. Add 10 μ l of human FcR blockage reagent (Miltenyi Biotech Inc., Auburn, CA) to each corresponding tube. Mix gently and incubate at room temperature for 10 min.
4. Add the desired admixtures (usually 10 μ l of cocktail antibody reagent) of fluorescence-conjugated antibodies at the recommended concentration to the correspondingly labeled tube.
5. Vortex gently and incubate the reaction mixture at room temperature for 20 min.
6. Lyse and fix the cells in each tube using the procedures recommended for the selected lysing method (TQ-Prep/Plus2 system, Beckman Coulter, Miami, FL or equivalent). As an alternative, use a Coulter Immunoprep lysing and fixing kit (Beckman Coulter, Miami, FL) (contains red blood cell [RBC] lytic agent, white blood cell [WBC] stabilizer, and cell membrane fixative) or its equivalent; if manual processing is needed, follow the instructions supplied with the reagent.
7. Add 100 μ l of Coulter Flow-Count beads or their equivalent to each tube and mix well. Otherwise, use the results gathered from both hematologic and cytometric analyses to calculate the absolute cell count.
8. Analyze the processed cells on a flow cytometer capable of multicolor fluorescence analysis immediately.

Note: Absolute cell counts can be determined by two methods.

1. The single-platform (direct) method depends on using Flow-Count beads to directly determine the absolute count of the cells and uses the following formula:

$$\text{Absolute cell count (cells}/\mu\text{l)} = \frac{\text{total number of cells counted}}{\text{total number of beads counted}} \times \text{known concentration of Flow-Count beads.}$$
2. The dual-platform (standard) method calculates absolute cell count by combining results from flow cytometry and hematology/cell counter and uses the following formula:

$$\text{Absolute cell count (cells}/\mu\text{l)} = \frac{\text{total WBC count (cells}/\mu\text{l)} \times \text{percentage of positively stained cells} \times \text{percentage of lymphocytes}}{10^4}.$$
3. Human FcR blockage reagent should be used to block nonspecific binding of antibodies to human FcR-expressing cells such as B cells, monocytes, and macrophages.

22.5.2 *Direct Immunofluorescence Staining of Lyse/Then-Wash Whole Blood Samples*

1. For each sample, label as many 12- \times 15-mm test tubes as need: one tube for the ordered antibody reagent (clinical panel) and one for the isotype antibody reagent control.
2. Pipet 100 μ l of the sample with EDTA as the anticoagulant into its correspondingly labeled tube.
3. Add 10 μ l of human FcR blockage reagent to its corresponding tube. Mix gently and incubate at room temperature for 10 min.
4. Add the desired admixtures (usually 10 μ l of cocktail antibody reagent) of fluorescence-conjugated antibodies at the recommended concentration to each tube.
5. Vortex the mixture gently and incubate the reaction mixture at room temperature for 20 min.
6. Lyse and fix the cells in each tube using the procedures recommended for the selected lysing method (TQ-Prep/Plus2 system or equivalent). As an alternative, use a Coulter lysing/fixing kit (contains RBC lytic agent, WBC stabilizer, and cell membrane fixative) or its equivalent; if manual processing is needed, follow the instructions supplied with the reagent.
7. Wash the cells by adding 2 ml of phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) staining buffer at room temperature.
8. Centrifuge the single-cell suspension at 500 \times g for 5 min. Rapidly invert the tube or discard the supernatant by aspiration.
9. Repeat the wash in steps 7 and 8 one time.

10. Resuspend the stained cell pellets in 0.5 ml of PBS/1% BSA staining buffer or PBS/1% PFA (paraformaldehyde) fixing buffer at room temperature, if necessary.
11. Analyze the processed cells on a flow cytometer capable of multicolor fluorescence analysis immediately.

22.5.3 Indirect Immunofluorescence Staining of Single-Cell Suspension

1. Label as many 12- \times 15-mm test tubes as needed.
2. Prepare the single-cell suspension appropriately (see above for cell type preparation) and adjust the single-cell suspension to a concentration of 1×10^7 cells/ml with PBS/1% BSA staining buffer. Analyze the processed cells on a flow cytometer capable of multicolor fluorescence analysis immediately.
3. Aliquot 100 μ l of the single-cell suspension (i.e., 1×10^6 cells) into 12- \times 15-mm test tubes.
4. Add 10 μ l of human FcR blockage reagent to each tube. Mix gently and incubate at room temperature for 10 min (optional).
5. Add the primary antibody at the recommended concentration to each tube. Mix well and incubate on ice or at 4°C for 20 min.
6. Wash the cells by adding 2 ml of PBS/1% BSA staining buffer at 4°C.
7. Centrifuge the single-cell suspension at $500 \times g$ for 5 min. Rapidly invert the tube or discard the supernatant by aspiration.
8. Resuspend the stained cell pellet in 0.1 ml of PBS/1% BSA staining buffer.
9. Add an appropriate secondary antibody at the recommended concentration. Mix well and incubate at room temperature for 20 min.
10. Repeat the wash described in steps 5 and 6 one time.
11. Resuspend the stained cell pellet in 0.5 ml of PBS/1% BSA staining buffer or PBS/1% PFA fixing buffer, if necessary.
12. Store the stained single-cell suspension on ice or at 4°C.
13. Perform flow cytometric analysis immediately.

22.5.4 Indirect Immunofluorescence Staining of Whole Blood Samples

1. For each sample, label two 12- \times 15-mm test tubes: one test tube for the ordered antibody reagent (panel) and another tube for the secondary reagent control.
2. Pipette 100 μ l of the sample with EDTA as the anticoagulant into correspondingly labeled tubes.
3. Add 10 μ l of human FcR blockage reagent to its corresponding tube. Mix gently and incubate at room temperature for 10 min.
4. Add the primary antibody at the recommended concentration to each tube. Mix well and incubate at room temperature for 20 min.
5. Wash the cells by adding 2 ml of PBS/1% BSA staining buffer at room temperature.
6. Centrifuge the single-cell suspension at $500 \times g$ for 5 min. Rapidly invert the tube or discard the supernatant by aspiration.
7. Resuspend the stained cell pellet in 0.1 ml of room temperature PBS/1% BSA staining buffer.
8. Add an appropriate secondary antibody at the recommended concentration. Mix well and incubate at room temperature for 20 min.
9. Lyse and fix the cells in each tube using the procedures recommended for the selected lysing method (TQ-Prep/Plus2 system or equivalent). As an alternative, use the commercial Coulter

lysing/fixing kit (containing RBC lytic agent, WBC stabilizer, and cell membrane fixative) or its equivalent; if manual processing is needed, follow the instructions supplied with the reagent.

10. Repeat the wash in steps 5 and 6 one time.
11. Resuspend the stained cell pellet in 0.5 ml of PBS/1% BSA staining buffer or PBS/1% PFA fixing buffer, if necessary.
12. Keep the stained single-cell suspension at 4°C and dark environment which is preferred for 24 h.
13. Perform flow cytometric analysis.

22.5.5 Direct Immunostaining of Intracellular Antigens Using Whole Blood

1. Collect the whole blood samples mixed with EDTA and treat them with the appropriate activator, if applicable.
2. Aliquot the 100- μ l samples into the appropriate number of tubes.
3. Add 10 μ l of human FcR blockage reagent to its corresponding tube. Mix gently and incubate at room temperature for 10 min.
4. Perform immunostaining for the cell surface antigens by adding fluorescent antibodies and then incubate the mixture for 15 min.
5. Add 2 ml of 1 \times ammonia chloride lysing buffer, gently mix, and incubate the mixture for 10 min.
6. Centrifuge the cells at 500 \times g for 5 min. Rapidly invert the tube or discard supernatant by aspiration.
7. Add 2 ml of PBS/1% PFA fixing buffer and incubate for 5 min at room temperature.
8. Isolate the cell pellet by repeating step 5.
9. Permeabilize the cells by adding 100 μ l permeabilization/staining buffer to each tube and incubate the mixture for 15 min at room temperature.
10. Incubate the cells with the required anti-intracellular antibodies at the recommended concentration for 30 min at room temperature.
11. Wash the cells by adding 2 ml of PBS/1% BSA staining buffer at room temperature.
12. Isolate the pellet of antibody-labeled cells by repeating step 5.
13. Resuspend the cell pellet in 0.5 ml PBS/1% BSA staining buffer.
14. Use the capture beads to set proper compensation controls and analyze the intracellular immunofluorescence of stained cells by flow cytometry immediately.

Note: The detection of intracellular antigens requires a cell permeabilization step prior to staining. Antibodies for intracellular compartments cannot be obtained if staining buffer without saponin is used because saponin-based permeabilization is reversible.

22.5.6 Direct Immunostaining of Intracellular Antigens Using Cultured Cell Lines

1. Harvest the cells from cultured plates and determine the total cell number in the cell collection.
2. Wash the cells once in PBS/1% BSA buffer and adjust the cell concentration to 1×10^7 cells/ml.
3. Aliquot 100 μ l of the single-cell suspension (i.e., 1×10^6 cells) into an appropriate number of tubes.
4. Immunostain the cell surface antigens using appropriate directly conjugated monoclonal antibodies at the recommended concentration(s). Incubate for 15 min at 4°C.
5. Wash the cells once by adding 2 ml of PBS/1% BSA staining buffer at 4°C.
6. Centrifuge the cells at 500 \times g for 5 min. Rapidly invert the tube or discard the supernatant by aspiration.
7. Resuspend the cells in 2 ml of PBS/1% PFA fixing buffer for 5 min at 4°C.

8. Wash the cells once by repeating steps 5 and 6.
9. Permeabilize the cells by adding 100 μ l of permeabilization/staining buffer to each tube and incubate for 15 min at room temperature.
10. Add fluorochrome directly conjugated antibodies to the intracellular antigens at the recommended concentration(s). Incubate for 30 min at 4°C.
11. Wash the cells twice by repeating steps 5 and 6 and then resuspend the cells in 0.5 ml of PBS/1% PFA fixing buffer.
12. Store the processed cells at 4°C and in a dark environment until flow cytometry analysis, which is performed preferably within 24 h.

Note: Appropriate standards serving as positive and negative control should always be included, e.g., an isotype-matched control sample for each flow cytometry run.

22.6 Clinical Applications of Flow Cytometry

22.6.1 *Quantitative Flow Cytometric Analysis of Peripheral Blood T-Lymphocyte Subsets*

The outbreak of acquired immunodeficiency syndrome (AIDS) accelerated the acceptance of flow cytometers as routine laboratory instruments because of the tremendous demands for testing the absolute CD4+ T-lymphocyte count with T-helper (CD4+): T-suppressor (CD8+) cell ratio as an early screening technique [13]. A principal molecular basis whereby human immunodeficiency virus (HIV) destroys normal immune function is infection and depletion of a subpopulation of T-cell expressing the CD4 surface antigen [14].

Enumeration of the CD4 T-helper lymphocyte count has many roles in HIV infection. First, the CD4 T-helper count can contribute to diagnosis in a symptomatic patient. In individuals with HIV, a CD4+ T-helper lymphocyte count below 500 cells/ μ l indicates that steps must be taken to prevent an opportunistic infection; a decrease in the level to less than 200 cell/ μ l meets the clinical definition of AIDS [15]. Second, the extent of depletion of CD4+ T-helper lymphocyte counts in patients with HIV is closely associated with clinical prognosis, facilitating estimates of disease progression to AIDS or death. Third, in combination with molecular measurement of HIV viral loads, CD4+ T-helper lymphocyte counts provide a quantitative assay for monitoring the efficacy of antiretroviral treatment administered to patients as well as for preventing opportunistic infections [16].

Nationwide guidelines for CD4+ T-helper and other subset enumerations in patients with HIV have been published by the Centers for Disease Control and Prevention [17]. T-lymphocyte subpopulation analysis is used to evaluate HIV status. Absolute counts and percentages of T-helper (CD4+), T-cytotoxic (CD8+), B-cell (CD19+), and natural killer (NK, CD56+) lymphocytes are measured as the subpopulations (Table 22.4). Typically, flow cytometry is used to calculate the percentage of WBCs that are CD3+ T-lymphocytes that also express the T-helper (CD3+CD4+) and T-cytotoxic (CD3+CD8+) subsets (Fig. 22.9).

22.6.2 *Immunophenotypic Analysis of Leukemia and Lymphoma*

During the typical life cycle of hematopoietic cells, a particular expression pattern of cellular markers can be characterized at every development stage. Flow cytometry-based detection of hematologic neoplasms depends on the principle that malignant cells express cellular molecules in different patterns

Table 22.4 CDC recommended panels for human lymphocyte subpopulation analysis

Testing tubes	Monoclonal antibody combination
	Four-color
Tube 1	CD45, CD3, CD4, CD8
Tube 2	CD45, CD3, CD19, CD16-CD56
	Three-color
Tube 1	CD45, CD3, CD4
Tube 2	CD45, CD3, CD8
Tube 3	CD45, CD3, CD19
Tube 4	CD45, CD3, CD16-CD56

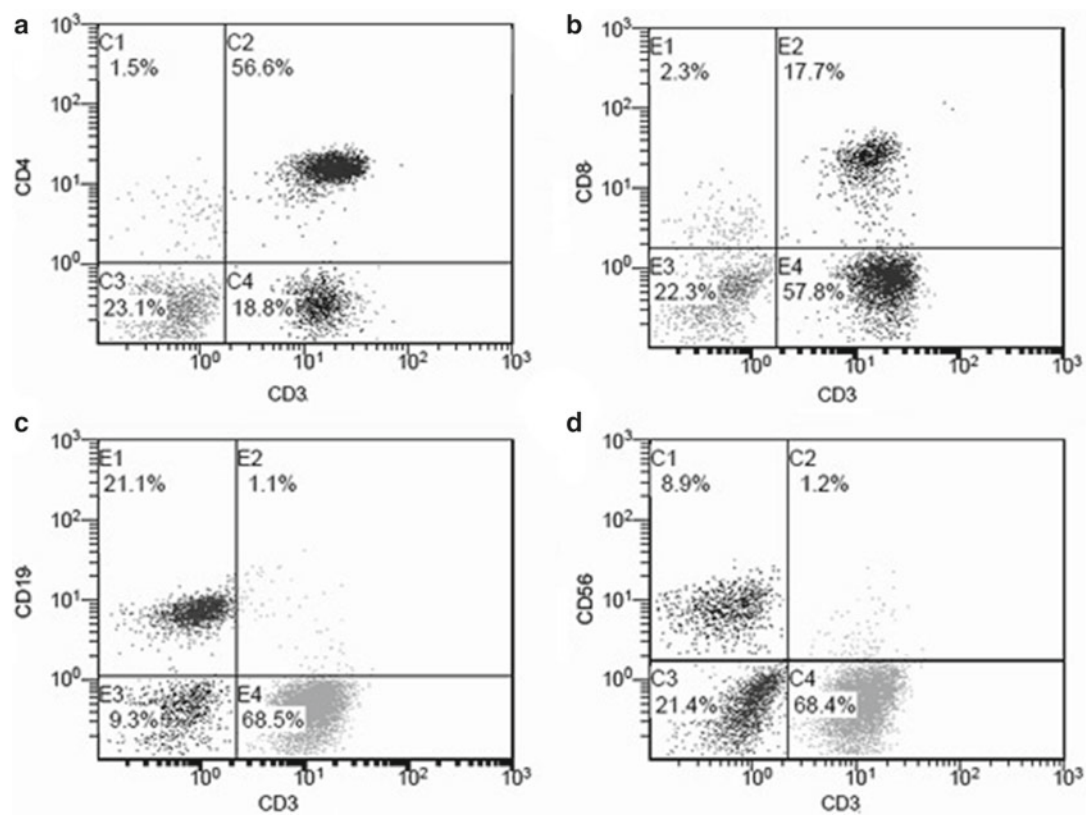


Fig. 22.9 Automated four-color panel for lymphocyte subpopulation analysis by flow cytometry. Enumeration results for lymphocyte subset are regularly expressed as percentage (%) of lymphocyte gated and absolute count (cells/ μ l). (a) T-helper, CD3+CD4+. (b) T-suppressor, CD3+CD8+. T-helper/T-suppressor ratio is routinely available (normally, between 1 and 1.5). (c) B-cell, CD19+CD3-. (d) NK-cell, CD56+(CD16+)CD3-

than normal cells [18]. Abnormal patterns of those cellular antigens in hematological neoplasms include:

1. Expression of a nonlineage-specific antigen
2. Abnormal absence or change in expression levels of normal antigens
3. Undifferentiated (homogenous) expression of antigens in a cell population that normally has more differentiated expression
4. Expression of an antigen at the wrong stage of development [19]

Table 22.5 Minimal antibody panels for diagnostic flow cytometry of hematological neoplasia

Flow cytometry panels	Individual cellular markers
T-cell leukemia/lymphoma	CD3/CD4, CD3/CD8, CD5, CD7, CD25, CD30
Chronic lymphocytic leukemia (CLL)/B-cell lymphoma	Surface kappa/lambda, CD5/CD19, CD23, CD10, FMC-7
Acute lymphoblastic leukemia (ALL)	Kappa/lambda, cytoplasmic μ , CD7, CD10/CD19, CD34, TdT
CLL prognostication	CD2/CD7, CD10/CD34, CD13-CD33, CD38, ZAP-70
Natural killer cell leukemia/lymphoma	CD2, CD3/CD4, CD3/CD8, CD16, CD56, CD57
Hair cell leukemia (HCL)	Surface kappa/lambda, CD11c/CD22, CD25, CD103, FMC-7
Myeloma/marcroglobulinemia	CD5/CD19, CD38/CD138, CD56, surface, and cytoplasmic kappa/lambda
Acute myeloid leukemia (AML)	CD14, CD13-CD33/CD7, CD34, CD117, HLA-DR, MPO
AML-M6 (erythroleukemia)	AML panel coupled with CD235a (glycophorin A)
AML-M7 (megakaryoblastic leukemia)	AML panel coupled with CD41, CD42b, CD61

MPO myeloperoxidase, *TdT* terminal deoxynucleotidyl transferase, *FMC-7* B-lymphocyte antigen CD20

Clinically, immunostaining panels of monoclonal antibodies to maturation-specific antigens and lineage-specific (e.g., lymphoid or myeloid) are used to identify cell populations with abnormal patterns of antigen expression (Table 22.5). The Clinical Cytometry Society has recommended essential antibody panels to be used for immunophenotyping hematological neoplasms [20]. Owing to the complexity of leukemia and lymphoma, flow cytometry is performed using a normal control. In order to visualize all populations, the forward scatter channel (FCS)/side scatter channel (SSC) of the instrument should be adjusted according to each patient's sample. The use of CD45 gating (CD45 vs. SSC plot) in combination with additional lineage-specific markers has been accepted as a standard approach for detecting hematopoietic neoplasia cells from normal heterozygous populations [9]. Therefore, flow cytometry, in conjunction with various laboratory studies (cytomorphologic, cytogenetic, and molecular), is used to reach a definitive diagnosis because many hematological neoplasms have unique immunophenotypic profiles that can be characterized by flow cytometry (Fig. 22.10).

22.6.3 Enumeration of CD34+ Hematopoietic Stem and Progenitor Cells

Hematopoietic stem cells are capable of proliferating and differentiating into WBCs, RBCs, and platelets through in vivo/ex vivo hematopoietic reconstitution and are being used in a wide range of therapies for malignant and nonmalignant diseases. Hematopoietic stem cells expressing CD34+ can be harvested either from the patient prior to therapy (autologous) or from an immunologically compatible donor (allogeneic). Successful transplantation and promotion of reconstitution require an adequate amount of CD34+ stem cells obtained from the recipient's bone marrow, peripheral blood, and cord blood [10]. Therefore, flow cytometry-based CD34 absolute count is needed for accurate enumeration of CD34+ stem cells to produce effective and reproducible clinical results.

Clinically, both single-platform and dual-platform CD34 assays are used for rapid enumeration by flow cytometry [11]. A straightforward immunophenotyping panel can be used to determine the proportion and absolute number of CD34+ stem cells in the total CD45+ WBC population of sample preparations (Fig. 22.11). To accurately measure rare CD34+ stem cells in hematopoietic specimens, it is important to use a class II or class III CD34 antibody that detects all glycosylation variants of the molecules and antibodies conjugated to fluorochrome phycoerythrin (PE). PE yields the strongest fluorescence brightness and enhances the sensitivity of CD34 stem cell assays. In addition, many total events are required for precise quantification because CD34+ stem cells are usually less than one percent (5–10 cells/ μ l) of peripheral blood and cord blood [12].

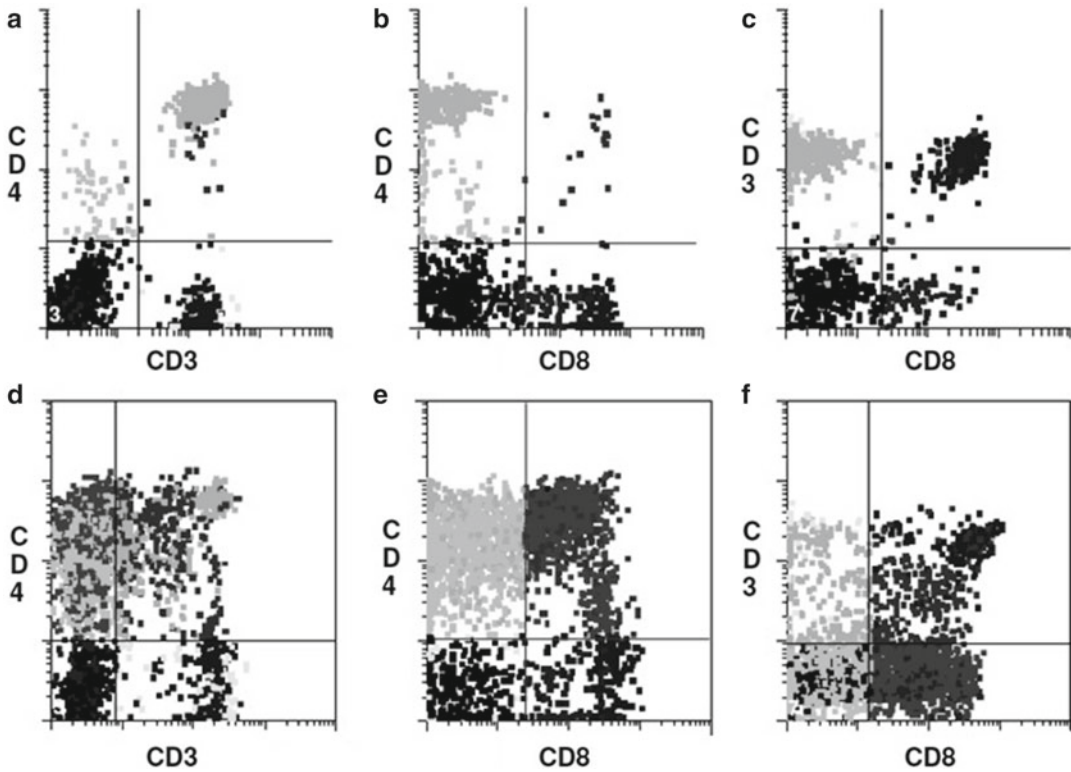


Fig. 22.10 The diagnostic immunostaining pattern of T-cell acute lymphocyte leukemia/lymphoma (T-ALL) in peripheral blood. The normal lymphoid subpopulations depicting in (a, b, and c) are highlighted for reference. (d) An abnormal cluster is significantly overexpressed CD3 + CD4^{dim-bright}. (e) An abundant CD4 + CD8+ double positive cells appear; that indicate T lymphoid precursor in an earlier stage. (f) Owing to noticeably increased number of CD3 + CD8+ T-cells. These three cellular markers are normally expressed in different maturation stages

The absolute number of CD34+ stem cells provides an estimate of the repopulating capacity of a stem and progenitor cell product [21]. The clinical efficacy of counting the absolute number of CD34+ stem cells has proven advantageous over the previously utilized approach of determining the total mononuclear cell dose that was performed for the quantity of stem cell preparation to transplantation [22].

22.6.4 DNA Content/Ploidy Analysis

DNA content/ploidy analysis by flow cytometers was originally developed to provide a rapid screening method for cervical cancer specimens to measure altered DNA content. Compared with immunophenotyping, DNA content analysis provides prognostic information to cancer patients and provides a diverse way to characterize hematological neoplasia cells. The cell cycle phase most often observed is S-phase. S-phase cells are in DNA replication, which is related to proliferation.

When a population of nuclei is stained with propidium iodide (PI) or a similar DNA-binding fluorescent dye, a histogram of the DNA content reflects the proportions of cells in different phases of the cell cycle. DNA ploidy analysis determines whether cells in a population contain a normal or abnor-

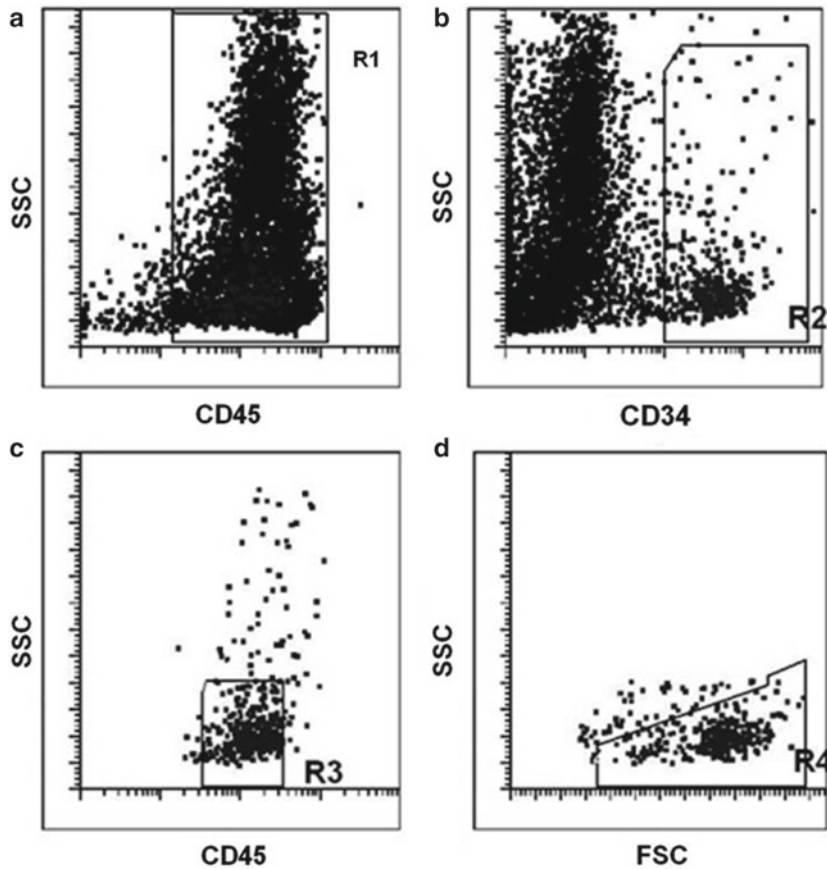


Fig. 22.11 Identification of CD34⁺ hematopoietic stem cells. (a) Using CD45 gating strategies, CD45⁺ WBCs in R1 are initially acquired, and non-WBCs as well as debris are excluded. (b) In order to obtain CD34⁺ cell absolute count, the second gate, R2, is drawn around CD34^{bright}-expressing cells. (c) The cells are further confined to CD45^{dim} and low SSC cells through the third gate, R3. (d) CD34⁺ stem cells are displayed in R4 with a scatter pattern, confirming the cellular features of heterogeneous size and low SSC

mal amount of DNA compared with a normal control. Cells with a normal content are referred to as DNA diploid[§], whereas those with an abnormal content are referred to as DNA aneuploid (Fig. 22.12). Currently, DNA histograms can be easily analyzed by automated software packages. The lengths of cell cycle phases, coefficient of variation (CVs) of the G0/G1 and G2/M peaks^{§§}, diploid percentage, aneuploid percentage, and the DNA index (DI) are statistically calculated and made available for reporting. A DI of 1.0 denotes diploidy, and a DI other than 1.0 denotes aneuploidy. For example, a DI of 2.0 indicates that the tumor cells tested are aneuploid and, more specifically, tetraploid [23].

Note: [§]The amount of DNA content (2n vs. n) from which cell cycle statistics can be calculated via a software model (ModFIT or FlowJo, see Fig. 22.12) correlates fluorescence intensity of DNA dye, such as the PI, when the single-cell is stained with the PI and exhibits stoichiometric binding.

^{§§}CVs of the G0/G1 and G2/M peaks are used to quantitate the DNA content of the PI-staining cells of interest.

In the determination of ploidy and analysis of cell division kinetics, such as percentage of S-phase fraction and doubling time, the DI has important implications in areas such as clinical

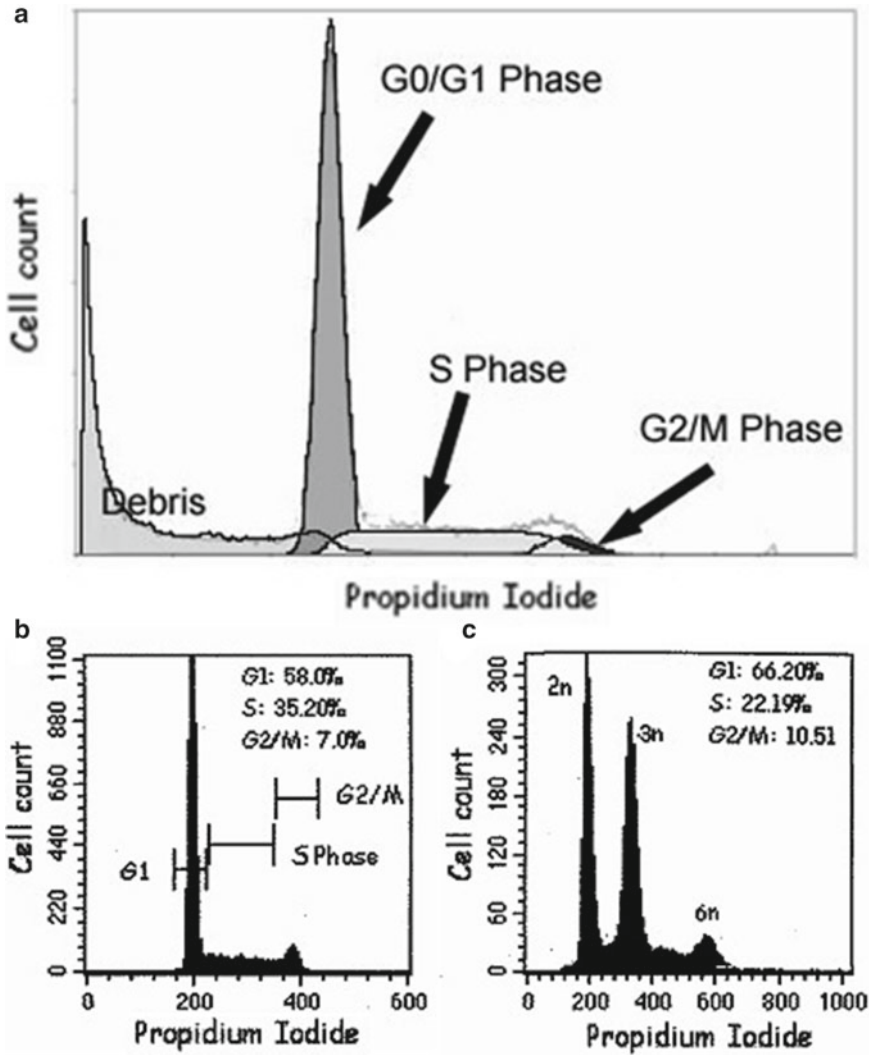


Fig. 22.12 (a) A schematic 1-parameter histogram of cellular DNA ploidy/cell cycle assessed by propidium iodide (b) Normal DNA ploidy/cell cycle model. Fluorescence peak of G0/G1-phase, 2n DNA is near channel 200; however, that of G2/M-phase, 4n DNA is near channel 400. (c) Abnormal DNA ploidy/cell cycle pattern. At about channel 200, there is a normal G0/G1 peak (2n DNA), whereas close to channel 300, one more G0/G1 peak of the triploid (aneuploid), 3n DNA cell subset is identified. Another aneuploid population of 6n DNA cells, which represents as the G2/M peak, is located near channel 600. The statistics are calculated via a software model of either ModFIT (Verity Software House, Topsham ME) or FlowJo (Tree Star Inc., Ashland, OR)

tumor prognosis [24]. DNA ploidy is also typically evaluated at the time of diagnosis in patients with precursor B-cell acute lymphoblastic leukemia/lymphoma (ALL), in which a hyperdiploid karyotype is associated with a favorable prognosis [25]. Some hematolymphoid neoplasms exhibit low rates of proliferation, and this can make tumor cells less susceptible to cytokine chemotherapy. Therefore, monitoring the percentages of S-phase fraction and ploidy is influential to the decision of when to start chemotherapy following cytokine treatment [26].

22.7 Troubleshooting

22.7.1 *Quality Control*

To ensure that a flow cytometer provides consistent results, optical and fluorescent standards are necessarily performed on a daily basis, and results are recorded daily in the quality control logbook. First, the flow cytometer must be evaluated with standardized fluorescent beads to determine if it is functioning properly [27]. These beads generate very precise, reproducible patterns, instrument settings, and signal intensity. The values obtained are used to determine cytometer error and produce a Levy-Jenning plot. Second, an appropriate compensation setting is established using antibody-stained cells and/or standardized beads; consequently, either the cells or the beads should imitate the staining, which should be the same as that of the actual specimens. Third, a control substance that mimics actual specimens should be used to assess the sensitivity of the flow cytometer. The control substances usually contain normal, stabilized blood cells, although some protein characteristics of specific cancer cells will not be present. The checkpoints for daily quality control in a flow cytometry laboratory are listed below [28]:

1. Ensure the flow cytometer is properly aligned and standardized for FSC/SSC and fluorescence intensities according to the manufacturer's instructions.
2. The fluorochromes emit at different wavelengths; however, they also have some overlap that should be appropriately corrected by electronic or software compensation.
3. Analyze a pair of positive and negative assay controls before running samples to verify compensation and antibody reactivity.
4. If the assay allows, consider using a tube of nonstained cells to establish the background level of the cells' autofluorescence instead of using a tube of isotype control-stained cells.

22.7.2 *Advantages and Limitations of Flow Cytometry*

Flow cytometry has the advantage of being more efficient, sensitive, accurate, and reproducible than other manual techniques, such as cytochemistry and immunohistochemistry. With modern a flow cytometry, multiple specimens can be processed simultaneously with a four- to six-color panel, and the testing can be completed within several hours; thus, the data obtained can be stored for further analysis [27, 28]. Electronic gating appliances also enable the separation of a particular cell subpopulation from a heterogeneous cell mixture without requiring tedious isolation techniques. Furthermore, flow cytometry provides a quantitative measurement of cellular marker expression which can be momentous in identifying malignant cells or other subpopulation of interest on that cellular markers are abnormally expressed.

At present, the major disadvantages of flow cytometry are the lack of a simultaneous morphologic record to correlate with markers of interest, the special skills required to operate the cytometer, and the relatively high cost of the instrument. Therefore, it cannot be emphasized enough that correlating flow cytometry results with clinical features, morphologic findings, and other relevant testing (such as immunohistochemistry, molecular biology, and cytogenetics) is needed to reach a correct diagnosis [2, 28].

22.7.3 *Checklist*

If any polemic issues related to a flow cytometric testing procedure occur, please review a checklist that is designed to ensure the quality of testing results (Table 22.6). Identify a checkpoint for the possible root cause and perform troubleshooting, as appropriate.

Table 22.6 Problematic issues affecting flow cytometry result quality, possible roots of cause, and proposed solutions

Types of error	Causes and suggested actions
Sampling error	<ol style="list-style-type: none"> 1. Large numbers of degenerated or dead cells 2. Specimens with necrosis and fibrosis 3. Delayed processing of splenectomy specimens 4. tumor cell/normal cell ratio
Reagent problems	<ol style="list-style-type: none"> 1. Inappropriate antibody dilutions 2. Surface immunoglobulins or cytophilic antibody. Solution: Incubating the cells in RPMI 1640 medium at 37°C for 1 h 3. Unsuitable or inappropriate fluorochrome conjugate. Solution: Select antibody without cross-reactivity and correct the conjugation.
Unexpected staining	<ol style="list-style-type: none"> 1. Some reagents may affect certain antigens and therefore may need to be reviewed, e.g., EDTA will affect some platelet markers 2. Lysing solutions may affect certain antigens. Select a method that does not interfere with antigen detection 3. Some antigens are expressed intracellularly, and therefore, cell permeabilization methods may be required
Nonspecific staining	<ol style="list-style-type: none"> 1. Nonspecific staining may be due to autofluorescence. Solution: Check levels of autofluorescence by including a tube of cells only 2. Antibody binds to FcR on certain cells, including tumor cells. Solution: Use human FcR blockage reagent or Fab fragment antibody 3. Ensure that a sufficient number of washing steps have been included
No staining	<ol style="list-style-type: none"> 1. Ensure that all antibodies have been stored correctly according to the manufacturer's instructions and have not exceeded their expiration date 2. Ensure that appropriate primary or secondary antibodies have been added 3. Check target antigen expression on cells tested and incorporate a test material of known antigen expression as a positive control 4. Confirm the antibody selected is conjugated to a correct fluorochrome. Make sure that the correct laser and channel are being used for excitation and emission of the fluorochrome
Weak staining	<ol style="list-style-type: none"> 1. Weak staining may be due to overdilution of antibodies. Ensure that antibodies are used at their correct concentration by titrating the antibodies before use 2. Weak staining in indirect staining systems may be due to a prozoning effect, where highly concentrated antibodies may give weak results. Titrate antibodies carefully 3. Weak staining may be due to an excessive number/amount of cells. Adjust the cell population to the recommended density 4. Weak staining may be due to the antigen expression. Check the literature for expected levels of expression
Instrument problems	<ol style="list-style-type: none"> 1. Inappropriate photomultiplier adjustment for isotypic control 2. Inappropriate color compensation for double labeling 3. Inappropriate gating for data analysis

22.8 Summary

Flow cytometry is a well-established molecular diagnostic technology that provides clinical scientists with a way to gather statistical data on many cells and then use that information to correlate multiple parameters within a cell population of interest. Because of its ability to collect thousands of data points in short periods of time, flow cytometry provides enhanced statistical power when used to efficiently analyze complex data to a busy clinical timeline. Software programs allow the gating and manipulation of all acquired parameters (FSC, SSC, and all fluorescent channels) for large data sets to provide statistically relevant results. Because four- to six-color flow testing is becoming the predominant type of cellular immunological analysis and because it is easy to use in clinical practice, fluorescence-based flow cytometry continues to expand its novel applications in the area of molecular diagnostics.

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Part IV
Clinical Molecular Pre/Postnatal Testing

Chapter 23

Molecular Prenatal Diagnostic Testing

Joanna Wiszniewska and Christine M. Eng

Keywords Amniocytes • CVS • Targeted analysis • Maternal cell contamination

23.1 Introduction

We have witnessed a tremendous acceleration in the field of human molecular genetics that has translated into a rapidly growing number of diagnostic tests developed and validated by clinical molecular laboratories. The diagnostic repertoire includes hundreds of tests for Mendelian disorders and traits. Some of them are relatively frequent, for instance, fragile X or cystic fibrosis; others are very rare and frequently referred to as orphan diseases. In most cases, prenatal diagnostic testing is also made available for these disorders if the molecular defect is identified in an affected proband. Families may consider prenatal testing to assess the risk of the fetus and to determine potential outcomes. Such testing gives families an opportunity to make informed decisions about management of pregnancy and plan for intervention or possible complications at delivery. While the molecular diagnostic assays are the same for prenatal or postnatal samples, special procedures are generally implemented to assure the utmost in quality assurance and control of these important samples.

23.1.1 *Direct and Indirect Mutation Analysis*

Typically, prenatal molecular DNA testing is focused on a specific single gene disorder for which the fetal risk is high (typically 25% or greater due to family history). There are two approaches that can be used to detect disease-causing mutations: direct and indirect mutation testing. Direct prenatal testing is the most accurate way to assess fetal risk, but it requires prior knowledge of the disease-causing mutation or mutations in the family. Preferably targeted analysis for known familial mutations in the

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fetal DNA should be performed at the same laboratory where the index case was analyzed. If testing is performed elsewhere, the sample from the index case should be made available to the laboratory performing prenatal analysis and run as a control. If a sample from an affected proband is not available, parental samples can substitute in most circumstances. For autosomal recessive disorders, the carrier status of the parents should be confirmed prior to prenatal diagnosis, particularly to demonstrate that two identified mutations are in *trans* configuration in the affected child, as well as to rule out the possibility of hemizygosity due to a deletion on one chromosome. It is important that the laboratory assures that the assay used in such cases can detect the familial mutation as often techniques evaluating the same genes may differ between laboratories.

Indirect mutation analysis may be applied in cases where the familial mutation has not been identified despite extensive molecular analysis of the gene responsible for the disorder, i.e., dystrophin in case of Duchenne muscular dystrophy or in rare occasions when the chromosomal *locus* is known but the specific gene has not yet been identified. In such scenarios, linkage analysis of genetic markers from the region of interest is performed to assess if the fetus carries a high-risk haplotype and is therefore predicted to be affected with the disease. Indirect testing requires analysis of DNA samples from multiple family members across two to three generations in addition to fetal DNA, and the interpretation of results relies on the accuracy of the clinical diagnosis in the family and accurate determination of affected status of each individual. Furthermore, the possibility of recombination between markers makes indirect testing a less precise option for prenatal diagnostic testing. If indirect testing is used, the location of linkage markers with respect to the disease gene should be stated as well as the risk of misdiagnosis due to recombination.

23.1.2 Invasive and Noninvasive Molecular Testing

Amniocentesis and chorionic villi sampling are the most commonly used invasive techniques to obtain fetal sample for analysis. Amniocentesis is most often performed between 15 and 20 weeks of gestation, whereas chorionic villus sampling allows for analysis of fetal status typically between 10 and 13 weeks gestation. In addition, DNA can be extracted from fetal blood obtained by cordocentesis. Current recommendations are that prenatal testing be performed in duplicate whenever possible. The initial analysis may be performed on the fetal DNA extracted from the original direct sample (direct chorionic villi or amniocytes spun from amniotic fluid), and confirmatory testing should follow on cultured specimens, or alternatively, two separate cultured specimens may be analyzed. The backup cultures of fetal cells have to be maintained until all studies are complete and final [1]. An optimal testing approach would be to split the sample and send it to two laboratories for concurrent independent testing to provide final answer within much shorter turnaround time. However, such approach is difficult to achieve due to limited sample availability, logistic of sample processing, and insurance considerations.

Invasive fetal sampling procedures are associated with fetal loss rates ranging from 0.5% to 1% [2]. The discovery that cell-free fetal DNA circulates in maternal plasma has led to the consideration of its use as a source of fetal material, thereby providing a noninvasive approach to determining prenatal genetic status. Cell-free fetal DNA is detectable around 4 weeks of gestation and comprises approximately 6% of total free plasma DNA by the third trimester of pregnancy [3]. However, the limited amounts of fetal DNA in maternal plasma and the need to differentiate fetal genotype from the maternal background present challenges that have hindered the implementation of noninvasive prenatal genetic testing in clinical diagnostics. Currently, the clinical noninvasive molecular testing is limited to assessment of fetal RhD status in RhD negative mothers and fetal sex determination [4, 5].

23.2 Protocols

23.2.1 *Extraction of Fetal DNA*

Fetal DNA can be extracted directly from amniotic fluid and chorionic villi tissue or from cultured samples. Typically, the DNA yield from a direct sample is sufficient to perform PCR-based testing. Prenatal testing by methods requiring larger amounts of DNA such as Southern blot analysis is generally performed on cultured fetal samples. On average, one T25 flask of 100% confluent fetal cells yields approximately 5–10 µg of DNA.

23.2.2 *Maternal Cell Contamination*

Contamination of fetal sample with maternal cells (MCC) poses a risk for error in molecular diagnosis especially if PCR-based testing is performed. The standards and guidelines for molecular testing recommend that clinical laboratories perform analysis to exclude the presence of maternal DNA in the fetal sample for every prenatal molecular test [1]. The risk of MCC depends on the sampling procedure, sample quality, and methods of sample processing. The MCC is more likely to be detected in cases where bloody amniotic fluid was obtained or when dissection of maternal decidua from chorionic villi sample was not complete. Culturing of the sample may result in over- or undergrowth of specific populations, i.e., culturing of the bloody amniotic fluid enhances only growth of the amniocytes, whereas culturing of CVS also enhances population of maternal cells. Most clinical laboratories perform the MCC analysis using one of the commercially available sets of polymorphic markers, and some laboratories opt to use home brew assays [6]. Regardless of the assay type used, the sensitivity to detect low level of contamination must be established during validation process, and positive controls must be included in each run. The maternal and fetal samples are run concurrently and the data analyzed for inheritance of one maternal allele with no indication of the presence of the second maternal allele. Thus, only informative maternal markers are useful in this analysis. A paternal sample is not necessary for this analysis.

23.2.3 *MCC Data Interpretation*

Fetal STR profiles are compared to maternal profiles. To rule out significant maternal cell contamination, at least two informative *loci* must be present. If the AmpFISTR Identifiler STR markers are uninformative, then analysis of additional markers should be performed (Figs. 23.1 and 23.2).

Occasionally, nonsharing of alleles may be observed. If such an anomaly is detected for a single marker, possible explanations include allele drop out or expansion/contraction of repeats. In cases where multiple markers are affected, the integrity of the sample should be assessed, i.e., sample mix-up, sample mixture, use of egg donor, etc., should be ruled out. It should be also noted that in addition to detection of maternal cell contamination, analysis of STR markers may also suggest trisomy or triploidy in the fetal sample that should be confirmed by other methods (Fig. 23.3).

23.2.4 *Special Considerations for Prenatal Samples*

In addition to procedures described above, additional measures implemented for prenatal samples include pretest and posttest discussion of the case with genetic counselors and independent review of

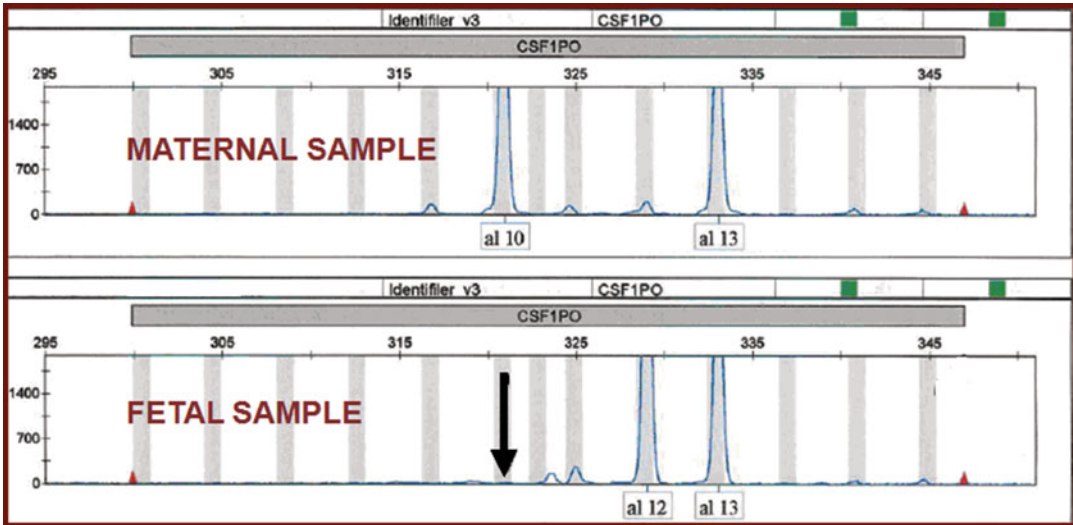


Fig. 23.1 Comparison of fetal and maternal CSF1PO marker that shows no indication of MCC at this informative site

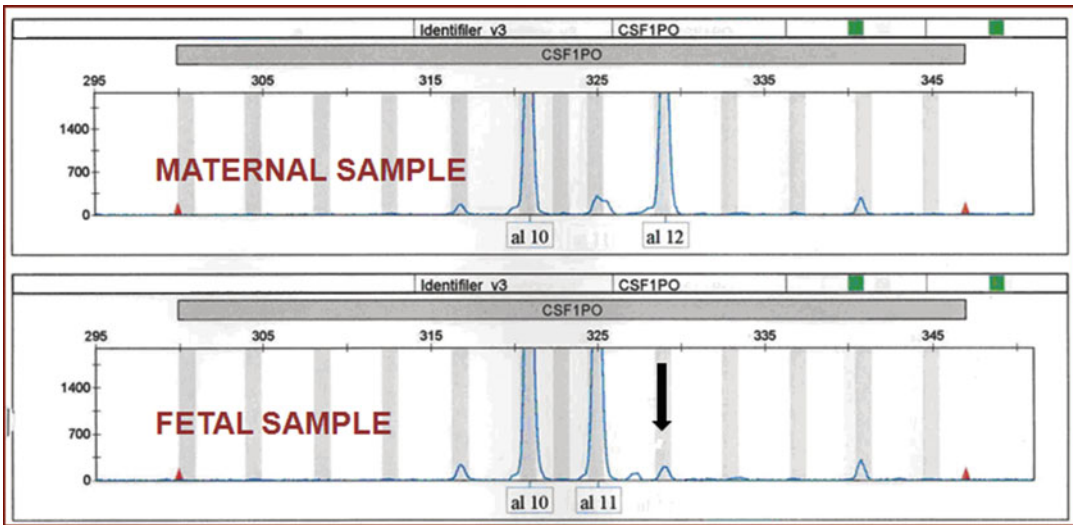


Fig. 23.2 Comparison of fetal and maternal CSF1PO marker that shows presence of MCC of approximately 5% at this informative site

laboratory results and interpretation by two lab directors prior to reporting. The laboratory should be attentive to turnaround times for prenatal analyses as time constraints may be significant. Finally, the laboratory should have procedures in place to deal with the possibility of detection of nonpaternity.

23.2.5 Protocol 1 – DNA Extraction of Prenatal Samples

Note: Sample protocols (These protocols are examples and should be optimized for each laboratory and validated prior to clinical implementation).

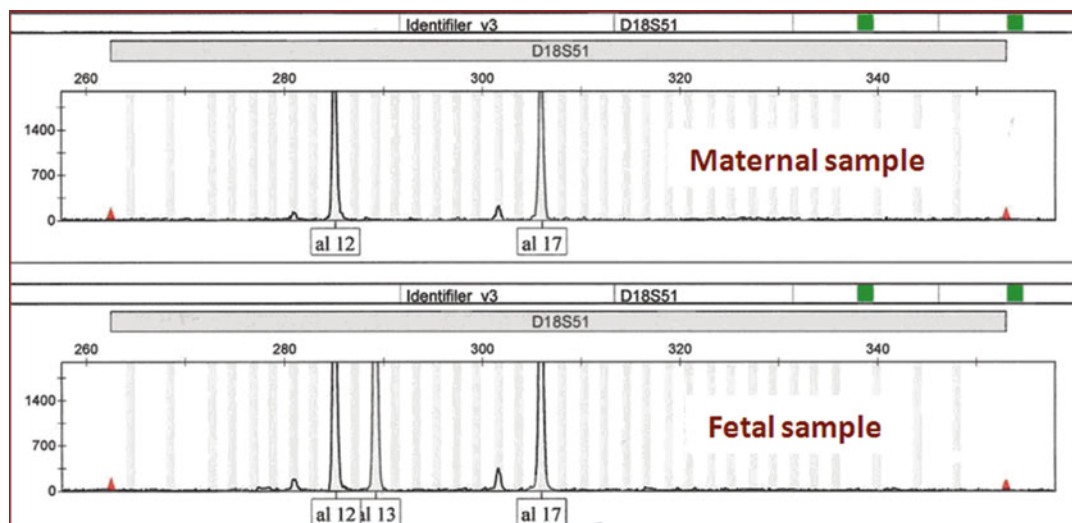


Fig. 23.3 Comparison of fetal and maternal D18S51 marker that shows presence of both maternal alleles in the fetal sample indicating trisomy 18

23.2.5.1 Reagents, Supplies, and Equipment

- Gentra Puregene Kit (QIAGEN)
 - Cell lysis solution
 - RNase A solution
 - DNA hydration solution
 - Protein precipitation solution
- Puregene proteinase K [20 mg/ml] (QIAGEN)
- Trypsin
- PBS
- Isopropanol
- 70% Ethanol
- 1.5-ml microcentrifuge tubes
- 15-ml conical centrifuge tubes
- Microcentrifuge (Beckman Coulter Microfuge[®]22R)
- Bench top centrifuge (Beckman Coulter Allegra[™] 6)
- NanoDrop Spectrophotometer
- Sterile cotton swabs
- Pipetman (10 μ l, 200 μ l, 1 ml)
- Fine tip, 1-ml and 3-ml sterile plastic pasture pipette

23.2.5.2 Cultured Cells Preparation Procedure

Note: Trypsin is used to lift cells off the bottom of the culture flask. One millilitre of aliquots are made and stored at -20°C . For each T25 flask, 1.0 ml trypsin is used, and for each T75 flask, 2.0 ml trypsin is used.

1. Remove appropriate number of trypsin aliquots from the -20°C freezer to thaw at room temperature. Label all tubes.
2. Pour off culture medium into waste container and remove as much of the medium as possible with a 3-ml pasture pipette.
3. Add 1 ml PBS solution to the flask and wash the surface of the cells by rocking the flask. Remove and discard all of the PBS solution.
4. Add 1.0–2.0 ml trypsin. Use the 3-ml pasture pipette to spread the trypsin evenly by rocking the flask.
5. Place the flask in the 37°C ($\pm 1^{\circ}\text{C}$) incubator for 10–15 min or until cells lift from the bottom of the flask. Tap the flask to see if the cells have been lifted.
6. Remove the flask from the incubator and transfer the *trypsin* containing the cells to a labeled 1.5-ml tube.
7. Add 0.5 ml PBS to the flask and wash down the cells by rocking. Transfer the PBS wash to the same 1.5-ml tube in step 6.
8. Centrifuge at $13,000\text{--}16,000\times g$ (on the Beckman Coulter Microfuge[®]22R) for 3 min. to pellet cells. Remove the supernatant leaving behind 10–20 μl residual liquid.
9. Vortex on maximum setting vigorously to resuspend the pellet in the residual supernatant; this greatly facilitates cell lysis in step 10 below.
10. Add 300 μl of *cell lysis solution* to the resuspended pellet and add 1.5 μl *proteinase K* solution (20 mg/ml). Mix by inverting 25 times and incubate at 37°C ($\pm 1^{\circ}\text{C}$) for 3 h to overnight. Samples are stable in *cell lysis solution* for at least 18 months at room temperature.
11. Add 1.5 μl *RNase A* solution to the cell lysate. Invert the sample at least 25 times to mix and incubate at 37°C ($\pm 1^{\circ}\text{C}$) for 15 min. Allow samples to cool on ice.
12. Add 100 μl of *protein precipitation solution* to the cell lysate.
13. Vortex **vigorously** at high speed for about 20 s to mix the *protein precipitation solution* with the cell lysate. At this stage, small protein clumps may appear throughout the lysate. This is a good indication of protein precipitation.
14. Centrifuge at $13,000\text{--}16,000\times g$ (on the Beckman Coulter Microfuge[®]22R) for 3–5 min using the microcentrifuge. The precipitated proteins will form a tight white pellet; however, if the pellet does not appear tight, repeat steps 13 and 14.
15. Pour the supernatant containing the DNA into a labeled 1.5-ml microcentrifuge tube containing 300 μl of cold *isopropanol*.
16. Mix the tube by inverting gently 50 times or until the white threads of DNA form a nice visible clump.
17. Centrifuge at $13,000\text{--}16,000\times g$ (on the Beckman Coulter Microfuge[®]22R) for 2–3 min. The DNA will form a small white pellet.
18. Pour off the supernatant and add 1 ml of 70% *ethanol*. Invert the tube several times to wash the DNA pellet.
19. Centrifuge at $13,000\text{--}16,000\times g$ (on the Beckman Coulter Microfuge[®]22R) for 1 min. Carefully aspirate off the ethanol.
20. Allow pellet to air-dry for 10–15 min.
21. Estimate the size of the pellet and add *DNA hydration solution*.
22. Allow DNA to rehydrate overnight with rocking at 37°C ($\pm 1^{\circ}\text{C}$) or 2 h at 55°C ($\pm 1^{\circ}\text{C}$).
23. Mix samples thoroughly by taping or pipette the solution SLOWLY up and down using the 1-ml transfer pipette. Quantitate DNA by using the NanoDrop Spectrophotometer. Store DNA at 4°C .

23.2.5.3 Direct CVS Preparation Procedure

1. Centrifuge the CVS for 3–5 min. Pour off supernatant.
2. Wash CVS with 1 ml PBS. Centrifuge for 3–5 min. Pour off supernatant.
3. Break up pellet and follow steps 10–23 (31.2.5.2).

23.2.5.4 Direct Amniotic Fluid Preparation Procedure (1–3 ml)

1. Transfer 1–3 ml of amniotic fluid to a 1.5-ml tube. If using 3 ml of amniotic fluid, add 1.5 ml to the microcentrifuge tube, centrifuge as indicated in step 2, add the remaining 1.5 ml to the tube, and repeat the centrifugation.
2. Centrifuge at 13,000–16,000 × g (on the Beckman Coulter Microfuge® 22R) for 2 min to pellet the cells. Remove the supernatant, leaving behind 10–20 µl residual liquid.
3. Vortex on maximum setting vigorously to resuspend the pellet in the residual supernatant; this greatly facilitates cell lysis in step 4 below.
4. Add 300 µl of *cell lysis solution* to the resuspended pellet and pipet the solution SLOWLY up and down. At this stage, no incubation is required; however, if cell clumps are visible, incubate at 37°C (±1°C) or room temperature until the solution is homogeneous. Alternatively, if maximum yield is required, 1.5 µl *proteinase K* solution (20 mg/ml) may be added to the lysate. Mix by inverting 25 times and incubate at 37°C (±1°C) for 3 h to overnight. Samples are stable in *cell lysis solution* for at least 18 months at room temperature.
5. Follow steps 11–23 (23.2.5.2).

23.2.6 Protocol 2 – Maternal Cell Contamination Studies Using Commercially Available AmpFISTR Identifiler PCR Amplification Kit Provided by Applied Biosystems Inc. [7]

23.2.6.1 Reagents

- AmpFISTR Identifiler PCR Amplification Kit (Applied Biosystems Inc.)
- HPLC grade water
- Hi-Di formamide
- GeneScan-500 LIZ Size Standard (Applied Biosystems Inc.)

23.2.6.2 Controls

- Always use at least one control for each marker in the multiplex.
- Controls must be a 90:10 mixture of DNA from two individuals.
- One control must be a 95:5 mixture DNA from two individuals.
- Controls are made as follows:
 1. Dilute two DNA samples with HPLC grade water to 10 ng/µl ±10%
 2. Dilute to 5 ng/µl using 9.5 µl water and 5 µl of 10 ng DNA or alt. method – 1 ng/µl using 9 µl water and 1 µl of 10 ng DNA.
 3. For 90:10 controls, mix 90 µl of the first DNA sample dilution with 10 µl of the second dilution. Dilution to be used in later runs until finished.
 4. For 95:5 controls, mix 95 µl of the first DNA sample dilution with 5 µl of the second dilution.
 5. Use 1 µl of each control DNA mixture for analysis.

23.2.6.3 PCR Setup

1. Dilute DNA samples to 0.5 ng/µl with HPLC grade water and aliquot into PCR plate.
2. For MCC controls, use premade dilutions (see above).
3. For negative PCR Control, use master mix alone.

4. Make PCR master mix for exact number of reactions plus controls.

PCR master mix	Per reaction (μl)
AmpFISTR PCR reaction mix	10.5
AmpliTaQ Gold	0.5
AmpFISTR Plus primer set	5.5
Water	10
Total	26.5

5. Add 24 μl PCR master mix to each PCR tube then add 1 μl of DNA at 0.5 ng/μl for a total volume of 25 μl.
6. Place the samples in the PCR machine.

1 cycle	95°C 11 min
28 cycles	94°C 1 min
	59°C 1 min
	72°C 1 min
1 cycle	60°C 45 min
	4°C hold

- (a) Make the GeneScan-500 LIZ standard mix per reaction as follows:

Deionized formamide	20 μl
GeneScan-500 LIZ Standard	0.5 μl

Calculate total number of reactions as follows:

Number of PCR reactions X 1.10

+

AmpFISTR Identifier Allelic Ladder Control X2

- (b) Add 2.5 μl PCR product or 2 μl AmpFISTR Identifier Allelic Ladder to 20 μl of GeneScan-500 LIZ standard mix.
- (c) Denature in a PCR machine for 3 min at 95°C.
- (d) Cool on ice for 3 min.
- (e) Load samples on the ABI Genetic Analyzer and run as a GeneScan plate according to the manufacturer's protocol (Applied Biosystems Inc.).
- (f) Analyze results with GeneMapper Software (Applied Biosystems Inc.).

Applied Biosystems <http://www.appliedbiosystems.com/absite/us/en/home.html>

23.2.7 *Protocol 3 – Analysis of Additional STR Markers Located in the DMD Gene Region*

23.2.7.1 Reagents

- AmpliTaq Gold PCR Amplification Kit (Applied Biosystems Inc.)
- HPLC Grade Water
- Hi-Di formamide
- GeneScan-500 LIZ Size Standard (Applied Biosystems Inc.)

Table 23.1 Primer sets (Adapted from [8–10])

Multiplex 1	
3' DYS MS-F	NED -GAA AGA TTG TAA ACT AAA GT
3' DYS MS-R	GTG TCT TGG ATG CAA AAC AAT GCG CTG
STR45-F	6FAM -GAG GCT ATA ATT CTT TAA CT
STR45-R	GTG TCT TCT CTT TCC CTC TTT ATT CAT
5' DYS II-F	VIC -GTG TAT ATC TTG ATA TAT AGG G
5' DYS II-R	GTG TCT TAT TAT GAA ACT ATA AGG AAT
Multiplex 2	
5' DYS1-F	NED -ACT GTA AAT GAA ATT GTT TT
5' DYS1-R	GTG TCT TCT TAA CAA AAT GTC CTT CAG
STR50-F	6FAM -AAG GTT CCT CCA GTA ACA GA
STR50-R	GTG TCT TTA TGC TAC ATA GTA TGT CCT
STR44-F	VIC -TCC AAC ATT GGA AAT CAC AT
STR44-R	GTG TCT TTC ATC ACA AAT AGA TGT TTC
Multiplex 3	
STR49-F	NED -CGT TTA CCA GCT CAA AAT CT
STR49-R	GTG TCT TCA TAT GAT ACG ATT CGT GTT
DXS1214-F	6FAM -TAG AAC CCA AAT GAC AAC CA
DXS1214-R	GTG TCT TAA GAT AGC AGG CAA CAA TAA
IVS44SK21-F	VIC -CAG CTC CAG AGG GTT AAT AAG
IVS44SK21-R	GTG TCT TGC TCA GTG AAT GAA TAG CAC

- Primer sequences for multiplexes 1, 2, and 3 STR: 5 μ M; **NED**, **FAM** and **VIC** denote fluorescently labeled primers in yellow, blue, and green colors, respectively. A seven base tail GTG TCT T is added to the 5' end of the reverse primers to minimize band stuttering (Table 23.1).

Note: Refer to Protocol 2 section 2 for control preparation

23.2.7.2 PCR Setup

1. Dilute DNA to 1 ng/ μ l.
2. Prepare reaction mixture per one reaction, as shown in Table 23.2.
3. Add 2.5 μ l of the prepared DNA to PCR tubes, distribute 12.5 μ l aliquots of reaction mixture to each tube, and mix well.
4. Run reactions in the thermal cycler using following conditions:
 - (a) Initial denaturation for 4 min at 94°C.
 - (b) Denature for 15 s at 94°C.
 - (c) Anneal for 15 s at 55°C.
 - (d) Extend for 30 s at 72°C.
 - (e) Repeat steps b–d ten times.
 - (f) Denature for 15 s at 89°C.
 - (g) Anneal for 15 s at 55°C.
 - (h) Extend for 30 s at 72°C.
 - (i) Repeat steps f–h 25 times.
 - (j) Extend for 30 min at 72°C.
 - (k) Incubate at 4°C.

Table 23.2 Reaction mixture

PCR master mix	Per reaction (μl)
10×PCR buffer	1.5
2.5 mM dNTP	1.2
25 mM MgCl ₂	1.5
AmpliTaq Gold	0.2
Multiplex 1	
5 μM NED-3' DYS MS	1.0
5 μM FAM-STR 45	1.15
5 μM VIC-5' DYS II	4.05
Water	1.9
Multiplex 2	
5 μM NED-5' DYS I	1.25
5 μM FAM-STR 50	0.28
5 μM VIC-STR 44	0.65
Water	5.92
Multiplex 3	
5 μM NED-STR 49	0.3
5 μM FAM-DXS 1214	0.3
5 μM VIC-IVS44 SK21	0.79
Water	6.71

5. Make the GeneScan-500 LIZ standard mix per reaction as follows:

Deionized Formamide	10 μl
GeneScan500-LIZ Standard	0.25 μl

6. Pipette 1 μl of each diluted sample into respective wells on 96-well plate.
7. Add 9 μl of sizing standard/formamide mix to each well. Spin down, if needed, in plate centrifuge to remove bubbles.
8. Denature plate for 3–5 min in thermocycler.
9. Place plate on ice to cool for 3–5 min before placing in machine.
10. Run GeneScan on ABI analyzer and analyze STRs using GeneMapper Software according to manufacturer's instructions (Applied Biosystems Inc.).

23.3 Summary

Prenatal DNA diagnosis employs the same genetic concepts and general molecular techniques as postnatal DNA testing; however special procedures for quality assurance are recommended. Analysis of fetal DNA can be performed on chorionic villi cells, amniocytes, cord blood, or free cell fetal DNA derived from maternal plasma. Typically, the amount of DNA extracted from direct CVS or amniotic fluid samples is sufficient for analysis using PCR-based assays; however, culturing of cells may be necessary if molecular technique requires large quantities of DNA. It is recommended that the laboratory performs analysis to exclude the presence of contamination of fetal samples with maternal cells to minimize the risk of misdiagnosis and that the confirmatory testing is performed on a separate DNA sample whenever possible.

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Chapter 24

PCR Based Diagnosis of Fragile X Syndrome

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Keywords Clinical testing • Trinucleotide repeat • Ovarian failure • FXTAS • Denaturing • Amplification • Nonradioactive • Methodology

24.1 Fragile X Syndrome

24.1.1 *Clinical Features and Molecular Pathogenesis of Disease*

Fragile X syndrome is an X-linked disorder that typically causes moderate to severe mental retardation or autism in males and variable learning disability in females [1]. The term, “fragile X”, relates to the cytogenetic observation of apparent breakage of the terminal portion of the long arm of the X chromosome when cells from affected individuals are cultured in folate-deficient medium [2]. This syndrome is one of the most common genetic causes of developmental delay in males with a frequency of about one in 5,000 [3]. Seventy percent of fragile X females have some degree of mental impairment but rarely fall into a range as severe as affected males [4]. Approximately 2.5% of all males and 1% of females with mental retardation have an underlying fragile X mutation [5]. These estimates vary depending on criteria for patient selection.

Fragile X syndrome has an unusual inheritance pattern [6] in which obligate carrier males may be unaffected, and yet, their daughters have sons with the full-blown syndrome. In general, the penetrance of mental retardation among both male and female gene carriers increases with successive generations, a pattern called “anticipation.” These phenomena are explained by the unusual nature of the underlying mutations [7–11]. Cloning of the gene, FMR1, revealed a CGG trinucleotide repeat in the 5' untranslated region. Most normal individuals have approximately 30 repeats. Unaffected carrier males and many unaffected carrier females have “premutations” with more than 55 repeats but fewer than 200. These premutations are not associated with mental retardation but tend to expand when passed from mother to child and hence become longer in later generations. In alleles with greater than 200 repeats, the promoter region of the gene is often hypermethylated and FMR1 RNA expression is

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decreased or absent, resulting in mental retardation. This meiotic premutation expansion through generations to a size range greater than 200 repeats, the “full mutation” range, explains anticipation.

Individuals with full mutations almost invariably inherit them from their mothers either through premutation expansion in the female germ line or because their mothers carry full mutation alleles in all of their cells. Alleles in the full mutation range are not detected in sperm from affected individuals, and transmission of full mutation alleles from fathers to daughters is almost unknown [12].

Full mutations are mitotically unstable, and individuals with full mutations have somatic mosaicism with a broad range of repeat sizes greater than 200. While alleles slightly larger than 200 repeats may occasionally be active, those longer than 250 repeats are almost always hypermethylated and inactive. Rare individuals with X-linked mental retardation have other inactivating mutations in the FMR1 gene, most often large deletions [13].

Premutation carriers are believed to be completely free of the developmental delay associated with full mutations, but other features have become evident since the gene was cloned and molecular detection of the premutation state became possible. Premature ovarian failure is found in approximately 20% of female carriers [14–16]. Among male premutation carriers, there is a 50% lifetime risk of an adult-onset neurodegenerative disorder leading to tremor, ataxia, and cognitive decline (acronym: FXTAS) [1, 17]. This syndrome typically occurs after age 50 in males and is less common, later in onset, and milder in females.

24.1.2 Clinical Uses of Fragile X Testing

The medical indications for the bulk of fragile X testing currently being performed are diagnosis in mentally retarded males and screening for premutations and full mutations in adult women of child-bearing age who are considering use of prenatal diagnosis [18]. Other major uses of testing include prenatal diagnosis, diagnosis of females with developmental delay, diagnosis of females with premature ovarian failure, and diagnosis of adult males or females with FXTAS-like features.

Given the complex inheritance pattern of fragile X syndrome, genetic counseling is recommended for interpretation of test results. Screening of normal women for fragile X premutations and full mutations in the prenatal clinic setting remains somewhat controversial due to counseling issues that arise and questions of cost-effectiveness. Still, this testing is driven by the high frequency of premutation alleles – 1 in 86, for those with a family history of mental retardation and 1 in 257 for women with no known risk factors for fragile X syndrome [19]. Those screening positive for premutations need counseling for related disorders such as premature ovarian failure and late-onset neurodegeneration (FXTAS) in addition to prenatal counseling on risk of a child with fragile X syndrome.

24.1.3 Subtleties of DNA-Based Testing and Interpretation of Results

There remain two types of “borderline” results that bear discussion. Some alleles in the size range between 45 and 55 repeats may be meiotically unstable and prone to minor degrees of expansion. Although there are no reports of expansion to a full mutation when passed from mother to child, these alleles may expand into the true premutation range. Hence, descendants of individuals carrying these “borderline premutations” may be at risk for fragile X syndrome. Furthermore, the immediate offspring of individuals with borderline alleles may be at risk for the phenotypes associated with premutations. For these reasons, there is clinical justification for reporting borderline premutations. However, it is essential to make clear, especially in the prenatal carrier screening setting, that these alleles have no chance of expanding to a full mutation within one generation.

Alleles at the smaller end of the full mutation size range may or may not cause typical fragile X syndrome. In some cases, these alleles are unmethylated and active, at least in a portion of an individual's cells. Males who are mosaic for an active allele are less severely affected than those with only inactive alleles. Therefore, methylation status may have prognostic value in the few cases in which an allele of this size (or smear of bands including this size range) is identified [20, 21]. Unfortunately, methylation in utero may not entirely correlate with methylation postnatally, particularly when prenatal diagnosis is done by early CVS [20, 22, 23], and the ability to predict developmental outcome based on prenatal DNA findings may be limited in the presence of a borderline full mutation. Of the CLIA-approved laboratories in the USA, participating in CAP-sponsored proficiency testing for fragile X, 28 out of 92 performed methylation assays as part of their fragile X testing in the 2009 cycle.

Another area that requires nuanced reporting is the relationship between the size of a premutation allele in a female and its probability of expanding to a full mutation in her offspring [14, 24]. Health-care providers involved in prenatal genetic testing and counseling may find useful a qualitative estimate of this probability. Although estimates of risk vary from study to study, alleles between 55 and 70 repeats have a risk of expansion to a full mutation that is probably less than 20%, while larger alleles have a very high risk of expansion. In many studies, alleles with 100 repeats or more invariably expanded to full mutations. The size of the premutation allele also correlates with the probability of premature ovarian failure [25] and FXTAS [26].

24.2 Methodology

In the initial period following cloning of the FMR1 gene, diagnostic testing was performed by Southern blotting of restriction-digested genomic DNA and probing with a radiolabeled fragment that fell between the CGG repeat region and the closest restriction site. To simultaneously identify methylation and allele size, a methylation-sensitive restriction enzyme was often included in the protocol [8–11].

The use of PCR to analyze this region was challenging due to technical difficulties in amplifying GC-rich regions. Early PCR protocols succeeded in overcoming this obstacle by using 7-deaza-dGTP as one of the nucleotides in the reaction mix [7, 27]. However, these methods did not robustly amplify full mutations and required incorporation of a radioactive label into the product or Southern blotting of the product after gel electrophoresis because 7-deaza-dGTP precludes staining with ethidium bromide.

The use of Pfu DNA polymerase and a denaturing amplification mixture has overcome this obstacle, allowing for detection of premutation and full mutation alleles by normal agarose gel electrophoresis with ethidium bromide staining to visualize products [28, 29]. Due to the somatic mosaicism typical of full mutations, these alleles often yield a diffuse smear of bands, and reliable diagnosis still requires Southern blotting of the PCR products and hybridizing with a probe specific for CGG repeats, particularly in female heterozygotes.

24.3 Protocol

24.3.1 Specimen Types

Genomic DNA extracted from peripheral blood leukocytes, fibroblasts, oral epithelial cells obtained in saliva or by cheek brush, or a fetal sample from direct CVS tissue, direct amniotic fluid cells, or cells cultured from one of these sources.

24.3.2 Reagents

Required Reagents

24.3.2.1 PCR Reagents

- 2 mM dNTP
- Dimethyl sulfoxide (DMSO)
- 5 M Betaine
- Cloned Pfu Polymerase (Stratagene)
- KlenTaq (AB Peptides)
- 10× KlenTaq PCR Buffer (AB Peptides)
- Agarose GPG/LE (American Bioanalytical)

Forward primer: FMR1 5' GCTCAGCTCCGTTTCGGTTTCACTTCCGGT 3'
Reverse primer: FMR2 5' AGCCCCGCACTTCCACCACCAGCTCCTCCA 3'

13681 cagcggggccg ggggttcggc ctcagtcagg **cgctcagctc cgtttcgggt tcaattccgg**
13741 tggagggccg cctctgagcg ggcggcgggc cgacggcgag cgcgggcggc ggcggtgacg
13801 gaggcgccg tgcaggggg cgtgcggcag cgcggcgggc gcggcgccgg cggcgccggc
13861 ggaggcgccg gcggcgccgg cggcgccggc ggctgggcct cgagcgccc cagcccacct
13921 ctcggggggc ggctcccgc gctagcaggg ctgaagagaa **gatggaggag ctggtggtg**
13981 aagtgcgggg **ctccaatggc** gctttctaca aggtacttgg ctctaggga ggcacctct
14041 tcgcccttc ttccctcct ttctcttg gtgtcggcg gaggcaggcc cggggccctc

Location of PCR primers within 5' UTR of FMR1 (based on accession number L29074)

24.3.2.2 Labeling Reagents Table 24.1

Table 24.1 Components of labeling reagent mixture	
CGG10 oligo (10 μM concentration, chemically synthesized)	10 μl
Terminal transferase (20 u/μl concentration) (Roche)	1 μl
Terminal transferase buffer (5× concentration) (Roche)	4 μl
CoCL ₂ (25 mM concentration) (Roche)	4 μl
DIG ddUTP (1 mM concentration) (Roche)	1 μl
	20 μl Reaction

Blotting Reagents (Table 24.2)**Table 24.2** Components of Church & Gilbert hybridization solution

Church & Gilbert hybridization solution	
1 M Sodium phosphate, pH 7.2 ^a	500 ml
0.5 M EDTA, pH 8.0	2 ml
BSA, fraction V	10 g
Herring sperm DNA, 10 mg/ml	20 ml
20% SDS	350 ml
Double distilled water	Bring volume to 1 l
^a 1 M Sodium phosphate, pH 7.2 – 420 ml of dibasic 1 M solution and 80 ml of monobasic 1 M solution	

- DIG Block and Wash Buffer Set (Roche)
- Antidigoxigenin-AP, Fab Fragments (Roche)
- CSPD (Roche)
- Digoxigenin-11-ddUTP (Roche)

Controls

In addition to amplifying patient samples, amplify a patient with 55 repeats and a female carrier of a full mutation as positive controls.

24.3.3 Procedure**24.3.3.1 Setup of PCR Reaction**

	1×
10× PCR Buffer	2.50 μl
DMSO	1.25 μl
2 mM dNTPs	2.50 μl
5 M Betaine	10.0 μl
1:5 Cloned Pfu Polymerase and KlenTaq	0.2 μl
20 μM FMR1 primer	0.5 μl
20 μM FMR2 primer	0.5 μl
ddH ₂ O	7.0 μl
	24.45 μl

Add 1 μl of genomic DNA per reaction.
Include positive and no template controls.

24.3.3.2 PCR Program

98° C	2 min	} cycle 35 times
98° C	30 second	
65° C	1 minute	
72° C	2 min	

Note: No temperature “hold” used following cycling.

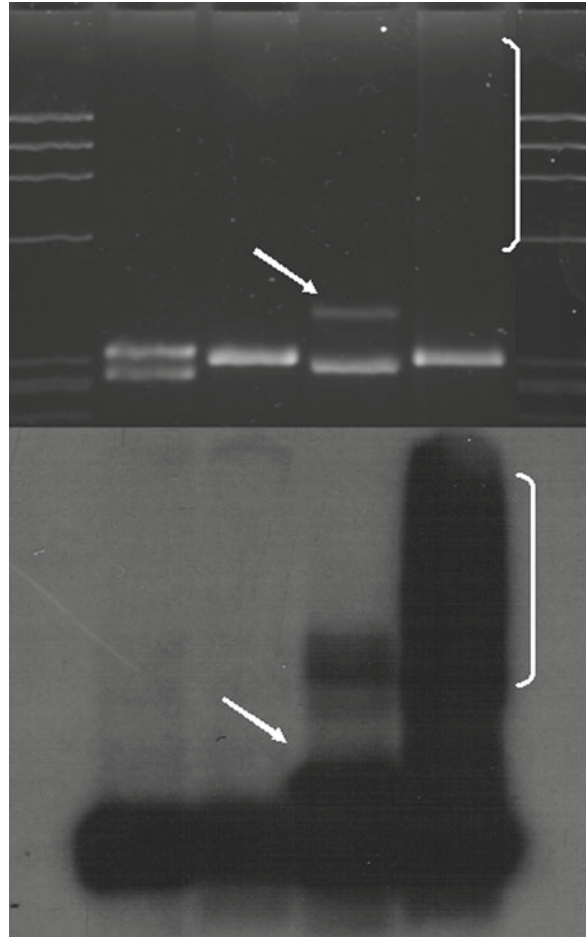
24.3.3.3 Gel Visualization

1. Make a 2% agarose gel using GPG 9 (or other standard purity) agarose.
2. Mix 5 µl of each reaction with 2 µl of bromophenol blue and load into the 2% gel, using ΦX Hae III as a size standard. A size standard should be run on either side of the gel to ensure that all samples have traveled the same distance.
3. Run the gel at 75 V for 3 h.
4. Photograph the gel with a fluorescent ruler next to the size standard.

24.3.3.4 Southern Blot Setup

1. Set up Southern blot on the gel per standard practice for capillary transfer of nucleic acids or electroblotting. *Note:* Use nylon membranes ONLY. Bake the blot in a vacuum oven following the transfer.
2. Label a CCG 30-mer oligo with digoxigenin (DIG) using terminal transferase (see Table 24.1 for reaction mixture).
3. Incubate at 37°C for 1 h.
4. *Note:* Store unused probe at –20°C. Add 4 µl (20 pmol) of the terminal transferase-labeled oligo to 10 ml of Church & Gilbert solution (see Table 24.2 for solution preparation). This probe is good for at least 4 weeks.
5. Probe with the oligo at 45°C for 2 h. Wash in 2× SSC, 0.1% SDS at room temperature for 15 min, followed by a 0.1× SSC, 1% SDS room temperature wash for another 15 min.
6. Rinse the blot for 1–5 min in washing buffer (Roche Block and Wash Buffer Set).
7. Incubate for 30 min in 100 ml of blocking solution (Roche Block and Wash Buffer Set).
8. Equilibrate for 3 min in 20 ml of detection buffer (Roche Block and Wash Buffer Set).
9. Prepare a fresh CSPD solution by adding 100 µl of CSPD to 900 µl of detection buffer. Mix with pipette. (For smaller blots, 500 µl of diluted CSPD solution can be used.)
10. Place the blot into a clean hybridization bag. Then add the CSPD solution that you have just prepared to the face of the blot. Gently spread the solution over the face of the blot by rolling a 25-ml pipette over it. Roll any air bubbles off of the face of the blot.
11. Incubate the blot for 5 min at room temperature (approximately 25°C).
12. After incubation, roll out the excess CSPD solution, then seal the hybridization bag. **DO NOT** dry the blot.
13. Incubate the blot for 10 min at 37°C to enhance the luminescence reaction.
14. Expose for approximately 20 min. *Note:* The light produced by the reaction of CSPD and AP will increase for up to 48 h after this incubation. Therefore, waiting before exposing the film for as much as 24 h is recommended and will result in shorter exposure times.

Fig. 24.1 Ethidium bromide–stained gel and corresponding Southern blot probed with a CGG oligonucleotide. *Arrow* indicates premutation allele with 55 CGG repeats. *Bracket* indicates smear of bands from mitotically unstable full mutation allele



24.4 Interpretation of Results

24.4.1 PCR

Normal X chromosomes yield a band of approximately 300 bp (20–40 CGG repeats). Chromosomes bearing a premutation yield a discrete band equal or larger than that of the premutation control and sometimes yield a smear of bands larger than the premutation band as well due to heteroduplex formation (Fig. 24.1, upper panel).

24.4.2 Southern Blot of PCR Products

A smear of DNA larger than the premutation control with no discrete premutation band is detected in chromosomes with a full mutation (Fig 24.1, lower panel). Southern blot with the CGG oligonucleotide probe will detect full mutation alleles, and the smear of bands will be seen in the positive control. As noted in the introductory section, rare individuals may be mosaic and have both a large premutation allele and a smear of bands in the full mutation range.

24.5 Troubleshooting

24.5.1 PCR

The PCR reaction is robust, but the common precautions for PCR reactions apply. Perform the PCR in a biosafety cabinet or other environment clean of amplified DNA. All reagents for PCR must be dedicated. Keep the reaction mixture cold by working on ice, and do not add the polymerase until the reaction is cold.

One aspect worth noting is the volume of enzyme added to the reaction. Adding too much enzyme to the reaction can cause spurious bands. Because of the viscous nature of enzyme preparations, setting a pipette to the prescribed volume will invariably deliver a significantly larger volume. Become familiar with the appearance of different volumes pipetted using a less viscous liquid (such as water) so you can approximate the correct volume when aspirating an enzyme solution.

24.5.2 Agarose Gel Electrophoresis

Electrophoresis for this assay is generally trouble-free. Keep in mind that a 2% agarose cannot be run at high voltage. Establish a range of voltage at which your gel does not overheat or fail to run straight. Use fresh buffer and a recently cast gel for the assay.

24.5.3 Southern Blot

In most cases, the general problems that occur with southern blots fall into two related categories: (1) low signal and (2) high background.

The comments below pertain to this particular assay and lists the most likely causes for the problems that occur.

24.5.3.1 Low Signal

- Check the age of the probe. Although the DIG does not decay like a radiochemical, it will not last indefinitely. If you have used a probe longer than 1 month, consider using a fresh one if you are having problems.
- Confirm that you are using all of the reagents in the proper quantities and concentrations specified by the manufacturer and protocol.
- Confirm that the incubations are being done correctly, particularly the steps using CSPD.
- Check that your DNA is transferring well by post-staining your gel with ethidium bromide and photographing the stained gel under UV light.

24.5.3.2 High Background

Estimate the hybridization temperature for your probe on paper prior to experimentation, then hybridize duplicate blots at different temperatures to optimize this condition. Too low of a hybridization temperature will result in nonspecific hybridization and high background.

The blot should not be dried at any point after step 13. This can result in high background. Too high of a probe concentration can cause background. The most common source of this problem is too low of a hybridization volume. After many uses of a probe, the volume may be too low to continue using that preparation. Inadequate washing or blocking can cause background. Follow the protocol and manufacturer's instructions with regard to these steps. Use of nonnylon transfer membranes is not recommended and is cited to be a source of background. Never handle the blot with bare hands, or in any way that can transfer oils from the skin to the blot. The oil can cause dark spots where it contacts the blot.

Note: Since the light generated to provide a signal is a product of an enzymatic reaction, it is worth remembering that conditions that will perturb that reaction can affect your results.

For instance, the heat from a sealer used to seal hybridization bags can be too high for the reaction (the alkaline phosphatase in particular) and can cause rippling background. Seal hybridization bags at a safe distance from the edges of the blot to avoid this.

24.6 Methylation Detection Procedures

Methodology that detects methylation status of the FMR1 CpG island is used by some laboratories and may be important prognostically in patients with borderline full mutation alleles. Standard Southern blotting with genomic DNA digested with either a methylation-sensitive enzyme or methylation-sensitive PCR can be used. Numerous publications have reported on methods to determine whether the CpG island upstream from FMR1 is hypermethylated [3, 8–11, 22, 27, 30, 31].

24.7 Summary

Testing for fragile X syndrome is a common clinical laboratory procedure. The medical indications for this testing have expanded beyond the original use in diagnosis of mentally retarded males to include developmentally impaired females, females with premature ovarian failure, and adults with tremor and ataxia. Because premutations have a high frequency even among women with no family history of mental retardation, a very common use of this test is in prenatal or preconception counseling among women from the general population.

Even with the advent of clinical, whole-genome sequencing, PCR-based methods will likely continue as a mainstay of fragile X diagnosis for the foreseeable future because the current platforms for high-throughput sequencing are not suited to detection of long CG-rich repeats. The methods presented here are rapid and reliable and avoid the use of radioisotopes.

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Part V
Human Identity Testing

Chapter 25

Microsatellite Testing Short Tandem Repeat Profile Generation

Robin DeVille Guidry and Laura Gahn

Keywords RFLP • VNTRs • Degraded DNA • PCR • STRs • Human identification • Multiplex • Core loci • CODIS • Allelic ladder • Identifiler PCR amplification kit • Fragment analysis • Capillary electrophoresis • 3130xl • Genetic analyzer • Spatial calibration • Spectral calibration • Spectral separation • Peak base pair sizing • Allele designations • GeneMapper ID • Artifacts (electrophoretic) • Artifacts (amplification) • Random match probability

25.1 Introduction

The need for more and more information from less and less DNA sample, rapidly, has led laboratories from the use of minisatellites (variable number of tandem repeats, VNTRs) for human identification (HID) to the use of microsatellites (short tandem repeats, STRs). Commercially available STR amplification kits permit the rapid analysis of compromised DNA samples, yielding discriminating information that can be compared amongst laboratories. After a brief review of the Human identification technologies that preceded STR analysis, this chapter will discuss STR profile generation using the commercially available AmpFISTR Identifiler PCR Amplification Kit from Applied Biosystems by Life Technologies and subsequent data interpretation.

25.2 RFLP-Based DNA Testing

Restriction fragment length polymorphism (RFLP) DNA analysis made use of VNTRs in human identity testing [1]. Due to the high variability of VNTRs between individuals and the large number of distinguishable alleles at each locus, RFLP analysis afforded great discrimination power with only a few loci tested (as few as four loci can provide a frequency of 1 in a million or less) [2, 3]. The quality

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of the information regarding the source of a biological specimen was vastly improved with the adoption of RFLP analysis over conventional serological analysis [4].

DNA provided a much more stable molecule, as many of the proteins analyzed degraded within weeks. As the proteins degraded, so did the ability to identify the potential source of the biological material. False positives and false negatives often seen in serological examinations introduced an additional layer of difficulty at analysis [5]. The degradation of the DNA molecule may result in no interpretable results, but it will not cause a different profile to be detected [3, 5].

Despite these advantages, RFLP analysis was time-consuming, labor-intensive, and difficult for the novice analyst to interpret [2, 4]. RFLP analysis required: DNA extraction, fragmentation by restriction endonucleases, electrophoretic separation on an agarose gel, transfer to a membrane by Southern blotting, hybridization with radioactive probes, and development on x-ray film [5]. If a single-locus probe was used, this process, which took several days to a week, would be repeated for each additional RFLP probe that was to be introduced, potentially taking 6–8 weeks for complete results [4]. And while only a few loci were needed to obtain compelling frequencies when a “match” was obtained, examining so few loci presented problems when siblings or closely related individuals were compared to one another [2, 5]. Chemiluminescent detection was later introduced to alleviate the safety hazard of radioactive probes but at the cost of reduced sensitivity, particularly with smaller alleles [4].

Besides the disadvantages presented in the technique, RFLP analysis had limited potential due to the need for high-quantity (>50 ng) and high-quality (intact, high molecular weight, >12 kb) DNA [2, 5]. Forensic samples in particular do not always offer the quality and/or quantity that RFLP analysis requires. DNA samples are subject to compromise, both intentionally and unintentionally. Potentially incriminating forensic DNA evidence might be destroyed in an effort to remove it prior to a collection by law enforcement. Alternatively, harsh environmental conditions, such as excessive sunlight [6] or improper packaging and storage by improperly trained individuals, could also reduce the likelihood of obtaining interpretable results. Furthermore, given the potential impact of the results of HID DNA analysis, whether to assist in sentencing a perpetrator to jail time or to confirm the donor of a cancer tumor, ample sample should exist for verification through reanalysis. The relatively large quantity of sample required for successful RFLP analysis did not lend itself well to sample conservation for reanalysis [2].

To complicate matters further, RFLP labs individually chose which restriction enzymes to use, making interlaboratory comparisons impossible. While the same VNTR probes may have been used, the differing restriction enzymes would have produced alleles of different lengths. *HaeIII* was used most often in the United States (by the Federal Bureau of Investigation (FBI) [1] and other public labs in the USA and Canada) to fragment DNA, but most European labs and Cellmark Diagnostics, a private US lab, used *HinfI* for digestion. Lifecodes Corporation, another private US lab, used *PstI* for its testing [2].

As this technology was introduced into the courtroom with potentially grave consequences for those implicated by the results, concerns over the quality and reliability of analysis in a preaccreditation world were inevitable [2]. The Technical Working Group on DNA Analysis Methods (TWGDAM), now known as the Scientific Working Group on DNA Analysis Methods (SWGAM), did not introduce guidelines for establishing validation criteria for RFLP analysis until 1991 [7], years after RFLP analysis was first introduced in the courts [8].

25.3 STR-Based DNA Testing

Since the mid-1990s, labs enjoy the sensitivity of PCR-based testing as well as its ability to develop interpretable results with degraded samples. PCR technology requires as little as 250 pg of template DNA for the detection of a complete DNA profile [9]. The 2–7 bp-sized repeat units of STRs permit the amplification of degraded DNA more readily than similarly structured VNTRs, which are generally

Table 25.1 Application of STRs in human identification [17]

<ul style="list-style-type: none"> • Forensic DNA casework • DNA databases (convicted offenders, forensic crime scene evidence) • Mass disasters • Missing persons investigations • Military casualties • Parentage and kinship testing • Genetic genealogy and answering historical questions • Authenticating human cell lines • Detecting genetic chimeras • Monitoring transplants • Monitoring needle sharing • Detecting cancer tumors • Disease locus mapping • Carrier diagnosis of disease states • Mapping genetic diversity • Agricultural genetics • Examining human population diversity
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about 10–100 bps in size [8, 10]. And while other PCR-based testing methods preceded the widespread use of STRs, none matched the discrimination power offered by the highly polymorphic VNTRs examined in RFLP analysis [11].

In addition to requiring less template DNA (approximately 1 ng or less) [12], the introduction of multiplexing, or coamplifying multiple STR loci simultaneously, allows for a further reduction in sample consumption [10]. More samples remain available for retesting with the same technology, or perhaps for additional testing with a technology that is not yet available. Many US forensic laboratories routinely multiplex 15 or more loci [2]. STR analysis is also automatable, providing a significantly increased throughput potential [13]. With the aid of an “autosampler,” the analyst simply tells the machine to start and walks away. The analyst need not be present for fragment separation to occur [8].

In 1997, the FBI selected the following 13 core loci to be used by forensic laboratories in the USA: CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11 [14]. Selection of common loci for laboratories to study not only permits comparison of data among laboratories without retesting (and further consumption of sample) but it also makes possible the use of a national DNA database – see Table 25.1 for the application of STRs in human identity testing [10]. A national database can serve as an investigative tool, possibly linking crime scene evidence to another crime scene, or crime scene evidence to a convicted offender whose profile has been entered into the database. A database may also serve to link the remains of an individual to his or her parents or child(ren) through DNA analysis in a missing person investigation [14, 15]. Combined DNA Index System (CODIS) software permits the submission, the search, and the storage of STR profiles for eventual comparison [16].

Limitations do exist for PCR-based STR analysis. STR loci are less variable than VNTR loci; this requires the use of more locations during examination to yield a similar discriminating power [12]. The start up and maintenance of this technology can be cost-prohibitive [12]. Interpretation of STR data requires knowledge of and experience with both amplification and electrophoretic artifacts, both of which can complicate mixture analysis. The greater sensitivity provided by the PCR-based technology allows for the increased potential of non-target amplification – contamination [2]. Laboratory policies must be preventative and reactionary to adequately address unintended DNA amplification. And finally, PCR is subject to inhibition. Inhibitors such as hemoglobin or denim’s indigo dyes can bind the active site of the Taq polymerase, impeding elongation during the PCR [2]. Failure to completely remove inhibitors which may coextract during sample purification may result in a partial or a complete failure

to amplify the template DNA. Bovine serum albumin (BSA), a standard component of commercially available PCR amplification reaction mixes, increases PCR efficiency by binding inhibitors [11, 16].

25.4 Commercially Available STR Amplification STR Amplification Kits

While laboratories have the ability to create the PCR tests in-house, commercially available kits remove the burden of quality control from that laboratory, allowing individual labs to focus more on casework analysis, particularly as the demand for DNA testing has increased and continues to grow [10]. The kits provide the consumer with a primer cocktail; a reaction mixture that contains the dNTPs, buffers, and various amplification components used to enhance the PCR; and DNA polymerase [26]. Most importantly, the premade kits also provide labs with allelic ladders for calibrating the amplified product during genotyping [10].

Allelic ladders are artificial mixtures of the most common alleles found in the population for each locus. The fragments contained within the ladders migrate as the fragments created in the PCR migrate during electrophoresis. By using the same primers for amplification, ladder alleles are the same size and sequence as the alleles synthesized in PCR. Having the ladders enables precision and reliability, both essential for interlaboratory comparisons [12]. The introduction of differently colored fluorescent tags at PCR allows for fragments of similar length, but from different loci, to be independently detected despite simultaneous separation via electrophoresis. During data analysis, analysis software assigns an allele designation to detected fragments, based upon not only its fragment length as it relates to the allelic ladder but also the color of the fluorescent label associated with that fragment.

The first commercially available kit was released in 1993 by the Promega Corporation. This kit included just three tetranucleotide STR loci, each of which was ultimately included in the 13 US core loci: TH01, TPOX, and CSF1PO [8]. Today, there are three major commercial suppliers of STR multiplex kits: Promega Corporation (Madison, WI), Biotype® (Dresden, Germany), and Applied Biosystems by Life Technologies™ (Foster City, CA) [18]. The AmpFlSTR® Identifiler® PCR Amplification Kit, which will be examined here, uses four dye labels for the loci, as well as a fifth dye for an internal size standard, to coamplify the following: the sex-determining gene Amelogenin, the 13 US core tetranucleotide loci, and two additional tetranucleotide loci contained within the 10 core loci of the UK and much of Europe (D2S1338 and D19S433) [19–21].

25.4.1 DNA Amplification with AmpFlSTR® Identifiler® PCR Amplification Kit [20]

Specimen types

Purified, inhibitor-free DNA samples that have been previously quantified

Equipment and Materials

- Calculator
- Gloves, disposable
- Marker pen, permanent
- Vortex
- Microcentrifuge or pulse spinner
- 1.5-mL microcentrifuge tube, sterile
- Tube rack

- Pipettors, adjustable
- Pipet tips, sterile, disposable hydrophobic filter-plugged
- Tube decapper or Kimwipes for opening tubes

Items from Life Technologies	Part numbers
1. MicroAmp® 96-Well Trays for Tubes with Caps ^a	N801-0541
2. MicroAmp® Reaction Tubes with caps, 0.2 mL ^a	N801-0540
3. MicroAmp® Reaction Tubes (8 tubes/strip) ^a	N801-0580
4. MicroAmp® Caps (8 caps/strip) ^a	N801-0535
5. MicroAmp® 96-Well Tray/Retainer Set ^a	403081
6. MicroAmp® Optical 96-Well Reaction Plate ^a	N801-0560
7. MicroAmp® 96-Well Base ^b	N801-0531
8. DNA Thermal Cycler (e.g., GeneAmp® PCR System 9700) ^c	N805-0001
9. AmpFISTR® Identifiler® PCR Amplification Kit User Manual ^c	4323291

^aSuggestions for usage: either 1 and 2; or 3, 4, and 5; or 4 and 6

^bItem 7 is recommended

^cItems 8 and 9 are required

25.4.1.1 Reagents

AmpFISTR® Identifiler® PCR Amplification Kit (Applied Biosystems, P/N 4322288), which includes:

- Control DNA 9947A (female)
- AmpliTaq Gold® DNA Polymerase
- Primer set
- PCR reaction mix (which includes MgCl₂, dATP, dGTP, dCTP, dTTP, bovine serum albumin, and 0.05% sodium azide in buffer and salt)
- Identifiler® Allelic Ladder (to be used postamplification)
- TE buffer (10 mM Tris–HCl containing 1 mM EDTA–Na₂) or sterile, deionized water

Controls

An amplification positive control (Control DNA 9947A from kit, 0.10 ng/μL). This positive control will consist of all amplification reagents with control DNA in place of sample DNA. 5–10 μL may be used, depending on the laboratory's optimal target, as established by validation. If using less than 10 μL, add the difference in TE buffer or sterile, deionized water.

An amplification negative control (TE buffer or sterile, deionized water). This negative control will consist of all amplification reagents with 10 μL of TE buffer or sterile, deionized water in place of sample DNA.

25.4.2 Procedure

25.4.2.1 Setup PCR Reaction

1. Label PCR tubes with sample name, or 96-well plate with appropriate identifying information. If using a 96-well plate, also create a worksheet that indicates the location of each sample on the 96-well plate.
2. Vortex PCR reaction mix, primer set, and AmpliTaq Gold® DNA Polymerase. Briefly spin tubes to remove any liquid from the caps.

Table 25.2 Thermal cycling for AmpFlSTR® Identifier® PCR Amplification Kit [20]

Initial incubation step	Denature	Anneal	Extend	Final extension	Final step
Hold		Cycle (28 cycles)		Hold	Hold
95°C	94°C	59°C	72°C	60°C	4–25°C
11 min	1 min	1 min	1 min	60 min	(forever)

3. Prepare a master mix in a 1.5 mL sterile microcentrifuge tube as follows:

Reaction mix	10.5 × # of samples	μL
Primer set	5.5 × # of samples	μL
AmpliTaq Gold ® DNA Polymerase	0.5 × # of samples	μL
	16.5	μL

Note: While creating a master mix is not necessary, it is recommended to ensure consistency in reagent content for all samples being amplified.

- 4. Mix by vortexing for approximately 5 seconds.
- 5. Briefly spin to remove liquid from cap.
- 6. Dispense 15 μL of master mix into each tube or well.
- 7. Add approximately 1 ng of sample DNA to the appropriate tube or well, not to exceed 10 μL in total sample volume. Addition of more or less template DNA may be appropriate to get optimal results. While the manufacturer suggests a range of 0.5–1.25 ng (IDF user manual; [20]), your laboratory validation may indicate a different target amount of DNA. Samples with high concentrations of DNA may be diluted with TE buffer prior to adding to the tube or well containing master mix.
- 8. Vortex Control DNA 9947A and spin briefly to remove liquid from cap. Add 5–10 μL of Control DNA 9947A from kit to the appropriate tube or well, adding an appropriate amount of TE buffer or sterile, deionized water if <10 μL is used. This will serve as your amplification positive control. You must include at least one positive control per PCR. Expected results for this sample indicate that the PCR components functioned properly (during fragment analysis, expected results also indicate proper genotyping).
- 9. Add 10 μL of TE buffer or sterile, deionized water to the appropriate tube or well to serve as the amplification negative control. Use of this control demonstrates that the PCR kit components were free of contaminating DNA prior to master mix distribution.
- 10. Cap the amplification tubes or wells.
- 11. The thermal cycler should be programmed according to Table 25.2 above. If using the GeneAmp® PCR System 9700, select the 9600 emulation mode so that temperature ramp speeds are set for 1°C/s, rather than 4°C/s, which is the 9700 default. Place the tubes or 96-well plate onto the thermal cycler and select the appropriate program and the appropriate sample volume. Start the program.
- 12. After amplification, remove the samples from the thermal cycler and store away from light. Store the amplification product refrigerated for short periods (<2 weeks) and frozen for long periods (<2 weeks).

25.5 Fragment Separation and Detection (AB 3130xl Genetic Analyzer)

The AB 3130xl Genetic Analyzer – see Fig. 25.1 below – separates and detects fluorescently labeled DNA amplification product using capillary electrophoresis with 16 capillaries operating in parallel [22, 23]. DNA amplicons are diluted with a mixture of deionized formamide (to ensure single-stranded



Fig. 25.1 *AB 3130xl Genetic Analyzer*: Applied Biosystems 3130xl Genetic Analyzer is a 16-cappillary electrophoresis instrument for medium throughput laboratories. Its automation capabilities allow for both sequencing and fragment analysis applications. Its dimensions, at 130 kg, are 74 cm in width (*closed door*), 54.8 cm in depth, and 81 cm in height

DNA after denaturation) and an internal size standard that enables fragment sizing during data interpretation [24]. This mixture is injected into a capillary by electrokinetic injection and separated by the sieving ability of polymer during electrophoresis. As the fluorescently labeled DNA fragments pass a laser beam and emit energy, a charge-coupled device (CCD) camera captures the fluorescence wavelength (525–680 nm) [24] and scan number, or data point from the start of the run [25]. This information is recorded and later converted into an electropherogram during data analysis [25]. By comparison with the internal size standard, the scan numbers of detected fragments are translated into base pair sizes for the associated DNA peaks or alleles. The use of multiple dyes allows for the simultaneous detection of fragments of multiple loci whose alleles are similar in length. Injection of allelic ladders alongside samples during electrophoresis allows the analysis software to assign the appropriate allelic designation by comparing the base pair size of a particular unknown fragment of a particular dye label to the corresponding known allele of the same length and color within the allelic ladder.

25.5.1 Specimen Requirements

Fluorescently labeled PCR-amplified STR DNA product that targeted 0.5–1.25 ng of template DNA, using the AmpFISTR® Identifier® PCR Amplification Kit.

25.5.1.1 Equipment and Materials

- Calculator
- Gloves, disposable
- Marker pen, permanent
- Lint-free tissue wipes

- MicroAmp® Optical 96-Well Reaction Plate
- MicroAmp® 96-Well Base
- 1.5-mL microcentrifuge tube, sterile
- Tube rack
- Pipettors, adjustable
- Pipet tips, sterile, disposable hydrophobic filter-plugged
- Tube decapper or Kimwipes for opening tubes
- 96-well plate septa
- Vortex
- Plate centrifuge
- DNA thermal cycler (e.g., GeneAmp® PCR System 9700)
- 96-well plate retainer
- 3130xl Genetic Analyzer (instrument, computer, and data collection software)
- 3130xl/3100 Genetic Analyzer Capillary Array, 36 cm
- 3130xl Buffer Reservoirs
- 3130xl Reservoir Septa
- 3130 Series Training DVD
- Applied Biosystems 3130/3130xl Genetic Analyzers Getting Started Guide
- Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide

25.5.1.2 Reagents

- POP-4™ Polymer for 3130/3130xl Genetic Analyzers
- 10× Genetic Analyzer Buffer with EDTA
- DS-33 Matrix Standard Kit (Dye Set G5)
- GeneScan™ 500 LIZ™ Size Standard
- Identifiler® Allelic Ladder (contained within PCR Amplification Kit)
- Hi-Di™ Formamide (highly deionized)
- Water (molecular biology grade)

Controls

The Identifiler® Allelic Ladder will be used to calibrate fluorescently labeled STR fragments during data analysis. The amplification positive control (Control DNA 9947A from kit, 0.10 ng/μL), if typed correctly, will demonstrate that the PCR components functioned properly and that analysis software genotyped correctly because proper fragment separation occurred.

Use of 10 μL of TE buffer or sterile, deionized water for the amplification negative control establishes that the PCR kit components were free of contamination. A “blank” amplification negative control also helps demonstrate that DNA is not transferred from sample to sample during the robotic injection of samples on the genetic analyzer (assuming a sample with DNA was injected using the same capillary, prior to the amplification negative control sample within that run). The GeneScan™ 500 LIZ™ Size Standard that is coinjected with each sample provides a curve of known fragment sizes to which unknown fragments will later be compared.

25.5.2 Procedure

25.5.2.1 Genetic Analyzer Preparation

1. Power on the monitor.
2. Power on the computer.
3. Log on to Windows.
4. Power on the instrument by pressing the on/off button on the front of the instrument.
5. Ensure the green status light is on and not flashing.
6. Start the 3130xl Data Collection software: Select **Start>All Programs>Applied Biosystems>Data Collection>Run 3130xl Data Collection v3.0** to display the service console. As each application proceeds from being off to activating to fully functioning, the shape adjacent to the application will progress from a red circle to a yellow triangle to a green square.
7. Click + to expand the subfolders of the left tree pane.
8. Perform the daily maintenance tasks – see Chap. 1 in the Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide.
9. To install a capillary array, select the **Install Array Wizard** and follow the prompts. Optional: Select Update Cap Array Info wizard to correct any errors or update capillary array and serial number information.
10. Add fresh polymer if insufficient polymer is available for your next run. Replace polymer on the instrument if it is the wrong type.
 - (a) Wear gloves while handling polymer, the capillary array, septa, or buffer reservoirs.
 - (b) Remove the polymer from 4°C storage.
 - (c) Loosen the cap and bring the polymer to room temperature.
 - (d) To dissolve deposits, tighten the cap and gently swirl the polymer.
 - (e) Select **Wizards>Replenish Polymer Wizard** to replenish polymer. Select **Wizards>Change Polymer Type Wizard** to change to a different polymer.
11. Prepare buffer for a single run.
 - (a) Add 5 mL of 10× Genetic Analyzer buffer with EDTA into a graduated cylinder.
 - (b) Add purified water to bring the total volume up to 50 mL.
 - (c) Mix well.
 - (d) Store 1× running buffer at 2–8°C for up to 1 month or at room temperature for 1 week.
12. Replace the 1× running buffer in the anode and cathode buffer reservoirs daily, or before each batch of runs. Failure to do so may lead to loss of resolution and data quality. The capillary will be left exposed to the air as the autosampler is brought to the forward position for cleaning and filling reservoirs. If left in this position for an extended time, the capillaries can dry out and damage the array.
13. Fill the water and cathode buffer reservoirs.
 - (a) Verify the oven and instrument doors are closed.
 - (b) Press the “Tray” button on the outside of the instrument to bring the auto sampler to the forward position.
 - (c) Wait until the auto sampler stop at the forward position, open the instrument door.
 - (d) Remove the cathode buffer and water reservoirs from the instrument.
 - (e) Dispose of remaining fluids and rinse out the reservoirs with deionized water.
 - (f) Rinse the cathode reservoir with 1× Genetic Analyzer Buffer with EDTA, and then fill to the line with 1× Genetic Analyzer Buffer with EDTA (about 16 mL).
 - (g) Fill the two water reservoirs to the line with quality deionized water (about 16 mL).
 - (h) Place a clean reservoir septa on each reservoir, and dry the outside of the reservoirs using a lint-free tissue wipe.

- (i) Place the cathode reservoir in the front left position on the autosampler. Place the two water reservoirs on the back left and right positions on the auto sampler.
 - (j) Close the instrument doors. (This will cause the autosampler to return to the last known position, returning the capillaries in water or in buffer.)
14. Fill the anode buffer reservoir. (Change the anode buffer every 24 h, before each run or batch of runs, or each time the polymer is replenished or changed.)
 - (a) Remove the anode buffer reservoir by firmly pulling down and twisting slowly.
 - (b) Discard the used buffer appropriately.
 - (c) Clean and rinse the reservoir with deionized water, and then rinse with buffer.
 - (d) Fill the anode buffer reservoir to the fill line with fresh 1× Genetic Analyzer Buffer with EDTA (about 16 mL). The meniscus should line up with the fill line.
 - (e) Put the anode buffer reservoir on the instrument.
 - (f) If the reservoir fills with fluid during bubble removal, repeat this procedure to discard and replace the running buffer.
15. Create a spatial calibration file. A spatial calibration provides information about the position of the fluorescence from each capillary on the CCD. It does not provide information about the performance of the capillaries. A spatial calibration must be performed each time you install or replace a capillary array or you temporarily remove the capillary array from the detection block.
 - (a) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Spatial Run Scheduler**.
 - (b) If the capillaries contain fresh polymer, select **Protocol>3130SpatialNoFill_1**.
 - (c) Otherwise, select **Protocol>3130SpatialFill_1**.
 - (d) Click start. Without filling the capillaries, the calibration run lasts approximately 2 min. If the capillaries are filled, the run takes approximately 6 min.
16. Evaluate the spatial calibration file.
 - (a) All peaks should be of similar heights.
 - (b) One orange cross should mark the top of every peak, with no misplaced crosses.
 - (c) Each capillary should have a single, sharp peak (small shoulders are acceptable).
 - (d) The difference between adjacent positions should be 13–16 pixels (theoretical spacing is 15 pixels).
17. Accept or reject the spatial calibration by clicking accept to write the calibration data to the database and .ini file or by clicking reject.
18. Perform a spectral calibration. The spectral calibration creates a matrix that is used during a run to reduce raw data from the instrument to the 5-dye data stored in the sample files. You should perform the spectral calibration when: a new dye set is used on the instrument; when the capillary array length is changed (or polymer type); after the laser or CCD camera has been realigned/replaced by a service engineer; if you begin to see a decrease in spectral separation (pull-up or pull-down) in the raw or analyzed data; or once every 6 months.
 - (a) Prepare spectral calibration chemistry.
 - (i) Remove the tube of matrix standard from the refrigerator.
 - (ii) Mix thoroughly then briefly spin in a microcentrifuge or pulse spinner.
 - (iii) Follow the matrix standard insert for the matrix and Hi-Di formamide ratios.
 - (iv) Vortex thoroughly. Briefly centrifuge the mixture.
 - (v) Add 10 µL of standard to wells A1 through H2 of a 96-well reaction plate.
 - (vi) Place a clean 96-well septa on the plate, aligning the holes in the septa with the wells of the plate, and then pressing down firmly.

- (vii) Briefly centrifuge the plate.
 - (viii) Heat the plate at 95°C for 5 min to denature the DNA.
 - (ix) Cool the plate at 4°C for 2 min.
 - (x) Place the reaction plate onto the plate base. Snap the plate retainer onto the plate and plate base.
- (b) Create a spectral instrument protocol.
- (i) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Protocol Manager**.
 - (ii) Click New to open the Protocol Editor dialog box.
 - (iii) Complete the Protocol Editor dialog box.
 - 1. Enter a name for the protocol.
 - 2. Optional: Type a description for the protocol.
 - 3. Select Spectral in the Type drop-down list.
 - 4. Select the correct dye set for your run.
 - 5. Select the Polymer and Array Length from the appropriate drop-down list.
 - 6. Select your chemistry file in the Chemistry drop-down list.
 - 7. In the Run Module drop-down list, select the run module Spec36_POP4.
 - 8. Click OK.
- (c) Create the plate record.
- (i) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Plate Manager**.
 - (ii) Click New to open the New Plate dialog box.
 - (iii) Complete the New Plate dialog box.
 - 1. Enter a name for the plate.
 - 2. Optional: Enter a description of the plate record.
 - 3. Select **Spectral Calibration** in the Application drop-down list.
 - 4. Select **96-Well** in the Plate Type drop-down list.
 - 5. Enter a name for the owner and the operator.
 - 6. Click OK.
- (d) Complete the Spectral Calibration Plate Editor dialog box.
- (i) In the Sample Name column of the Spectral Calibration Plate Editor dialog box, enter a sample name, and then click the next cell. The value 100 automatically displays in the Priority column.
 - (ii) Optional: In the Comments column, enter any additional comments.
 - (iii) In the **Instrument Protocol 1** column, select a protocol from the list or create a new protocol.
 - (iv) Highlight the entire row.
 - (v) Select **Edit>Fill Down Special** (Alt + D).
 - (vi) Click OK.
- (e) Place the plate assembly into the instrument.
- (i) Verify the oven and front doors are closed.
 - (ii) Press the Tray button and wait for the autosampler to stop at the forward position.
 - (iii) Open the front doors.
 - (iv) Place the plate assembly on the autosampler in position A or B.

- (v) Ensure the plate assembly fits flat in the autosampler.
 - (vi) Close the instrument doors.
- (f) Run the spectral calibration plate.
- (i) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Run Scheduler>Plate View**.
 - (ii) Search for your plate record. In the **Scan or Type Plate ID** text box, type in the plate name and click **Search** to find the plate. Or select **Find All**. All plates in the database will display in the plate record section.
 - (iii) Link the plate. Select the plate record that you want. Click the plate position indicator that matches the plate you want linked. The plate map color will change from yellow to green when it is successfully linked.
 - (iv) In the toolbar of the data collection software, click the green play arrow to begin the run. Note: The instrument may pause before running the plate to raise the oven temperature.
19. View the Pass/Fail Status after the run.
- (a) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Instrument Status>Event Log**.
 - (b) In the Event Messages section of the window, view the status of each capillary.
 - (c) Each capillary should have a Q value above 0.95 and a condition number range of 8.5–14.5.
20. Evaluate the spectral profile and raw data.
- (a) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Spectral Viewer**.
 - (b) In the Dye Set drop-down list, select the G5 dye set.
 - (c) In the plate diagram, select a well on the plate diagram to view the capillary spectral results.
 - (d) Evaluate the spectral profile and raw data for the selected capillary:
 - (i) Verify that the order of the peaks in the spectral profile from left to right is blue-green-yellow-red-orange.
 - 1. If yes, proceed to 25.5.2.1.20.d.ii.
 - 2. If no, the calibration has failed. See the Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide.
 - (ii) Verify that the order of the peaks in the raw data profile from left to right is orange-red-yellow-green-blue.
 - 1. If yes, proceed to 25.5.2.1.20.d.iii.
 - 2. If no, the calibration has failed. See the Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide.
 - (iii) Verify that the peaks in the spectral profile do not contain gross overlaps, dips, or other irregularities.
 - 1. If yes, the capillary has passed. Repeat this process for every capillary.
 - 2. If no, the calibration has failed. See the Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide.
21. Set an active spectral calibration.
- (a) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Spectral Viewer**.

- (b) In the Dye Set drop-down list, select a dye set.
- (c) In the List of Calibrations for Dye Set drop-down list, select the spectral calibration you want to use. The spectral profile and raw data is displayed.
- (d) Click Set.

Sample Preparation

1. Prepare a master mix of deionized formamide and GeneScan™ 500 LIZ™:
 - (a) 8.85 µL deionized formamide × number of samples, controls, and ladders
 - (b) 0.15 µL GeneScan™ 500 LIZ™ × number of samples, controls, and ladders
2. Vortex thoroughly. Briefly centrifuge the mixture.
3. Label 96-well reaction plate.
4. Aliquot 9 µL of mixture into plate.
 - (a) Note: All 16 wells of every two columns containing at least one sample must contain formamide, even if it does not contain sample, to prevent air bubbles from entering a capillary at injection.
5. Add 1.0 µL of PCR product or AmpFISTR Allelic Ladder per well. Mix by pipetting up and down.
 - (a) Note: PCR product may be diluted with TE prior to this step to achieve acceptable RFU (Relative Fluorescence Unit) values.
6. Place a clean 96-well septa on the plate, aligning the holes in the septa with the wells of the plate, and then pressing down firmly.
7. Briefly centrifuge the plate to remove bubbles.
8. Heat the plate at 95°C for 5 min to denature the DNA. Cool the plate to 4°C.
9. Place the reaction plate onto the plate base. Snap the plate retainer onto the plate and plate base.
10. Place the plate assembly in the instrument.
 - (a) Verify the oven and front doors are closed.
 - (b) Press the Tray button and wait for the autosampler to stop at the forward position.
 - (c) Open the front doors.
 - (d) Place the plate assembly on the autosampler in position A or B.
 - (e) Ensure the plate assembly fits flat in the autosampler.
 - (f) Close the instrument doors.
 - (g) The samples are ready to be run.

Start the Run

1. Create an instrument protocol.
 - (a) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Protocol Manager**.
 - (b) Click New to open the Protocol Editor dialog box.
 - (c) Complete the Protocol Editor dialog box.
 - (i) Enter a name for the protocol.
 - (ii) Optional: Type a description for the protocol.

- (iii) Select Regular in the Type drop-down list.
 - (iv) Select the run module HIDFragmentAnalysis36_POP4_1.
 - 1. To customize a run module, see the Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide.
 - (v) Select the dye set G5.
 - (vi) Select OK.
2. Create the plate record for analysis.
- (a) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Plate Manager**.
 - (b) Select New to open the New Plate dialog box.
 - (c) Complete the New Plate dialog box.
 - (i) Enter a name for the plate.
 - (ii) Optional: Enter a description of the plate record.
 - (iii) Select your GeneMapper application in the Application drop-down list.
 - (iv) Select 96-Well in the Plate Type drop-down list.
 - (v) Enter a name for the owner and the operator.
 - (vi) Select OK.
 - (vii) The GeneMapper software Plate Editor opens.
 - (d) Complete the plate record.
 - (i) In the Sample Name column of a row, enter a sample name then click the next cell. Optional: In the Comments column, enter any additional comments or notations for that sample.
 - (ii) In the Priority column, change the priority value, if desired.
 - (iii) In the Sample Type column, select a sample type from the drop-down list.
 - (iv) In the Size Standard column, select a size standard from the drop-down list.
 - (v) In the Panel column, select a panel from the drop-down list.
 - (vi) In the Analysis Method column, select a method from the drop-down list.
 - (vii) In the SNP Set column, select an SNP set from the drop-down list, if applicable. Otherwise, select None.
 - (viii) Enter text for User-Defined columns 1–3, such as the analyst's initials, the injection time, or a batch identifier (optional).
 - (ix) In the Results Group 1 column, create a new Results Group or select a group from the drop-down list.
 - (x) In the Instrument Protocol 1 column, create a new instrument protocol or select a protocol from the drop-down list.
 - (xi) For the same samples and protocols, highlight the entire row. Select **Edit>Fill Down Special (Alt + D)**.
 - (xii) To do more than one run, select **Edit>Add Sample Run (Alt + A)**.
 - (xiii) Complete the columns for the additional runs.
 - (xiv) Click OK to save then close the plate record.
 - (e) Link the plate.
 - (i) In the tree pane of the data collection software, Select **GA Instruments>ga3130xl>Run Scheduler>Plate View**.
 - (ii) Search for your plate record. Select **Find All**. All plates in the database will display in the plate record section.

- (iii) Link the plate by selecting the plate record that you want. Select the plate position indicator that matches the plate you want linked. The plate map color will change from yellow to green when it is successfully linked.
- (f) View the Run Schedule.
 - (i) In the tree pane of the data collection software, select **GA Instruments>ga3130xl>Run Scheduler>Run View**.
 - (ii) Select a row for any run. The corresponding wells to be injected for that run are highlighted in the plate diagram.
 - (iii) Start the Run. In the toolbar of the data collection software, select the green play arrow to begin the run. Note: The instrument may pause before running the plate to raise the oven temperature.

25.6 Fragment Analysis

25.6.1 Principle

Automated genotyping software can be used to analyze the raw data captured by the genetic analyzer during capillary electrophoresis. Allele assignment occurs through a three step process: spectral separation, peak base pair sizing, and allele designations. GeneMapper™ ID Software, Version 3.2, converts the electrical signal recorded during electrophoresis into an electropherogram that is used for data interpretation.

25.6.2 Spectral Separation

Identifiler® uses four dyes to fluorescently label the DNA fragments synthesized during the PCR. Those are 6-FAM (blue), VIC (green), NED (yellow), and PET (red). A fifth dye, LIZ (orange), is used to label the internal size standard, GeneScan™ 500 LIZ™. Each of these dyes emits its maximum fluorescence at a different wavelength ranging from 525 to 680 nm [24], but there is some overlap in the emission spectra between the dyes. By creating a spectral calibration (see Sect. 25.5.2.1.18), the analysis software attempts to separate the five different fluorescent dye colors into distinct spectral components. The spectral calibration creates a matrix file of numbers that represent the mathematical description of the spectral overlap among the five dyes. With a quality spectral calibration, DNA fragments of similar sizes, but from different loci, can be assigned to the proper locus during analysis based upon their dye [20].

25.6.3 Peak Base Pair Sizing

The internal size standard, GeneScan™ 500 LIZ™, is coinjected with each sample and normalizes injection-to-injection variations in migration. A calibration curve is created using the migration of the fragments of known size in the size standard. The unknown DNA fragments synthesized in the PCR are then compared to that curve and assigned a size value [24].

correctly, will demonstrate that the PCR components functioned properly and that analysis software genotyped correctly because proper fragment separation occurred. The GeneScan™ 500 LIZ™ Size Standard that is coinjected with each sample provides a curve of known fragment sizes to which unknown fragments will be compared.

25.6.4.2 Procedure/DNA Analysis

1. Login to GeneMapper with a username and password.
2. With a new project open, select **File > Add Samples to Project** to navigate to the disk or directory containing the sample files.
3. Select the data folder you wish to analyze and select **Add To List** at the bottom of the screen. If the wrong folder is selected, simply select that wrong folder and select **Clear** to remove it from your list.
4. Click **Add** to import files into the project and close the dialog box.
5. Verify the Sample Type, Analysis Method, Panel, and Size Standard are correct.
 - (a) Sample types: Allelic ladder or sample (The allelic ladder that will be used for analysis must be identified as “Allelic Ladder.” Failure to do so will prohibit successful analysis.)
 - (b) Correct Analysis Method
 - (c) Panel: Identifiler_v2
 - (d) Correct Size Standard
6. Verify the following within the Analysis Method window:
 - (a) The analysis range is set to exclude the primer peaks.
 - (b) The analysis smoothing option is “light.”
 - (c) The size calling method is Local Southern.
 - (d) The minimum peak threshold is 100 RFUs (or the value your laboratory’s validation has determined as acceptable).
7. Highlight a column heading and select the green arrow in the toolbar.
8. You will be prompted to name the project before analysis will take place.
9. Once the project has been analyzed, view the samples by selecting the **Display Plots Button** to open the Samples Plot window.
10. Deselect all colors and select only orange to examine the LIZ peaks. Injection quality can be easily assessed by examining the LIZ peaks.
 - (a) Verify that all of the following peaks are present in LIZ: 75, 100, 139, 150, 160, 200, floater (245–250), 300, 340, 350, 400, and 450 bps.
 - (b) Examine peak morphology. Verify that all peaks are well formed and similar in height.
 - (c) Verify that all nonladder floating peaks are within ± 0.5 bp of the ladder’s floating peak. If not, outlier samples may be analyzed with a different ladder from the run or reinjected.
11. Examine the ladder and controls.
 - (a) Verify that the allelic ladder was correctly labeled (see AmpFISTR® Identifiler® PCR Amplification Kit User Manual) [20].
 - (b) Verify that all amplification positive control peaks are present and labeled correctly. Each amplification set must have at least one acceptable positive control and each genetic analyzer run must have one acceptable positive control.
 - (c) Verify that all amplification negative controls and extraction reagent blanks are free of labeled allelic activity that cannot be readily attributed to an amplification or electrophoretic artifact. Each genetic analyzer run must have one acceptable negative control.

12. Examine all sample plots for data quality and allele calls. Refer to the laboratory's data interpretation guidelines for guidance with editing artifacts. Data that is inconsistent with quantitation results may be subject to reinjection, reamplification, or reextraction.
13. Print the plots of the allelic ladder used for analysis, the positive and negative controls, the associated reagent blank(s), and all samples for each case file.
14. Select **File>Close Plot Window** to return to the Project window and select **File>Save Project** to save any changes before exiting the GeneMapper ID software.

25.7 Troubleshooting: Artifacts

Artifacts can certainly make data interpretation difficult for the novice analyst, but sound and conservative interpretations are achievable with an understanding of the limitations of the multiplex PCR systems [26]. Without proper identification, artifacts could potentially lead to the incorrect exclusion of an individual when his/her known DNA profile does not contain the "false" allele [2]. Artifacts routinely observed in STR data analysis can be divided into two major groups: artifacts of amplification or biology and artifacts of electrophoresis or technology [10]. Generally speaking, amplification artifacts are reproducible upon capillary electrophoresis reinjection, while electrophoretic artifacts will not be reproduced upon reinjection. The most common artifacts are described below.

25.7.1 *Artifacts of Amplification*

25.7.1.1 Stutter Peaks

Stutter peaks are the most common artifacts observed. Stutter peaks result when there is slipped-strand mispairing during the extension phase of PCR, causing the amplification of a strand of DNA one repeat unit shorter ($n-4$) or larger ($n+4$) than the true allele [8]. Candidates for stutter qualify not only based upon their position, relative to the true allele, but also by their size. Stutter peaks are much smaller than the true allele. Applied Biosystems' stutter validation for the AmpFISTR® Identifiler® PCR Amplification Kit showed in their lab that stutter peaks are less than 15% of the true peak's height, at all loci except D18S51 (17.0%) [20]. Stutter in samples with evidence of a minor contributor should be interpreted with caution to prevent true minor alleles from being ignored. Stutter may be elevated when excessive template DNA is used [6].

25.7.1.2 Incomplete Nucleotide Addition

Incomplete 3' (+A) nucleotide addition occurs when excessive template DNA is added to the PCR reaction or when an inhibitor of the *Taq* polymerase is present in the reaction [6]. The *Taq* DNA polymerase used in most commercially available kits prefers to add an extra nucleotide, usually adenine, to the 3' end of the PCR product [2]. For consistency, the kits have been optimized for complete adenylation of all strands, making the detected fragments actually one base larger than the template DNA's original sequence [26]. When adenylation is incomplete, the result is a "split peak." The artifact will be one base pair unit shorter than the true allele. Moderate minus A may be resolved with a diluted reinjection, as the smaller peak goes undetected upon reinjection. Excessive minus A leads to two peaks roughly the same size. In the latter case, the sample should be reamplified using less template DNA.

Triallelic Patterns

Triallelic patterns are rarely observed but show up as three well-shaped peaks. They may possess similar RFU values, or one may be much smaller than the other two [2]. Triallelic patterns are the result of chromosomal or gene duplications. A three-banded profile may also be observed if somatic mutation occurred during embryological development [9]. Triallelic patterns can be distinguished from mixtures because mixtures generally contain more than two alleles at multiple loci [2].

25.7.2 Electrophoretic Artifacts

25.7.2.1 Pull-Up Peaks

Pull-up (“fluorescence bleed through” [26]) refers to the artifactual peaks that show up in a dye different than the true allele’s dye. More common when a true peak is particularly high in RFUs, pull-up occurs because the spectral did not fully separate the spectral overlap of the multiple dyes used to fluorescently label the DNA fragments [6]. The pull-up peak is either the exact base pair size of the true peak or very close to the base pair size of the true allele. Pull-up observed when true peaks are not particularly high may be an indication that a new spectral calibration should be created.

25.7.2.2 Spikes

Spikes are sometimes more easily recognized than pull-up peaks because their morphology is dissimilar to true allele peaks; spikes tend to be narrower and sharper than the nicely rounded true peaks. Though not always, they tend to occur in all five color channels. Spikes are attributed to electrical anomalies and should not appear in the same location upon reinjection [24].

25.7.2.3 Dye Blobs

Dye blobs, like spikes, have morphology so different from true alleles that diagnosing them is relatively easy. Dye blobs are generally broader than true alleles and are commonly seen earlier in the injection, around 120 bps [2]. Dye blobs occur when fluorescent dyes are detached from their primers and migrate independently through the capillary [16].

25.8 Reporting

The results and conclusions from DNA analysis must be scientifically supported by the analytical data using appropriate standards and controls. Below, conclusions will be limited to single-source DNA profiles.

25.8.1 Exclusions

A conclusion of “exclusion” generally applies in cases where the relevant reference sample contains an allele that is not observed among the interpretable alleles in the evidence profile, where dropout or loss of data is not suspected.

25.8.2 *Inclusions*

A conclusion of “inclusion” or “cannot exclude” or “match” generally applies in cases where all of the alleles contained in the reference profile are observed among the interpretable alleles in the evidence sample profile.

25.8.3 *Statistical Analysis*

If an individual cannot be excluded as the DNA donor to an item of evidence, results of a statistical analysis should accompany this conclusion [27]. Usually, a random match probability is estimated by comparing the alleles in the profile in question to a population database. This analysis provides an approximate frequency for the very specific combination of alleles observed. This frequency is intended to aid the end user of the DNA results with weight or significance of the match. Do the profiles match because they are from the same person, or do they match because two different unrelated individuals have the same DNA profile? [8] The formulas used in calculating the frequency of a DNA profile are described below.

25.8.3.1 *Heterozygote Formula*

Heterozygote: A_iA_j : $P_{ij} = 2p_i p_j$, $i \neq j$ (NRC-II formula 4.1b [27])

25.8.3.2 *Homozygote Formula*

Homozygote: A_iA_i : $P_{ii} = p_i^2 + p_i(1-p_i)\theta$, $\theta = 0.03$ (NRC-II formula 4.4a [27])

25.8.3.3 *Allele Frequencies*

Well-studied and generally accepted allele frequencies can be obtained from the FBI databases in the following articles:

Budowle B, Moretti T, Baumstark A, et al. Population data on the thirteen CODIS core short tandem repeat loci in African Americans, US Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians. JFS. 1999;44(6):1277–1286.

Budowle B, Collins P, Dimsoski P, et al. Population Data on the STR Loci D2S1338 and D19S433. Forensic Sci Commun. 2001;3(3). <http://www2.fbi.gov/hq/lab/fsc/backissu/july2001/budowle2.htm>.

To prevent the underestimation of or the inaccurate estimation of an allele frequency because it was either not represented in the database, or underrepresented in the database, a minimum allele frequency is used. The minimum allele frequency reported will be $5/2N$, where N is the number of individuals in the specified FBI population databases cited above, and $2N$ is the number of chromosomes counted because autosomes are in pairs [2]. When the observed allele frequency falls below the minimum allele frequency of $5/2N$, the minimum allele frequency will be used instead for the most conservative approach [27].

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Chapter 26

Parentage

Laura Gahn and Robin DeVille Guidry

Keywords Parentage testing • Paternity testing • Short tandem repeats • STRs • Paternity index • Combined paternity index • Probability of paternity

26.1 Introduction-Parentage Testing

26.1.1 *Autosomal DNA Inheritance and the Genetic Basis of Parentage Testing*

DNA-based determination of biological parentage makes use of the basic genetic premise that the autosomal chromosomes (and therefore autosomal loci) exist in pairs, with one of each allele pair at any given locus inherited from the biological mother and one from the biological father of the child. Therefore in examining an autosomal locus of a biological trio (mother, father, and child), we should be able to identify in the child an allele matching that of the biological mother and an allele matching that of the biological father. In a trio where either parent is in question, examining autosomal loci for alleles matching between the child and questioned parent can be informative in determining parentage, if there is significant heterogeneity in the population at the loci examined. The same genetic principles apply whether the paternal or maternal relationship is in question. Most often, such testing is carried out to determine paternity; therefore, the remainder of the chapter will discuss paternity determination, but it should be noted that the same laboratory analysis and statistical evaluation of the results can be used for maternity analysis as well.

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26.1.2 Autosomal Short Tandem Repeat (STR)–Based Parentage Testing Methods

Virtually all paternity testing is now performed using STRs (AABB Relationship Testing 2008 Annual Report). STRs are ideal for paternity analysis because they are spread throughout the genome, they are relatively easy to analyze, and there are many well-characterized loci available for use in testing. This last point is critical because characterization of allele frequencies in the population of interest is necessary for accurate statistical analysis of inclusionary paternity results.

26.1.3 Advantages of Using Commercial Multiplex Kits for Parentage Testing

Laboratories test 10–15 STR loci for a typical paternity test in order to be assured of sufficient discriminating power. This amount of analysis is obtained easily via numerous commercially available STR multiplex kits described in the previous chapter. These kits provide a reasonably priced test and, more importantly, extremely consistent results for paternity testing. Additionally, since these kits amplify many of the same loci and since there is standardization of the repeat nomenclature, results between labs and between kits can be compared. These kits also include amelogenin, a sex-determining marker, which provides an additional quality control check of both sample collection and laboratory processes, as an incorrect sex determination could indicate that a sample was incorrectly identified or switched.

26.2 Methodology

26.2.1 Starting Materials

- **DNA Profiles**

The Identifiler™ multiplex, or other commercially available multiplexes utilizing ten or more STR loci, is sufficient for most paternity testing situations. The multiplexes most commonly used in the USA currently, Identifiler™ and Powerplex16™, have 15 autosomal STR loci plus amelogenin for sex determination.

It is preferred that profiles be generated for the biological mother, child, and the alleged father. Testing can be performed in the absence of the mother; however, problems can arise in some situations in which the mother is not tested, such as cases with suspected mutations, or those in which the mother is biologically related to the tested man. Since these situations are not necessarily known prior to testing, it is preferable to include the mother in the testing when possible.

- **Allele Frequency Data**

Allele frequency data for the population (i.e., ethnic background) from which the alleged father comes should be used in the statistical analysis. Multiplex kit manufacturers provide population data that may be used in the statistical analysis of results when using their multiplex kits (see the individual kit User's Manuals). The FBI has also published allele frequency data that is widely used in these analyses [1]. Allele frequency information for the loci used in commercially available kits has been published by many authors worldwide for a large number of population groups; however, if individuals from a previously unstudied population will be subjected to paternity testing, the need for a population

Table 26.1 Obligate paternal alleles (obligate allele underlined)

Mother	Child
AB	<u>AC</u>
AB	<u>A</u>
AB	<u>AB</u>
A	<u>AB</u>
A	<u>A</u>
Not available	<u>A</u>
Not available	<u>AB</u>

study should be addressed. Some laboratories performing a large number of paternity tests have chosen to validate and use frequency data collected in their own laboratories.

26.2.2 Initial Analysis of Genetic Profiles

26.2.2.1 Identification of Obligate Paternal Alleles

The first step of analyzing DNA profiles in a paternity test is to determine if the tested man has the DNA that the actual biological father must have given to the child – the obligate paternal allele. This is determined at each locus independently.

When the biological mother is tested, at each locus, the mother’s alleles are compared to the child’s to determine what allele she donated to the child. In the case of a heterozygous child, the child’s allele not matching the mother must have been donated by the father (the obligate paternal allele). If the mother and child match both alleles, then it cannot be determined which allele came from the father, and both must be considered obligate alleles, i.e., potential matches.

In the absence of the biological mother (sometimes referred to as duo testing), it cannot be determined which of the child’s alleles came from the father, so each of the child’s alleles must be considered to be obligate paternal alleles.

Table 26.1 summarizes possible patterns of allele inheritance between the biological mother and child and resulting obligate paternal alleles.

Table 26.3, summarizes a hypothetical paternity test with a mother, a child, and two tested men, one who is excluded and one who is not. It should be noted that in this hypothetical DNA test, results for only five DNA loci are shown for simplicity. As discussed above, the Identifiler multiplex includes 15 autosomal loci plus amelogenin. In this table, the child’s obligate paternal alleles have been identified in bold.

26.2.2.2 Exclusions

Once the obligate paternal alleles have been determined, the tested man’s alleles are examined to determine whether either of his alleles at each locus matches the obligate paternal allele. If the tested man does NOT have an allele matching the child’s obligate paternal allele at three or more loci of the multiplex, it can be concluded that he is excluded as the biological father of the child. (See discussion below regarding results for one or two nonmatches.) Because the tested man is definitively excluded, no statistical evaluation of these results is required. In the example in Table 26.3, Tested Man 1 does not have an allele matching the child’s obligate paternal allele at four of the five loci tested. As a result of the four nonmatches, he can be definitively excluded as the biological father of the child.

26.2.2.3 Not Excluded, or Included

If the tested man has an allele matching the obligate paternal allele at each locus of the multiplex, then he is not excluded as being the biological father of the child. Alternatively, it may be stated that he is included as a possible biological father of the child. In Table 26.3, Tested Man 2 has an allele matching the obligate paternal allele at each of the five loci tested. Therefore, he is included as a possible biological father of the child.

26.3 Statistical Analysis

26.3.1 *Statistical Measurements of the Strength of an Inclusion*

A determination of included is only useful if some meaningful measure can then be made of the likelihood that the man in question is in fact the biological father. Therefore in paternity testing, when an initial determination of included is made, the statistical significance of the inclusionary result is determined.

Two statistical representations are typically used to express the strength or usefulness of paternity DNA evidence: the combined paternity index (CPI) and the probability of paternity [2].

These numbers take into account the pattern of alleles of the mother, child, and alleged father (including whether each is homozygous or heterozygous and which alleles the individuals share), as well as the frequency of the obligate paternal allele in the relevant population. The relevant population is considered to be the population group of the tested man. Therefore, the population group of the tested man as well as reliable population frequency information must be available. If the population group of the tested man is not known, one alternative is to calculate and report the CPI and probability of paternity for all major population groups commonly observed in the USA (or the country in which the testing is performed). Another alternative is to calculate all but report only the most conservative (lowest) resulting statistics.

26.3.2 *Paternity Index, Combined Paternity Index, and Probability of Paternity Formulas*

A paternity index is a specialized likelihood ratio that is calculated for each locus. It represents the likelihood of observing these alleles under the competing hypotheses that (1) the tested man is the biological father versus (2) the tested man is an unrelated individual of the same population group. Table 26.2 shows the paternity index formulas for each allele pattern that may be observed with or without the mother included in the testing.

In the inclusion example in Table 26.3 (Tested Man 2), for each locus, the allele pattern for the mother, child, and tested man has been matched to one of the patterns in the paternity index formula chart and a formula chosen for the PI calculation.

The loci used in paternity testing are independently inherited, therefore the product rule can be applied, and the individual PI values can be multiplied together to determine the combined paternity index (CPI).

Table 26.2 Formulas for paternity index calculations

M	C	AF	Paternity index formula
BD	AB	AC	$1/(2a)$
BC	AB	AC	$1/(2a)$
BC	AB	AB	$1/(2a)$
BC	AB	A	$1/a$
B	AB	AC	$1/(2a)$
B	AB	AB	$1/(2a)$
B	AB	A	$1/a$
AB	AB	AC	$1/[2(a+b)]$
AB	AB	AB	$1/(a+b)$
AB	AB	A	$1/(a+b)$
AB	A	AC	$1/(2a)$
AB	A	AB	$1/(2a)$
AB	A	A	$1/a$
A	A	AB	$1/2a$
A	A	A	$1/a$
Unknown	AB	AC	$1/(4a)$
Unknown	AB	AB	$(a+b)/(4ab)$
Unknown	AB	A	$1/2a$
Unknown	A	AC	$1/2a$
Unknown	A	A	$1/a$

Reference: Guidance for AABB Standards for Paternity Testing Laboratories, 9th ed.

Capital letters = allele pattern; Lowercase letters = associated allele frequency

Table 26.3 Example of paternity inclusion and exclusion

	D2S1338	D19S433	vWA	TPOX	D18S818
Mother	17, 18	13	16, 18	8	13, 16
Child	17, 20	13, 15	17 , 18	8	13 , 16
Obligate paternal allele	20	15	17	8	13 , 16
Tested Man 1 (excluded)	17, 24 (nonmatch)	12, 15	18 (nonmatch)	8, 11 (nonmatch)	12, 18 (nonmatch)
Tested Man 2 (included)	19, 20	15	17	8	16 , 19
PI formula for Tested Man 2 (see Table 26.2)	$1/2a$	$1/a$	$1/a$	$1/a$	$1/[2(a+b)]$
Frequency for PI formula ^a	0.135	0.173	0.171	0.230	0.126, 0.137
Locus PI	3.70	5.78	5.85	4.35	1.90

$CPI = 3.70 \times 5.78 \times 5.85 \times 4.35 \times 1.90 = 1,034$

Probability of paternity = $[1,034/(1,034 + 1)] \times 100 = 99.9\%$ (50% prior probability)

^aThe example frequencies used in PI calculations are for demonstration purposes only. Bold alleles are obligate alleles

Finally, the probability of paternity (expressed as a percentage) can be calculated as follows, assuming a 50% prior probability: probability of paternity = $[CPI/(CPI + 1)] \times 100$.

The prior probability of 50% assumes no prior information or knowledge of paternity in the case, and therefore a 50% chance that the tested man is (and 50% chance that the tested man is not) the father.

In Table 26.3, the five individual PIs multiplied together result in a CPI of greater than 1,000 and a probability of paternity of 99.9%.

26.4 Mutations

Because of the possibility of mutational events, a conclusion of excluded should never be based on a nonmatch at a single locus; if the tested man has alleles matching the obligate paternal allele at all but a single locus, a mutation event must be suspected. A mutation in an STR during replication most often results in the child's allele being a single repeat unit shorter or longer than the father's allele [2]. Mutations are observed in the STR loci with a frequency of about 0.1% but are more frequently observed at some loci than others (AABB Relationship Testing 2008 Annual Report). Cases with suspected mutations can be reported out as inclusions, but the nonmatching locus should be taken into account in the statistical calculation. See the AABB Guidance for Standards for Relationship Testing, 9th edition, Appendices 11 and 12, for mutation rates for the commonly used STR loci as well as an extensive discussion of handling of apparent mutations in paternity testing cases.

If two nonmatches are observed, both off by a single repeat as is characteristic of mutations, it may be impossible to determine whether the tested man is the biological father. Two mutational events would be rare but possible; on the other hand, such a large number of matches in a man who is unrelated would also be rare but possible. A third alternative is that the tested man is closely related to the actual biological father; this possibility should be investigated should this event occur.

26.5 Reporting

Paternity testing reports typically contain, at a minimum, the following information:

- Names and collection information for all tested parties
- Type of testing performed and loci tested
- Alleles detected for each tested party for all loci
- Statement of excluded or not excluded, including language explaining the conclusion
- For inclusions, individual locus PIs as well as the CPI and probability of paternity
- The signature of the reviewing director

Many states have specific requirements for accreditation of testing laboratories as well as chain of custody and minimum probability of paternity requirements. Therefore, before any testing for legal purposes (including child support, immigration, and probate) is conducted and reported, care should be taken to determine legal requirement specific to the jurisdiction in which the test results will be used.

26.6 Accreditation Requirements

In the United States, there is no federal legal accreditation requirement for paternity testing. The AABB (formerly the American Association of Blood Banks) accredits laboratories for relationship testing. This accreditation is voluntary; however, as noted above, many individual states require this accreditation for paternity tests intended for legal purposes. Information about their accreditation program can be found at <http://www.aabb.org>. Additionally, the state of New York requires that any laboratory performing paternity testing on any resident of the state be certified by the New York State Department of Health Clinical Laboratory Evaluation Program, regardless of whether the test will be used for any legal or medical purpose. More information regarding their certification program can be found at <http://www.wadsworth.org/labcert/clep>.

26.7 Summary

Paternity analysis utilizing STR profiles is relatively straightforward. While these PI, CPI, and probability of paternity calculations are not difficult, they are tedious to perform manually. Software programs can perform these calculations in an automated fashion. One example of such a program is DNA-View (<http://dna-view.com/dnaview.htm>). Some situations, such as suspected multiple mutations and inbreeding, can be more difficult to interpret and require far more complex statistical interpretation of the DNA results.

Other types of DNA analysis can be employed for certain types of relationship determinations where autosomal STR analysis cannot. Y STR analysis and mitochondrial DNA testing can be used to determine paternal and maternal lineage, respectively, over multiple generations. Mitochondrial DNA testing can also be used for maternal lineage determination on highly degraded samples. However, because neither Y STR nor mitochondrial DNA analysis provides enough information for individualization, standard autosomal STR analysis remains the primary method of choice for parentage analysis.

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Part VI
Clinical Molecular Training

Chapter 27

Diagnostic Molecular Training

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27.1 Introduction

Recent advances in molecular science and technology have brought diagnoses and treatment of diseases one step closer to the ultimate goal of personalized healthcare. As the field of clinical molecular genetics rapidly evolves, so does the need for more comprehensive clinical training programs. Currently, there are undergraduate and graduate accredited training programs designed to offer a robust curriculum of didactic learning and hands-on application experience. In this chapter, we will provide general descriptions on both types of programs.

27.1.1 Accredited Training Programs in Diagnostic Molecular Science

As the field of diagnostic genetics rapidly expands due to advancements in genomic testing and as the demand for such information increases to aid in diagnosis, prognosis, and treatment options, so does the need for proper training. Accreditation agencies that recognize the need and commitment to improve their level of standards of clinical care and testing in medical genetics have set high standards to ensure that quality and competency are required for the caregiver, as well as the institution providing the care. This chapter will address two levels of diagnostic genetics education: one for accredited undergraduate training programs and one for accredited graduate training programs [1, 2].

27.1.1.1 National Accrediting Agency for Clinical Laboratory Sciences

The National Accrediting Agency for Clinical Laboratory Sciences (NAACLS) is a nonprofit organization that independently accredits the education programs of diagnostic molecular scientists (DMS).

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This organization is also recognized by the Council of Higher Education Accreditation (CHEA). Having this recognition affirms that standards and processes of accrediting organizations are held in the highest regard, with consistency in quality improvement and accountability expectations that CHEA has established.

There are two main committees that govern the accreditation for DMS programs: The Review Committee for Accredited Programs (RCAP) and the Board of Directors. The program evaluation is based on a set of standards, which are the minimum criteria used when determining programmatic accreditation [1]. There are four primary aspects to the program accreditation process: (1) the self-study process; (2) the site visit process; (3) the review committee evaluation process; and (4) the Board of Directors evaluation process. The site visitors are made up of volunteers representing their field of practice, which may include laboratory practitioners or educators. The review committee is comprised of educators and practitioners representing the field of laboratory sciences, which include medical laboratory scientists, medical laboratory technicians, histotechnologists, histotechnicians, diagnostic molecular scientists, cytogenetic technologists, and pathologists' assistants.

27.1.2 Benefits of Accreditation and Eligibility

NAACLS uses an external peer-reviewed accreditation process. Participation in the accreditation process is strictly voluntary. Therefore, there is no legal requirement that specialized programs and institutions must participate. However, there are benefits to accreditation, including:

1. Recognition as a nationally accredited program that has met the established standards of educational quality
2. Continuous improvement for the educational program, as faculty and staff are required to provide ongoing self-evaluation, research, and planning
3. A better understanding of the goals of a professional education
4. Reasonable assurance that entry-level practitioners meet minimum educational standards
5. Assistance in achieving educational objectives

There are three basic eligibility criteria that must be met for a program to be accredited. The first requirement is that the sponsoring institution and its affiliates, either clinical and/or academic, must be accredited by a recognized regional and/or national agency. This may include, but is not limited to, the College of American Pathologists (CAPs), The Joint Commission (TJC), or the American Association of Blood Banks (AABBs). The second requirement is that the academic institution seeking sponsorship of a clinical laboratory science-related program must be empowered by a state authority to grant the appropriate degree. The third requirement is that the institution must have the legal authorization under applicable state law to provide postsecondary education.

27.1.3 The Accreditation Process

A program seeking initial accreditation must first submit a letter of intent, a completed initial application, an application fee, and an approved preliminary report (see Table 27.1). Additional information can be found on the main NAACLS website (www.naacls.org). Once these items are submitted and approved, five additional steps must be completed before full accreditation can be awarded.

Table 27.1 Accreditation process from the NAACLS handbook [1]

Step	Action	Responsible party	Time frame for the program
1. Initial application request	Submit letter to NAACLS requesting an application	CEO/President or other high-ranking administrator of sponsoring institution	Starting point
2. Submit application and initial application fee	Sponsoring institution submits: Application for initial accreditation form Initial application fee	Proposed program director	As soon as the program has obtained all the signatures required for the application
3. Submit preliminary report and program official approval form	Sponsoring institution submits: Preliminary report with required documentation Program official approval form, along with the required documentation requesting approval as program director	Proposed program director	As soon as the program has all the documentation prepared
4. Preliminary report approved ^a	Program encouraged to proceed with the self-study process. Program begins its first class	NAACLS	Review received within 2 months of the submission of the application for initial accreditation, preliminary report, program official approval form, and program director's CV

^aA program will not be considered for accreditation unless the preliminary report is approved, stating that NAACLS is reasonably assured that the program will meet the standards. If the program enrolls students too early in the initial application process and NAACLS does not approve the program's preliminary report, the program may risk having students graduate from their program before the program is accredited

27.1.3.1 The Self-Study Process

A self-study is a set of documents used by the program director (PD) to evaluate his or her own program. Completion of this process will require help from the PD's faculty, staff, and administration, as questions in the form of "standards" must be addressed and supported with documentation. Of the 22 standards, only a few do not need to be answered in the initial self-study. These standards can be found on the NAACLS website (www.naacls.org) and can be prepared using the electronic format (PDF) or the hard copy version. These standards are designed to help the program officials evaluate their program and will demonstrate whether the program complies with the standards. Once the self-study is prepared, it gives a snapshot of the program's present condition; it is hoped that through this process, the PD will have a better understanding of the status of their program and also will be able to determine whether their program is aligned with their institution's missions and goals.

27.1.3.2 The Paper Review Process

Once the self-study has been submitted, it is assigned to a paper reviewer. The paper reviewer is a NAACLS volunteer who is from the same profession as the program that is seeking accreditation. The paper reviewer could be an educator and/or a practitioner. The main purpose of the paper reviewer is to review the self-study and not the program. The assumption is that good practice processes are already being implemented and documented. This reviewer serves as the first part of the leg of the external review process for adequate compliance. When the paper reviewer is finished with this process, he or she generates a report and sends it to the PD for his or her response. At this time, the PD must address any areas that are found to be deficient. The purpose of the PD's response is to clarify any concerns identified by the paper reviewer and/or to develop new policies to address these concerns.

27.1.3.3 The Site Visit Process

The site visitors are NAACLS volunteers who are educators and/or practitioners in a related field. The site visit process is a fact-finding process, whereby site visitors visit the program's institution and verify the information submitted for the self-study. In addition, there are documents that require further verification because the self-study only requires a small sample size or a single unit of instruction as a sample submission. During this visit, program officials, students, faculty, graduates, and advisory committee members are interviewed through committee meetings and evaluations to ensure that there is sufficient input from each of the stakeholders and that there is a formal process for evaluation (which may reveal) → concerns (which may require further) → action (and re-evaluation by looking at the) → outcome. At the end of this process, a site visit report is developed on the basis of the information that the program has provided for alignment with NAACLS standards. Once the PD receives this report, he or she is asked to address any concerns with either additional explanations or new policies.

27.1.3.4 The Review by the Program Review Committee

Once the site visit has been conducted and the site visit report has been generated and given to the PD for his or her response, the report, the PD's response to the report, and paper reviewer's report are all sent to the Program Review Committee (PRC). The PRC makes a recommendation of compliance, marginal compliance, or noncompliance to the standards on the basis of the documentation submitted and then recommends accreditation actions to the NAACLS Board of Directors.

27.1.3.5 The Review by the NAACLS Board of Directors

The Board of Directors performs all accreditation actions on the basis of the recommendations from the PRC and also ensures that the decisions made are consistent throughout the process. The accreditation actions may be one of three outcomes: awarding, withholding, or withdrawing.

27.1.4 Competencies for Diagnostic Molecular Scientists

Achieving competency as a diagnostic molecular scientist requires proper theoretical knowledge as well as applied experiences. A training program should teach the following skill set and theoretical knowledge, regardless of whether the program is seeking accreditation.

27.1.4.1 Specimen Processing

1. Specimen collection, preservation, storage, and transport.
 - (a) Select containers, anticoagulants, transport media, and preservatives.
 - (b) Identify collection, transport, processing, and storage factors affecting specimen quantity, quality, and preservation.
 - (c) Transport/ship specimens using packaging that meets OSHA safety guidelines.
 - (d) Store specimens, considering time and temperature.
2. Select type of specimen for the test requested including: blood, bone marrow, other bodily fluids, tissues, cells in culture, and cell swabs.
3. Evaluate acceptability of specimens by checking: labeling and patient identification; type of specimen and method of collection; age of specimen; sufficiency of the quantity, transport, and storage method; quantity of the specimen; and appropriateness of test requested.
4. Discuss corrective actions such as salvage of any unsatisfactory specimens or notification regarding the inappropriate specimens.
5. Record pertinent data such as: patient demographics, specimens, test type, accession number, and any special requests.
6. Maintain records and laboratory database.

27.1.4.2 Basic Molecular Biology Principles

1. Describe DNA structure with respect to: base sequence, pairing, replication, and the packaging into chromosomes.
2. Describe the physical chemistry of nucleic acids and relate the base pairing to the nucleic acid hybridization.
3. Describe the DNA replication, DNA polymerase, and repair.
4. Differentiate between the prokaryotic and eukaryotic gene structures and mechanisms of transcription, and correlate these items with the cell cycle.
5. Describe the human genome organization, chromatin, chromosome structure, genetic variation, and polymorphism.
6. Describe the molecular basis of and how to test for inherited and acquired disease etiologies based on: deletion, duplication, insertions, missense and nonsense mutations, null and frame-shift mutations, mutations that affect RNA transcription and splicing, trinucleotide repeat mutations, gene rearrangements, and methylation/acetylation that leads to gene alterations.

7. Describe the basis of and how to test for different patterns of inheritance including: autosomal-dominant and autosomal-recessive disorders, sex-linked disorders, multifactorial, mitochondrial, and nonclassical (mosaicism, imprinting, uniparental disomy, and trinucleotide-repeat disorders).
8. Explain mechanisms of phenotype expressions with respect to: penetrance, variable expressivity, anticipation, and allelic and locus heterogeneity.
9. Explain, calculate, and evaluate quantitative genetics.
10. Explain the organization of the DNA/RNA genome of infectious agents.
 - (a) Explain and interpret the molecular and evolutionary relationships between organisms on the basis of genomic sequences and phylogenetic analysis.
 - (b) Explain and characterize species- and genus-specific sequences.
 - (c) Compare and contrast eukaryotic and prokaryotic molecular mechanisms of gene expression.
 - (d) Characterize plasmid DNA structure, transfer, and function.
 - (e) Explain prokaryotic acquisition of foreign DNA and relate it to the genetic basis of pathogenesis and drug resistance.
 - (f) Explain RNA polymerase function, transcription, splicing, translation, RNA processing, posttranslational modification, regulation of gene expression, and variation of gene expression among tissues.
 - (g) Explain genomic organization and gene structure including: L1 repeats, mini-satellites, regulatory regions, exons, introns, stop codons, and polyadenylation signal.
11. Describe recombinant DNA technology—vector properties, vector capacities, and cloning schemes.
12. Explain the action of the gene at a cellular level with respect to: dominant, dominant-negative, and recessive.
13. Explain gene expression at the cellular level: dominant, dominant-negative, and recessive.
14. Discuss the basic principles of genetic counseling, including pedigree analysis.
15. Describe the risk factors for mutations regarding: advanced maternal age and nondisjunction, advanced paternal age and new autosomal dominant mutations, mutagens, and carcinogens.
16. Correlate molecular genetics results with cytogenetic results for prenatal diagnosis, family studies, and cancer diagnostics or cancer-risk assessments.
17. Describe the clonal origin of neoplasms and the clonal evolution.
18. Describe the multistep pathogenesis of neoplasia involving inherited predisposition, activation of oncogenes, and inactivation of tumor-suppressor genes, alteration of gene-regulating apoptosis, and mutation of DNA repair genes.
19. Relate molecular testing to the diagnosis of inherited disease, leukemia and lymphoma, monitoring solid-tumor analysis, infectious disease testing, and human leukocyte antigen (HLA) allele identification for transplantation, identity, and paternity determination.
20. Describe the major histocompatibility complex on chromosome 6, and the nature and consequence of the extensive polymorphism of the Class I and Class II genes.
21. Describe nucleic acid-based methods for identifying HLA alleles and clinical applications.
22. Relate HLA alleles to antigens expressed, serologic markers, and clinical applications to organ transplantation, null alleles, and graft rejections.

27.1.4.3 Molecular Techniques

1. Describe and perform preparation of nucleic acid.
 - (a) Choose appropriate nucleic acid isolation method, giving consideration to specimen type and test requested.

- (b) Describe various methods for isolation of nucleic acid.
 - (c) Perform nucleic acid isolation.
 - (d) Resuspend and dilute the isolated nucleic acid sample.
 - (e) Evaluate quality, purity, and quantity of nucleic acid.
 - (f) Store nucleic acids with consideration for nucleic acid type and test requested.
 - (g) Evaluate and identify the need for corrective actions and necessary documentation.
2. Discuss and complete the nucleic acid manipulation.
- (a) Perform the restriction enzyme digestion.
 - (b) Assess and document the digestion.
 - (c) Select and document the corrective action, if required.
 - (d) Describe the probe design, specificity, and sensitivity.
 - (e) Describe and compare the methods for direct nucleic acid hybridization/detection.
 - (f) Prepare the nucleic acid probes.
 - (g) Perform the cloning procedures.
 - (h) Label the nucleic acids.
 - (i) Determine the appropriate label for the sample type and test requested.
 - (j) Determine the labeling efficiency.
 - (k) Purify the labeled nucleic acid probes.
 - (l) Prepare the cDNA.
3. Describe and perform the in vitro nucleic acid amplification.
- (a) Evaluate the target nucleic acid sequence.
 - (b) Select the appropriate primers.
 - (c) Choose the appropriate amplification technique on the basis of the sample type and test requested.
 - (d) Optimize the reaction components and conditions for sensitivity and specificity.
 - (e) Prepare the master mixes and primer dilutions at the correct concentrations.
 - (f) Perform nucleic acid amplification.
 - (g) Prevent amplicon contamination.
 - (h) Monitor and document all phases of amplification.
 - (i) Troubleshoot the reaction conditions/components for failed or nonspecific reactions.
 - (j) Employ and interpret positive and negative controls to detect contamination.
 - (k) Describe the amplification techniques.
4. Describe and perform electrophoresis.
- (a) Select the technique on the basis of specimen type and study requested.
 - (b) Determine the matrix type, buffers, concentrations, and running parameters.
 - (c) Choose the markers, loading dyes, and controls.
 - (d) Identify the factors that affect the rate of migration and the resolution of different specimen types.
 - (e) Perform the separation techniques.
 - (f) Detect the separated sample.
5. Interpret the results.
- (a) Label the marker band sizes.
 - (b) Determine the sample fragment sizes.
 - (c) Assess the resolution.
 - (d) Evaluate the controls.

6. Identify, troubleshoot, and document the problems associated with separation and detection.
7. Describe and perform the transfer of nucleic acids and proteins to the solid matrix.
 - (a) Select the appropriate technique for the transfer.
 - (b) Identify the factors affecting the rate and completeness of the transfer.
 - (c) Prepare the nucleic acid and protein samples for the transfer.
 - (d) Perform the transfer.
8. Describe and perform the hybridization methodology.
 - (a) Equilibrate and/or block the membrane/slide for prehybridization.
 - (b) Prepare the probe.
 - (c) Hybridize the probe to the sample.
 - (d) Perform the post-hybridization washes.
 - (e) Detect the hybridization results.
 - (f) Record conditions of the hybridization technique in an organized manner.
 - (g) Identify, troubleshoot, and document any problems associated with the hybridization reactions.
9. Describe and perform the nucleic acid sequencing.
 - (a) Describe the chemical and technological principles and the practical aspects of manual and automated sequencing.
 - (b) Perform the automated sequencing.

27.1.4.4 Summarize Results and Report Them to the Designated Authority

1. Evaluate the interpretability of the results.
 - (a) Quantity and quality of the nucleic acid extracted
 - (b) Restriction enzyme digest
 - (c) Adequacy of the amplification
 - (d) Gel electrophoresis
 - (e) Adequacy of the transfer of nucleic acid to the solid support
 - (f) Hybridization procedure
 - (g) Evaluation of autoradiographs for the background and intensity of the bands
2. Interpret the results.
 - (a) Assign the base-pair sizes.
 - (b) Determine that controls are within range.
 - (c) Determine the significance of the pattern.
 - (d) Quantitate the results, if necessary.
 - (e) Determine the necessity of repeating analysis.
3. Record the results.
 - (a) Determine that specimen/case numbers are consistent in all phases of analysis.
 - (b) Resolve all questionable or inconsistent data.
 - (c) Correlate the test results with other laboratory results and/or clinical information.
 - (d) Prepare the preliminary report.
 - (e) Properly store the data.

27.1.4.5 Laboratory Practices

1. Prepare the reagents.
2. Select, operate, and maintain the laboratory equipment and instruments.
3. Perform and document the calibration, performance, and maintenance checks.
4. Identify and document any malfunctions and corrective actions.
5. Design and implement a system to maintain an adequate stock of laboratory supplies and chemicals, including monitoring of their expiration dates.
6. Use correct procedures to store, handle, and dispose of biological, chemical, radioactive, sharp, and glass materials/waste.
7. Implement the established procedures for general laboratory safety.
8. Employ the appropriate cleaning procedures for laboratory glassware, instruments, and equipment.
9. Ensure laboratory quality control and continuous quality improvement in all areas and comply with any federal, state, and/or local regulations.
10. Demonstrate ethical behavior and professional standards.

27.2 Training for M.D. and Ph.D. Level Scientists Seeking a Career in Clinical Practice and Laboratory Testing

The American Board of Medical Genetics offers fellowship programs in Clinical Genetics, Clinical Cytogenetics, Clinical Molecular Genetics, and Clinical Biochemical Genetics. The ABMG certification is recognized by the American Board of Medical specialty (ABMS) as a primary medical specialty. The ABMG program was approved by ABMS in 1991. Individuals certified before 1993 were grandfathered into the programs. The ABMG certification is open to individuals with M.D. or Ph.D. in genetics or related biological sciences and whose credentials the ABMG credentialing committee has verified. The program involves training in the candidate's primary specialty for 24 months. Training in an additional specialty can be completed in 12 months immediately following the training in the primary specialty. Candidates can spend up to 6 months in performing research in a period of 3 years. The research period does not have to be continuous and can be spread throughout the training period.

A logbook has to be completed for each specialty and the guidelines for each training cycle are posted on the ABMG website: www.abmg.org. Candidates have to collect 150 cases in each specialty and the guidelines and rules for case collection can differ between specialties. The cases collected for logbook represent the various areas of training covered in each specialty and are designed to give the candidates exposure and experience in their areas of certification.

27.2.1 Description of Specialties

Clinical genetics involves practice of genetics in medicine. In this training the focus is on inherited diseases covering all major areas of medicine.

The clinical biochemical, cytogenetic, and molecular genetics specialty training is a laboratory based program and requires candidates to train in performing laboratory tests using a range of methodologies.

Clinical biochemical genetics involves diagnosis of conditions such as inborn errors of metabolism, and other conditions, which include disorders in the metabolism of amino acids, organic acids, carbohydrates, lipids, and other metabolic compounds. Candidates are trained in all aspects of running routine samples.

Clinical cytogenetics is designed to train candidates in diagnosing chromosomal abnormalities. Some major technological advancement has taken place in this area and the program is designed to incorporate these changes and give candidates exposure to the newer techniques.

Clinical molecular genetics is focused on diagnosing conditions, which involve alterations in DNA sequence using a variety of methods. This area of genetics has also seen tremendous growth. The program is designed to give candidates exposure to the newer areas, and therefore the logbook requirements are evaluated for each cycle.

At the end of the training period and after successful completion of the logbook and the ABMG exam candidates can function as technical supervisors, laboratory directors, or as clinical consultants depending on their specialty.

27.2.2 Other Subspecialties

Two additional areas of specialty include medical biochemical genetics and molecular genetic pathology. The area of medical biochemical genetics primarily focuses on diagnosis and treatment of patients with metabolic disorders, whereas molecular genetic pathology includes diagnosis and treatment of heritable disorders, infectious diseases, and cancer.

27.3 Summary

The importance of proper clinical training cannot be emphasized enough especially with the rapid advancement of clinical molecular technology to address the need for personalized care. Although we have listed a curriculum based on existing published criteria and what we thought was relevant at this current time, other techniques and platforms may be in use by the time this is out for publication.

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Chapter 28

Molecular Diagnostics Point of Care Testing Training

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Keywords Point-of-care testing • Molecular diagnostics • POCT MDx • Turnaround time • Clinical Laboratory Improvement Amendments • Testing personnel training

28.1 Introduction

Point-of-care testing (POCT) is defined by the College of American Pathologists as any laboratory testing that takes place at or near the site of patient care [1]. POCT was practiced for hundreds of years before the advent of modern medical laboratory science. Indeed, the very first laboratory tests were performed at the point of care and were focused on urine and blood conditions [2, 3]. The growing complexity of medical technology has shifted medical laboratory science to a central laboratory setting staffed with highly trained personnel, leading to increased quality control of medical laboratory science procedures. Until recently, POCT was often overlooked by professionals in laboratory medicine, and as a result, POCT research and development were often limited [3].

In the 1980s, several companies introduced easy-to-use handheld glucose meters to the market. These devices replaced urine dipstick tests and significantly improved patients' ability to gauge their own glucose levels, ultimately allowing them to achieve superior management of their diabetes. In the 1990s, the successive generations of these devices (originally intended for home use) were introduced into hospitals to aid clinical staff in managing diabetic patients. This allowed the clinical staff to promptly adjust patients' insulin dosages, because the bedside capillary blood glucose tests provided convenient and rapid test results that central laboratories could not match [3]. Such tight and immediate control of diabetic patients' blood glucose led to a widely acknowledged reduction in the secondary complications of type I and type II diabetes [3–5]. In addition, the wide implementation of bedside

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blood glucose testing in the 1990s reminded the medical community that the current medical system had a delayed turnaround time (TAT) for laboratory test results, which was caused by the physical distance between the patients and central medical laboratories or reference laboratories.

The medical community has been keenly aware of certain health conditions for which the prolonged TAT of clinical test results has led to adverse outcomes or inferior recovery for patients. A surge in the demand for minimal TAT, positive reports on the operational and economic outcomes of POCT [6], and improvements in POCT systems have all led to enthusiasm for and substantial financial investment in modern POCT. Although the current POCT sector continues to be dominated by specific and limited applications (e.g., assessment of diabetes, pregnancy, and cardiac conditions) [2], scientists and investors alike are now actively engaged in attempts to expand and diversify the field of POCT. This chapter addresses the potential benefits and obstacles of point-of-care molecular diagnostics, with particular emphasis on the training of testing personnel.

28.1.1 POCT Molecular Diagnostics

POCT and molecular diagnostics (MDx) have been the only two fields within the global in vitro diagnostics sector that have shown significant growth over the last few years [2]. In the last decade, investments have gained momentum toward achieving an efficient and effective combination of the two fields (POCT MDx) to create powerful diagnostic tools with minimal TAT. Currently, POCT MDx techniques are being developed in several targeted areas, including genetic disorders, oncology, and microbiology [3, 7, 8]. The two most promising of the targeted areas in POCT MDx at this early stage are pharmacogenomics (the study of the genetic basis of the differing response of individuals to drugs [9]) and molecular microbiology concerning infectious diseases [3, 5]. In both fields, incorporating POCT will enhance patient care. Through POCT applications, clinicians in both fields will be able to quickly identify pathogens or patients' genotypes and subsequently initiate appropriate treatment almost immediately [7, 9].

As in most POCT fields, simplification of testing methods and equipment, accurate and precise testing methods, and short TAT are considered essential for POCT MDx devices. This trend has been prominent within the MDx sector, making the transition of MDx technology into POCT more plausible. Indeed, in addition to the goal of providing more information or results for each sample, delivering quicker results has been the main goal of MDx, as is evidenced by the increase in automated MDx platforms on the market today. In fact, many companies have already started the transition to making more automated platforms. For in-depth evaluations of specific companies and technologies involved in POCT pharmacogenomics and molecular microbiology of infectious diseases, we recommend reviews by Dobson et al., Holland and Kiechl, and Park et al. [7, 9, 10].

In a 2008 CAP TODAY interview, Daniel H. Farkas, the executive director of the Center for Molecular Medicine (Grand Rapids, Michigan), described the then-under-development POCT MDx kits that he was aware of to be "not ready for prime time" except for one—GeneXpert (Cepheid, Sunnyvale, California) [11]. In a recent clinical study of point-of-care detection of enterovirus (EV) RNA using the GeneXpert system, Ninove et al. found that the EV-specific GeneXpert kit, a real-time reverse transcriptase-polymerase chain reaction–based technology, demonstrated fantastically high sensitivity (100%) and specificity (99.3%) [12]. These kinds of results bode well for the future of the POCT MDx field.

Although a limited number of POCT MDx kits are already available, technological advances within the field continue to surface. One of the more significant developments comes from Lezhava et al., who described the development of an isothermal, single-tube, single-nucleotide polymorphism detection technique that works directly from blood samples [13]. Their technique provides potential

solutions to two of the major impediments to POCT MDx development: the miniaturization of thermal cyclers and the need for DNA purification. Assuming that technological advances continue at this pace, a substantial number of additional POCT MDx kits should soon become available.

28.2 Regulations for POCT MDx

28.2.1 *Applying the Clinical Laboratory Improvement Amendments to POCT MDx*

In 1988, the United States Congress passed the Clinical Laboratory Improvement Amendments (CLIA), a regulatory law intended to oversee and improve clinical testing performed on humans within the United States. Since its active implementation on September 1, 1992, CLIA has imposed uniform regulatory standards on all clinical laboratory testing, regardless of the testing site, including POCT [3, 7, 14].

Before any POCT site can perform tests, the site must acquire a CLIA certificate. If the POCT site is located within a hospital complex, then the holder of the certificate can be the central laboratory, which holds a single CLIA certificate that addresses all performed tests, or the POCT site can request and maintain its own certificate. Both options are viable, and the choice between the two options should be made at the individual institution's discretion. The most critical issue to consider when applying for a CLIA certificate is choosing the appropriate certificate to cover all testing performed at the POCT site; CLIA certificates are largely determined by the complexity of the tests offered at the certified site.

When a POCT kit is initially produced, the US Food and Drug Administration (FDA) classifies the testing method into one of three categories of complexity: waived, moderate complexity, and high complexity. Accordingly, prospective POCT sites can apply for one of three types of certificates: (1) a certificate of waiver, which allows the site to perform only waived test methods; (2) a certificate for provider-performed microscopy procedures, which allows only physicians, midlevel practitioners, or dentists to conduct a select subset of moderate-complexity tests along with all waived tests; or (3) a certificate of registration, which enables the laboratory to conduct moderate- and/or high-complexity tests, provided that the laboratory also applies for a certificate of compliance or a certificate of accreditation and meets all the requirements. Naturally, the waived category has the least stringent requirements, and the high-complexity category has the most stringent requirements. Because of the strict personnel and quality requirements that come with high-complexity testing, POCT usually adapts only waived or moderate-complexity testing methods [14]. For any new test to qualify as a waived testing method, CLIA requires that the testing method under consideration be "simple." 42 U.S.C. 263a(d)(2), (3). For a test to be considered simple, the FDA recommends that it possess the following properties listed on Section III (Demonstrating "Simple") of the FDA's Recommendations: "Clinical Laboratory Improvement Amendments of 1988 (CLIA) Waiver Applications for Manufacturers of In Vitro Diagnostics Devices" [15]:

1. Is a fully automated instrument or a unitized or self-contained test.
2. Uses direct unprocessed specimens, such as capillary blood (finger stick), venous whole blood, nasal swabs, throat swabs, or urine.
3. Needs only basic, nontechnique-dependent specimen manipulation, including any for decontamination.
4. Needs only basic, nontechnique-dependent reagent manipulation, such as "mix reagent A and reagent B."
5. Needs no operator intervention during the analysis steps.

6. Needs no technical or specialized training with respect to troubleshooting or interpretation of multiple or complex error codes.
7. Needs no electronic or mechanical maintenance beyond simple tasks, e.g., changing a battery or power cord.
8. Produces results that require no operator calibration, interpretation, or calculation.
9. Produces results that are easy to determine, such as “positive” or “negative,” a direct readout of numerical values, the clear presence or absence of a line, or obvious color gradations.
10. Provides instructions in the package insert for obtaining and shipping specimens for confirmation testing in cases where such testing is clinically advisable.
11. Has test performance comparable to a traceable reference method, as demonstrated by studies in which intended operators perform the test. If a reference method is not available for a test you are proposing for waiver, please contact OIVD to discuss your proposed plan prior to submitting your application.
12. Contains a quick reference instruction sheet that is written at no higher than a 7th grade reading level.

The largest obstacle for POCT MDx is thus the contrast between the complexity levels of POCT and MDx. The aforementioned disinclination of POCT sites to adapt high-complexity testing may limit their adapting of POCT MDx methods; nucleic acid testing methods are currently classified as highly complex [7, 14]. However, the classification criteria and the accompanying technological advances change over time, as evidenced by the growing list of waived POCT procedures (which included only 8 analytes in 1992 but now features more than 100 analytes measured by multiple kits). With the majority of the research efforts in MDx and POCT MDx spent in simplifying test equipment and methods, it is not difficult to foresee POCT MDx kits being classified as moderate-complexity tests.

28.2.2 POCT MDx Personnel Requirements (CLIA Subpart M)

POCT MDx sites must meet the regulatory requirements for moderate-complexity testing under CLIA, assuming the MDx procedures are classified as moderate complexity. We will focus on one aspect of the CLIA requirements: personnel qualifications and assigned responsibilities. For POCT sites holding their own CLIA certificates and performing moderate-complexity testing, individuals with the proper qualifications must be identified to assume the roles of director, technical consultant, clinical consultant, and testing personnel. Some of those personnel requirements may be satisfied by one individual who can fulfill all the qualifications. In contrast, for POCT sites operating under the institution’s certificate from their central laboratory, typically, only the testing-personnel requirements need to be addressed because the required oversight can be attained from the central laboratory. Consequently, POCT MDx may be best performed at POCT sites that are extensions of centralized hospital laboratories. Regardless of the source of oversight, the following positions must be filled before a POCT MDx site can operate.

28.2.2.1 Director (CLIA Section 493.1403-07)

Above all else, the director must be able to manage and direct the personnel and the performance of the tests. The director can be a physician (MD or DO) with anatomic or clinical pathology certification or 2 years of experience directing nonwaived laboratory testing. Alternatively, individuals with degrees in a chemical, physical, biological, or clinical laboratory science can become directors with varying amounts of nonwaived laboratory testing experience, depending on the level of their degree. Prospective directors with a doctorate degree need 1 year of experience directing nonwaived laboratory testing, those with a master’s degree need 2 years of experience, and those with a bachelor’s degree need

4 years of experience (2 years of laboratory training or experience and 2 years of experience directing nonwaived laboratory testing).

The director is responsible for all aspects of POCT test performance, site operations, and personnel management. The director must ensure that all personnel receive appropriate education and training so that they can perform the tests properly, and the director may delegate responsibilities and duties to testing personnel.

28.2.2.2 Technical Consultant (Section 493.1409-13)

The POCT site also must have one or more individuals qualified by education and training or experience to provide technical consultation for each of the specialties and subspecialties of service in which the POCT performs moderate-complexity testing. The technical consultant can be a physician (MD or OD) with board certification or at least 1 year of laboratory training or experience in nonwaived testing procedures in the designated specialty. Alternatively, as in the director qualifications, individuals with a doctoral or master's degree in chemical, physical, biological, clinical, or medical technology science and 1 year of training or experience in the designated specialty of nonwaived testing, or those with a bachelor's degree and 2 years of training or experience in the designated specialty of nonwaived testing, can also become technical consultants.

The technical consultant is responsible for the technical and scientific oversight of the POCT site. The person in this position need not be onsite at all times but must be available to the testing site as needed to provide requested consultations. The technical consultant is also responsible for establishing and maintaining quality standards by enrolling the site in a proficiency testing program, evaluating the competency of testing personnel, and identifying training needs for each member of the POCT site.

28.2.2.3 Clinical Consultant (Section 493.1415-19)

The clinical consultant must be a physician (MD, DO, or DPM) or must possess a doctorate degree in chemical, physical, biological, clinical, or medical technology science. The clinical consultant determines the appropriateness of the testing ordered and interprets the test results. Unlike the technical consultant, who advises POCT staff, the clinical consultant mainly interacts with clients or patients directly to discuss test quality, diagnosis, possible treatments, and management of patient care.

28.2.2.4 Testing Personnel (Section 493.1421-25)

Each individual performing a moderate-complexity POCT test must have a high school diploma or its equivalent, along with director-approved training for appropriate testing procedures. Testing personnel are responsible for processing specimens, performing tests, and reporting test results. Given the relatively few minimum requirements, training for testing personnel is critical in the complex field of POCT MDx. The last section of this chapter will focus solely on potential materials and methods to train vital testing personnel.

28.3 Training for Testing Personnel

Despite the strong trend toward simplification of methods in POCT MDx, the field will likely continue to demand specific knowledge and experience from those conducting the tests. Indeed, minimally trained personnel in this field may struggle to successfully conduct testing because of the

sensitivity of the nucleic acid material and the ever-present threat of contamination outside of sterile laboratory conditions. Although certified molecular biologists are the most appropriate and qualified testing personnel, such a stringent personnel requirement may substantially reduce the viability of POCT MDx care, owing to the insufficient number of qualified individuals in less-developed regions and the increased salaries required to maintain certified medical scientists. Therefore, the remainder of this chapter will focus on possible methods to develop and maintain sufficient competency levels of testing personnel.

28.3.1 Content of POCT MDx Training

Before any training begins, the POCT site or central laboratory director and the technical consultant should make sure that they are strictly following CLIA regarding the training of testing personnel in the specified skill sets. According to Section 493.1423, testing personnel must complete documented training programs that ensure that each trainee has the following:

1. Skills required for proper specimen collection, including patient preparation and labeling, handling, and processing or preparation of the patient's sample
2. Skills required to implement all standard POCT MDx procedures
3. Skills required to perform the assigned POCT MDx kit
4. Skills required to perform preventive maintenance, troubleshooting, and calibration procedures related to each test assigned
5. A working knowledge of reagent stability and storage
6. Skills required to implement the quality control policies and procedures of the POCT site
7. An awareness of the factors that influence test results
8. Skills required to verify the validity of patient test results through the evaluation of quality control sample values prior to reporting patient test results

To sufficiently cover all the requirements stated above, the training sessions should resemble the outline for achieving competency for diagnostic molecular scientists, listed in the previous chapter (27.2.4.1–27.2.4.5), with additional reference to the Technologist in Molecular Biology [MB(ASCP)] certification examination guideline by the American Society for Clinical Pathology. Attempting to teach testing personnel the entire outline may be unreasonable because of the time and financial constraints involved. However, the complexity of the field will require that extensive lessons be provided to the trainees on the basic molecular science principles (e.g., principles of polymerase chain reaction), regardless of the type of POCT MDx kit assigned. Providing trainees with fundamental concepts will improve their understanding of a kit's limitations and emphasize to them the necessity of strictly adhering to the proper sample handling guidelines. For these reasons, the director and technical consultant at each POCT site should create a lesson plan that includes some of the most critical items from the outline. The sample guideline below illustrates one potential derivative of the outline, selecting only those items critical for POCT MDx:

1. Specimen collection
 - (a) Explain procedures for patient preparation, specimen collection, labeling the specimen, and processing the specimen
 - (b) Explain how to collect and maintain the patient's test record

2. Basic molecular biology principles

- (a) Describe basic facts about DNA structure and chemistry of nucleic acids as it relates to the proper understanding of base pairing and replication.
- (b) Describe the process of DNA replication and repair.
- (c) Describe the human genome organization, chromatin, chromosome structure, genetic variation, and polymorphism.
- (d) Describe the molecular basis of and how to test for inherited and acquired disease etiologies based on the various types of mutations.
- (e) Describe the basis of and how to test for different patterns of inheritance.
- (f) Explain basic knowledge of applying molecular genetics results to diagnosing patients using the POCT MDx kits.
- (g) Relate molecular testing to the diagnosis of the disease that the assigned kit is designed to identify.

3. Molecular techniques

- (a) Describe the concept behind proper isolation of a nucleic acid, with particular emphasis on the potential errors that testing personnel could make to contaminate the patient sample or degrade the nucleic acid.
- (b) Describe the particular processes used by the assigned kit.

4. Summarizing and reporting the results

- (a) Describe the proper protocol for recording test results.
- (b) Explain how to interpret results as instructed by the assigned kit's manufacturer's protocol.
 - (i) Determine the significance of the results.
 - (ii) Identify erroneous/unacceptable results.
- (c) Describe and recommend actions in troubleshooting potential problems associated with the kit.
- (d) Determine the necessity of repeating the test.
- (e) Prepare a preliminary report for the assigned physician and site director.

5. Laboratory practices

- (a) Explain basic safety guidelines in addition to those provided by the manufacturer of the testing kit.
- (b) Describe the proper methods of handling and storing the assigned kits, including how to identify the expiration date of the kits.
- (c) Demonstrate ethical behavior and professional standards.

Additionally, the POCT MDx committee at each institute or independent site should create a comprehensive collection of lessons, each one tailored to a particular POCT MDx kit. This portion of the training content should be largely based on the manufacturer's procedures or on the manual from the POCT MDx kit [16]. Adapting the manufacturer's protocol should not only save time but will also likely enhance the performance of the testing personnel. Trainees should be given ample opportunity to thoroughly familiarize themselves with the testing material and its limitations and ask questions regarding specific steps mentioned in the protocol. Teaching the procedures any other way may inadvertently encourage the testing personnel, especially those with previous experience with similar kits, to slightly deviate from the manufacturer's protocol. In fact, failure to follow the manufacturer's protocol explicitly has been listed as one of the ten most cited deficiencies in current POCT sites [17]. Regardless of the depth of basic knowledge featured in the training content, the final and largest portion of the training content must be dedicated to practical or hands-on instruction [16].

28.3.2 *Organizing and Managing Training Sessions*

As previously mentioned, the director of a POCT site is required by CLIA to organize training sessions for testing personnel with the assistance of the technical consultant. POCT MDx training must be part of the testing personnel orientation course, and the training program must be assigned as a mandatory regular requirement. When organizing training sessions, each POCT MDx committee must address two key issues on the basis of their institutional policies and resources: selection of the lead trainers and certification of training.

28.3.2.1 Selection of the Lead Trainers for POCT MDx

The complexity of POCT MDx demands that the leading educators and trainers of POCT MDx testing personnel be certified technologists in molecular biology, MB(ASCP). Ideally, the lead trainers will have years of clinical laboratory experience, along with some practical knowledge of or experience with issues faced by healthcare professionals at the point of care. Individuals who are MB(ASCP)-certified technologists already have teaching and training responsibilities (as listed in the certification examination guidelines), and often senior clinical MDx technologists have already trained entry-level laboratory personnel and other healthcare professionals.

Hence, the main concern is the source of the lead trainers. Specifically, should these qualified trainers come from the manufacturers of the kits or should they come from the central laboratory at the POCT site's institute? Central laboratory technologists (in-house trainers) presumably present less financial burden on the institution, while also providing a continuous support system in the immediate vicinity of the POCT MDx site. Nevertheless, manufacturer-representative technologists are able to provide trainees with more definitive answers to their questions regarding the kit's procedures, while also presenting the most accurate demonstration of handling and operation of the kit. Even if the POCT site committee or its institutional committee determines that hiring manufacturer representatives to lead training sessions is financially feasible, it is essential that POCT MDx sites maintain some central laboratory technologists as in-house trainers to provide solutions for day-to-day issues [16].

28.3.2.2 Certification of Training

Training certification must be conducted at the end of the training program for POCT MDx personnel. The purpose of this assessment is to ensure that the minimum standards of competence are defined and that those failing to meet the standards are provided with additional training and support before entering patient care [16]. The specific content of such an assessment will depend on the POCT MDx kit assigned to the testing personnel. Regardless of the content of the written portion of the competence assessment, the assessment must include conducting, or at least observing, actual testing procedures on training samples and troubleshooting realistic issues with the kit to ensure that the trainees are fully prepared. At the conclusion of the initial training session, a certificate of training should be issued with the name of the trainee, the names of POCT MDx kits the trainee is allowed to perform, and the expiration date of the certificate. The expiration date must be within 1 year (the maximum length of time allowed by federal regulations) of the issue date and is meant to strictly enforce attendance of update sessions by testing personnel. In addition to satisfying legal requirements, update sessions or certification-renewal sessions provide trainers with the opportunity to share new technologies and techniques with testing personnel. Moreover, laboratory best practices dictate that continual retraining is the only way to ensure the continual competence of the trainees and their continual strict adherence to manufacturer protocols.

28.4 Summary

There are clear and compelling benefits for establishing POCT MDx in medical institutes. However, each institute opting to introduce POCT MDx kits into its practice must consider not only the disincentives described in this chapter but also individualized and specified institutional obstacles. Likewise, POCT MDx site members involved in the process of designing a training program for testing personnel should use this chapter only as a starting point toward tailoring unique lesson plans fitting institutional needs and, equally importantly, arranging lesson plans according to the set qualifications of testing personnel within the particular POCT MDx site.

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