Molecular Pathology Methods

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Abstract

Molecular pathology is based on the principles, techniques, and tools of molecular biology as they are applied to diagnostic medicine in the clinical laboratory. Molecular biology methods were used to elucidate the genetic and molecular basis of many diseases, and these discoveries ultimately led to the field of molecular pathology. As molecular research identifies the most fundamental causes and markers of disease, clinical testing of human and pathogen genetic material has become routine in laboratory medicine. Underlying mutations responsible for genetic diseases, including cancers, are being discovered and used in clinical molecular tests. In this chapter, fundamental and more advanced molecular biology techniques, as practiced in the molecular pathology laboratory, are reviewed.

Keywords

Molecular pathology • PCR • Polymerase chain reaction • Sequencing • Primers • Probes • Nucleic acid • Polymerase • Microarrays • DNA • RNA • Electrophoresis • Capillary electrophoresis • Hybridization • Denaturation • Southern blot • Northern blot • FISH • Amplification • Cytogenetics • Ligation • Melt curve

Introduction

Molecular pathology is based on the principles, techniques, and tools of molecular biology as they are applied to medical practice in the clinical laboratory. These tools were developed in the research setting and perfected throughout the second half of the twentieth century, long before the Human Genome Project (HGP) was conceived. Molecular biology methods were used to elucidate the genetic and molecular basis of many diseases, and these discoveries ultimately led to the field of molecular pathology. Eventually the insights these tools provided for laboratory medicine were so valuable to the armamentarium of the pathologist that they were incorporated into pathology practice. Today, clinical molecular testing continues to grow rapidly as in vitro diagnostic companies develop new kits for the marketplace and as the insights into disease that have been gained as a result of the HGP develop into clinical laboratory tests.

Molecular pathology is a natural extension of anatomic and clinical pathology. As molecular research identifies the

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most fundamental causes and markers of disease, clinical testing of human and pathogen genetic material has become routine in laboratory medicine. Underlying sequences and variations responsible for genetic diseases, cancers and infectious diseases are being discovered and used in clinical molecular tests. Some have become routine tests in molecular pathology and are described in detail elsewhere in this book.

In this chapter, fundamental and more advanced molecular biology techniques, as practiced in the molecular pathology laboratory, are reviewed. The entire field of molecular pathology is relatively new, having begun in the 1980s and matured through the 1990s. The new century has brought important advances in automated nucleic acid preparation [1–3], polymerase chain reaction (PCR), real-time PCR, deoxyribonucleic acid (DNA) sequencing, genomic microarrays, and, most recently, massively parallel sequencing of large portions of the genome. More and more, the underlying biochemistry occurring in laboratory instruments may be invisible to the user. The goal of this chapter is to describe the principles of these methods so that practitioners have adequate information for instrument troubleshooting and test interpretation.

Gene products, such as proteins and polypeptides, are molecules and could technically be classified within "molecular pathology." This chapter focuses on nucleic acid methods, including investigation of DNA and ribonucleic acid (RNA). The field will eventually move beyond genomics to proteomics. Proteomics is not addressed in this chapter since clinical proteomics is in extremely limited practice in molecular pathology today (see Chap. 61).

Basic Science Discoveries: The Foundation

Molecular pathology techniques are rooted in fundamental molecular biology discoveries of the 1940s–1980s [4]. The clinical laboratory application of molecular biology techniques would not be possible without the discovery by Griffith and Avery that nucleic acid is the genetic material. The foundation of work by Chargaff and Franklin was capitalized on by Watson and Crick, who elucidated the structure of DNA. Understanding DNA structure is seminal to understanding nucleic acid hybridization, which is central to almost all molecular methods used in the clinical molecular pathology laboratory. Additionally, work by Nirenberg (unraveling the genetic code), Wilcox, Smith, Nathans, and others (use of restriction endonucleases for DNA manipulation), Baltimore and Temin (discovery of RNA-dependent DNA polymerase or reverse transcriptase), Britten and Davis (hybridization kinetics), Kornberg and Okazaki (work on DNA polymerases and DNA replication, respectively), Southern (development of solid-phase DNA hybridization, i.e., the Southern blot), Sanger, Maxam, and Gilbert (development of DNA sequencing), Mullis (discovery of PCR for in vitro nucleic acid amplification), and their scientific collaborators and competitors led to a refined understanding of how DNA may be manipulated in vitro for research and ultimately clinical molecular testing purposes.

General Methods

Nucleic Acid Isolation

The first step of most molecular pathology tests is isolation of DNA or RNA from a patient specimen, by either manual or automated methods. Nucleic acid purification begins with lysis of the cells in the sample. Cell lysis liberates cellular macromolecules including proteins, lipids, and nucleic acids. Cell lysis can be accomplished using a detergent solution to break cell membranes and remove lipids. Proteins are enzymatically degraded with protease or selectively precipitated. Protein digestion is performed at about 56 °C which permanently denatures many proteins but does not affect nucleic acids. This process is followed by selective nucleic acid extraction that takes advantage of the physical and chemical differences between nucleic acids and other cellular molecules, forming the basis for their isolation. The nucleic acid is then purified from the soluble contaminants produced in the extraction method by precipitation in an ethanol–salt solution. Variations on this theme that combine extraction and purification are the selective adsorption of nucleic acids to silica columns under chaotropic salt conditions or magnetic bead chemistry. The isolated nucleic acid is resuspended in a dilute salt buffer, for example, 10 mM Tris/1 or 0.1 mM EDTA pH 7.6–8.0 (TE buffer).

The initial lysis step is modified according to the specimen. If the specimen is fresh or frozen solid tissue, the tissue is first homogenized in an appropriate buffer (often TE buffer). If the specimen is formalin-fixed, paraffin-embedded (FFPE) tissue, the paraffin is removed with an organic solvent such as xylenes, followed by rehydration through an alcohol series to a dilute salt buffer before protease digestion. FFPE tissue also is heated during the prolonged protease digestion step to reverse formalin cross-linking between proteins (primarily histones) and nucleic acids. Although this reduces the degree of nucleic acid shearing in subsequent vortexing or centrifugation steps, DNA longer than that packaged into a nucleosome (about 200 base pairs [bp]) is difficult to recover from FFPE tissue. Whole blood specimens may require Ficoll separation and a centrifugation step to separate the DNA-containing white blood cells (WBCs) from the erythrocytes prior to recovery of nucleic acids from WBCs because of the inhibition of PCR by hemoglobin. This blood fractionation step generally is not performed in automated nucleic acid extraction instruments.

Organic (Phenol) Extraction

Nucleic acids have a strong net negative charge because of the phosphate groups in the sugar–phosphate backbone, and thus are highly soluble in an aqueous environment. By contrast, proteins, lipids, and carbohydrates contain varying proportions of charged and uncharged domains producing hydrophobic and hydrophilic regions. This difference makes proteins entirely soluble in organic solutions or confines them to the interface between the organic and aqueous phases during an organic extraction. This characteristic forms the basis for phenol–chloroform extraction, in which phenol is added to an aqueous solution containing cellular constituents, mixed, and then centrifuged to separate the aqueous and organic phases. If the pH of the extraction is near neutral, both DNA and RNA stay in the aqueous phase, while proteins are in the phenol–chloroform phase or aqueous-phenol interface. If the pH is acidic, the phosphate groups of DNA are preferentially neutralized, driving DNA into the organic phase (or interface) and allowing RNA to be selectively extracted. This method produces high-quality nucleic acids but is relatively labor-intensive, employs hazardous chemicals, and produces liquid organic waste.

Ethanol–Salt Precipitation

Nucleic acids can be precipitated in an aqueous solution by the addition of concentrated ethanol and salt. Ethanol makes the solution hydrophobic, while salt increases the ionic strength of the solution, thereby reducing the repulsion of the negatively charged sugar–phosphate backbone of nucleic acids. Centrifugation allows the nucleic acid precipitate to be collected and resuspended in a dilute salt buffer (TE buffer).

Chaotropic Salt-Silica Column Extraction

Chaotropic salts, such as sodium iodide (NaI) or guanidinium isothiocyanate (GITC), disrupt the structure of water, promoting the solubility of nonpolar substances, such as proteins, in polar solvents, such as water. Saturated chaotropic salts also promote the adsorption of nucleic acids to glass or silica columns. Nucleic acids are purified by a series of washing steps including reducing agents, such as sodium azide, to further remove contaminants and inhibit remaining enzymes. The nucleic acids are eluted from the column with a dilute, nonchaotropic salt buffer. Since the method is simple, fast, offered in commercial kits by several manufacturers and adaptable to high-throughput robotic nucleic acid isolation, this method is widely used by clinical molecular laboratories.

Magnetic Bead Extraction

Another solid phase extraction method uses ligand-coated magnetic beads to capture nucleic acids. After cell lysis, DNA molecules are attracted to the ligands on the magnetic beads. The beads are immobilized by a magnet, allowing multiple washings of the bound nucleic acids to remove proteins and other contaminants. The nucleic acids then are eluted from the ligands on the magnetic particles with an elution buffer. Magnetic bead extraction chemistries are widely available commercially, and ideal for automation and use in high test volume settings.

RNA vs DNA Isolation

DNA is the repository of genetic information, which is then transcribed into RNA. RNA is the major constituent of ribosomes (ribosomal RNA or rRNA), forms transfer RNA (tRNA) and messenger RNA (mRNA) which are central in protein translation, and plays a regulatory role in gene expression as microRNA (miRNA) and long-noncoding RNA (lncRNA). DNA is a hardy molecule present at stable cellular levels (with the exceptions of gene amplification and deletion in tumor cells). In contrast, the level of RNA corresponding to a gene can fluctuate dramatically within a very short time in response to changes in a cell's microenvironment and functional needs. This fluctuation results from changes in both the rate of transcription and degradation of RNA species.

DNA is relatively easy to isolate and store because deoxyribonucleases (DNases) are easily denatured by heating or inhibited by sequestration of divalent cations needed for their function. RNA, by contrast, is rapidly degraded by a variety of ribonuclease (RNase) enzymes that are replete within the cell and on the skin surface. While this is necessary for cellular homeostasis, the ubiquity of RNases is problematic for the molecular analysis of RNA. RNases are very stable, active in virtually any aqueous environment, and can regain their activity after denaturation. As a result, RNA is subject to rapid degradation by RNases in most laboratory settings, making RNA a notoriously labile molecule. In addition, RNA is inherently chemically unstable under physiological conditions, primarily due to the spontaneous cleavage of the backbone phosphodiester linkages by intramolecular transesterification reactions involving the 2′-hydroxyl group of the ribose. This contrasts with DNA which lacks the reactive hydroxyl group in its deoxyribose sugar group, resulting in an inherently greater stability. The rate of degradation varies among RNA species, leading to further analytical complexity for the clinical molecular laboratory.

RNA analysis depends on successful RNA isolation and preservation. The overall techniques are similar to those described above for DNA isolation, but with the mandatory addition of steps to inhibit or degrade cellular RNases and prevent their reintroduction into the isolated RNA. RNA isolation must be performed promptly after specimen collection, particularly if RNA quantitation is desired. If RNA isolation is delayed, the sample should be stored at −80 °C, or at an

intermediary isolation point in a stable buffer with RNase inhibitors. Scrupulously clean laboratory technique is required, including careful cleansing of laboratory equipment with bleach, autoclaving of glassware, preparation of reagents with nuclease-free water, and wearing and frequently changing gloves. Addition of GITC or β-mercaptoethanol to the RNA isolation reagents inhibits or denatures RNases present in the sample. The isolated RNA is rehydrated in water or TE buffer that is nuclease free, and stored at −80 °C to further inhibit the activity of any residual RNases.

Despite these difficulties, RNA is valuable in the clinical molecular laboratory for several reasons. Many clinically significant viruses, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV), have RNA genomes. Quantitation of RNA provides an important measure of gene expression, which can be used in the diagnosis or monitoring of disease. In addition, mRNA does not contain introns, which is an advantage when analyzing neoplastic translocations with variable intronic breakpoints such as *BCR–ABL* [5]. An advancement is the availability of blood collection tubes containing a preservative solution that increases RNA stability at room temperature (PAXgene, Qiagen Valencia, CA), allowing a longer timeframe from collection to purification without RNA degradation.

Nucleic Acid Measurement for Quantity and Quality

Nucleic acid quantitation is optional for many protocols that utilize in vitro nucleic acid amplification. Some methods, however, require use of accurate quantities of nucleic acid necessitating assessment of the yield and concentration of purified nucleic acids, which is typically done using ultraviolet (UV) spectrophotometry. The absorbance of a nucleic acid solution is measured at several wavelengths. The maximal absorbance for nucleotides is at 260 nm of UV light (A_{260}), while for proteins the maximal absorbance is at 280 nm (A_{280}). Nucleic acids can therefore be quantified by the A_{260} measurement, while the A_{260}/A_{280} ratio provides an estimate of the purity of the sample. Pure DNA has an A_{260} of 1.0 at a concentration of 50 μg/ml and an A_{260}/A_{280} ratio of 1.8, while pure RNA has an A_{260} of 1.0 at a concentration of 40 μg/ml and an A_{260}/A_{280} ratio of 2.0. Lower A_{260}/A_{280} ratios indicate the presence of protein in the solution. Other contaminants can be detected by their absorbance at other wavelengths, such as phenol at A_{270} and guanidinium at A_{230}.

Ethidium bromide (EtBr) intercalates into DNA strands, causing DNA to fluoresce upon illumination with UV light. The fluorescence intensity of EtBr correlates with the number of base pairs of DNA in which the EtBr is intercalated, which is a function of both the size (length) and quantity of the DNA fragment. Therefore, by staining sample DNA with EtBr in an electrophoresis gel and comparing the brightness to mass standards in adjacent lanes, the quantity of DNA can be estimated. This provides a convenient system for estimation of post-PCR DNA quantity prior to sequencing. More importantly, the image of the EtBr-stained sample DNA can be used to assess DNA quality. High-quality, substantially intact genomic DNA forms a single band close to the well which serves as the origin of electrophoresis. In contrast, DNA degradation is apparent as a smear of EtBr-stained DNA extending downward from the well. EtBr is mutagenic and produces light background staining and, therefore, largely has been replaced in the clinical laboratory by other intercalating dyes such as SYBR Green.

Electrophoresis

In electrophoresis, an electric field is used to separate charged molecules by differential mobility in a sieving matrix that can be either liquid or solid (gel). The differential mobility is determined by the size of the molecule and its conformation, the net charge of the molecule (as modified by pH), temperature, and the pore size of the matrix. DNA, being negatively charged, migrates towards the anode (+) when an electric field is applied to an electrolyte solution. The size of DNA can be modified by restriction endonuclease digestion (see below), rendering DNA fragments small enough to be mobile in the matrix. Nucleic acid conformation can be modified with denaturing conditions prior to or during electrophoresis. Nucleic acids usually are electrophoresed at a slightly alkaline pH to ionize all phosphate groups in the backbone of the molecule, enhancing the negative charge which allows nucleic acids to be moved in the electrophoretic field.

The pore size of the matrix is determined by the composition and concentration of the polymer. For any given pore size, the mobility of a molecule through the matrix is inversely proportional to the log of its size. Therefore, for a given size difference between two molecules, the difference in the rate of migration will be substantially less if both molecules are large. The limiting mobility is defined as the rate of migration through the gel at which large molecules can no longer be separated for any given pore size. This may be related to the tendency of sections of long DNA fragments to "snake" through different pores in the gel, retarding the mobility of the fragment. The limiting mobility of gels can be overcome by using pulsed field gel electrophoresis (PFGE), in which the voltage gradient is periodically reoriented.

Polyacrylamide Gels

In clinical molecular laboratories, the matrices used most commonly are acrylamide and agarose. Polyacrylamide gels are formed by cross-linking acrylamide monomers with bisacrylamide in the same salt buffer used for electrophoresis and

pouring the solution in a thin space between two glass plates. A comb is inserted at one edge between the plates to form wells for sample insertion. After the gel has formed, the plates are mounted in a vertical electrophoresis unit such that the gel forms a bridge between two buffer chambers. Samples, controls, and sizing standards are mixed with a loading buffer containing a dye to track the progress of electrophoresis and glycerol to increase the density of the aqueous samples so the samples sink to the bottom of the wells, then loaded into the wells. Electrodes are attached to the buffer chambers and connected to a power supply providing constant voltage. After electrophoresis, the glass plates are separated and the gel is soaked in EtBr (or another intercalating agent) solution. DNA is visualized by EtBr staining under UV light. Polyacrylamide forms very small pores and is useful for high resolution of DNA fragments from 100 to 1,000 bp. However, polyacrylamide gels are thin and fragile, the glass plates are cumbersome to work with, and nonpolymerized acrylamide is a lung irritant and neurotoxin; therefore, alternatives to polyacrylamide gel electrophoresis are desirable in the clinical laboratory. Although more costly, precast acrylamide gels are commerically available to circumvent the biohazards of nonpolymerized acrylamide.

Agarose Gels

Agarose gels are formed by boiling an agarose gel powder until the agarose has completely dissolved in the same buffer used for electrophoresis, optionally adding EtBr (or another intercalating agent), then pouring the solution into a horizontal casting tray. One or more gel combs can be used to form rows of wells in the agarose. After cooling and polymerization, the gel is loaded in a horizontal electrophoresis apparatus and covered with buffer in a single chamber. DNA is mixed with a loading buffer, as described above for acrylamide gels, wells are loaded and electrophoresis performed as described above. Agarose gels have a larger pore size than acrylamide gels. Agarose gels with a concentration of 1 % are used to separate DNA fragments of 1–20 kilobases (kb), while higher concentration gels are useful to separate smaller DNA fragments. Agarose gels are thicker and more stable than polyacrylamide gels but do not provide the same degree of resolution. Agarose is safer than acrylamide but still must be handled and disposed of with care if the gel contains EtBr. Other modified agarose compounds are available that can be mixed in various ratios with standard agarose to increase the resolution of agarose gels. Like acrylamide gels, precast agarose gels are commerically available.

Capillary Electrophoresis

Capillary electrophoresis (CE) is a widely used separation technology for analysis of proteins, peptides, chemicals, natural products, pharmaceuticals, and DNA. CE systems are commercially available and generally provide more consistent and standardized results with less time and effort than gel electrophoresis. Using CE, DNA fragments are rapidly separated with a high-voltage gradient, because the capillary dissipates heat quickly. Therefore, one CE run takes approximately half an hour or less, and if 8, 16, or more capillaries are run simultaneously, the process reduces the time from standard electrophoresis, which requires 3–4 h. This is a significant time savings in the clinical laboratory for applications such as DNA sequencing. CE enables more standardized results, maximization of workforce efficiency, increased productivity and throughput, and the potential for error reduction. CE also uses smaller sample volumes. As such, CE has become the method of choice for most clinical molecular laboratories.

In CE, electrophoretic separation takes place in a capillary tube ranging in length from 25 to 100 cm and approximately 50–75 μm in diameter. Most capillary tubes are made of glass (silica) walls that often are covered with an external polyimide coating. Acid silanol groups impart a negative charge on the internal wall of the capillary. A low-viscosity acrylamide-based flowable polymer acts as the electrolyte solution and sieving matrix within the silica capillary, and is responsible for the conductivity of current through the capillary. Polymer concentration affects the pore size and movement characteristics of the DNA through the capillary.

A small section of the capillary coating is removed at one end of the capillary to create a detection window. The detection window is optically aligned with the detection system of the instrument. The detection system often includes either a diode or argon laser combined with a charge-coupled device (CCD) camera or filter wheel and photomultiplier tube. The opposite end of the capillary and electrode is used for sample injection by electrokinesis. In electrokinetic injection, the capillary and electrode are moved into the sample well. The sample enters the capillary when a voltage of 2–5 kV is applied for approximately 5–15 s. The voltage causes sample ions including DNA to migrate electrophoretically into the capillary in a flat flow profile. Electrokinetic injection produces increased resolution compared to hydrodynamic injection, which produces a laminar (curved) flow profile. After the injection, the capillary and electrode are returned to a buffer reservoir for the separation. The DNA fragments separate by size and charge during migration through the capillary (smaller fragments moving more quickly than larger fragments) and are detected through the window at the far end of the capillary.

In the clinical molecular laboratory, DNA sequencing and DNA fragment sizing and/or quantitation are the most common applications performed on CE instruments. One negative aspect of CE as opposed to older polyacrylamide gel technology is that CE is more sensitive to contaminants and DNA concentration. DNA, being negatively charged, migrates into the capillary when voltage is applied. If there

are any other charged particles in the sample, they also are injected into the capillary. For example, salt is an ionic competitor. If salt is present, the fluorescent signal intensity of the sample will be greatly reduced because of ionic competition during the brief injection. Proper sample preparation is therefore a key to successful CE.

After a post-reaction purification step, if needed for the specific CE application, DNA samples are resuspended in a sample loading solution. High-quality deionized formamide often is used as the sample loading solution. If DNA is denatured prior to CE, the formamide maintains the denatured state of DNA and provides a very stable environment for fluorescent dyes. Following the post-reaction purification and resuspension of products, the samples are ready for analysis on the CE instrument. The fragments are injected into the capillary and detected by laser-induced fluorescence, and data are generated for analysis using software supplied by the manufacturer for different CE applications.

Restriction Endonucleases

Restriction endonucleases (REs) cleave DNA at specific nucleotide recognition sequences. Restriction endonucleases are naturally occurring proteins produced by and purified from bacteria. Each bacterial species contains one or more REs, each recognizing a unique sequence of base pairs in double-stranded DNA, called recognition sites (most commonly 4–8 bp long). The natural function of REs within bacteria is to digest and inactivate foreign DNA (such as bacteriophage DNA). The frequency of recognition sites in target DNA for any given RE is inversely proportional to the size of the recognition site. Some REs do not cleave DNA when their recognition sites are methylated; this can be useful in certain clinical laboratory applications such as detection of imprinted genes in genetic diseases or promoter hypermethylation in tumors. Some mutations occur at RE recognition sites and can be detected by a change in the RE digestion pattern of a PCR product or genomic DNA. Unique DNA restriction fragment patterns are generated by digestion with different REs, creating a range of DNA restriction fragment sizes, which can be fractionated and detected using agarose gel electrophoresis. Restriction endonuclease digestion is commonly used as a component of clinical molecular tests.

Specific Methods

DNA Sequencing

The ability to sequence DNA has been essential to the field of molecular pathology because sequence information is a prerequisite for PCR, PCR alternatives, and hybridization with probes necessary for successful Southern blot analysis. The method for DNA sequencing developed by Sanger, Nicklen, and Coulson [6] is the basis for most DNA sequencing performed both in clinical laboratories and for the HGP.

The Sanger sequencing reaction uses a single DNA primer and DNA polymerase resulting in linear, rather than the exponential, PCR amplification (see below). Components essential to the Sanger sequencing reaction include: (1) DNA template that is purified and quantitated; (2) sequence-specific primers, complementary to the opposite strands and ends of the DNA region to be sequenced, which is desalted and usually purified by high-performance liquid chromatography (HPLC); (3) small proportions of dideoxynucleoside triphosphates (ddNTPs) in addition to the conventional deoxyribonucleoside triphosphates (dNTPs) used in DNA sequencing reaction; and (4) an electrophoresis technique capable of clearly distinguishing single nucleotide length differences in DNA strands dozens or hundreds of nucleotides in length. Dideoxynucleotides differ from deoxynucleotides by having a hydrogen atom attached to the 3′ carbon rather than an hydroxyl (-OH) group, which is present on the deoxynucleotide. Because the ddNTPs lack a 3′-OH group, elongation of the newly polymerized DNA chain cannot occur once a ddNTP has been incorporated (arabinonucleosides also can be used as inhibitors of elongation). The end result is a set of newly synthesized DNA chains that are complementary to the template DNA but that vary in length, with the length determined by the point at which the ddNTP was incorporated into the 3′ end of the chain.

In the original paper, four reactions were performed for each template, with the addition of a single inhibitor to each, ddGTP, ddATP, ddTTP, or araCTP [6]. The DNA chains were separated by polyacrylamide gel electrophoresis under denaturing conditions and visualized using (α-^{32}P)-dATP on a radio-autograph. The four reactions were run in consecutive lanes of the gel, and the complementary DNA sequence was determined by manual inspection based on the size of each chain, and the specific ddNTP in the reaction. As sequencing techniques evolved, radioactive labeling was replaced by fluorescent labeling. Two major categories of fluorescent labeling are used for sequencing. In dye-primer labeling, the sequencing primer is labeled, and the sequencing reaction requires four tubes differing only in the incorporation of the specific ddNTP. In dye-terminator labeling, the sequencing primer is unlabeled and instead each ddNTP is labeled with a different fluorophore, thus enabling the entire sequencing reaction to be performed in a single tube. Dye-primer labeling may be used in fragment analysis for detection of microsatellite instability, loss of heterozygosity, forensic identification, or allogeneic bone marrow transplantation monitoring using short tandem repeat polymorphisms. Automated sequencers recognize both the size of the DNA chain and the fluorescent color of the chain to assign the

nucleotide sequence, and also function as precise detectors for fragment analysis [7].

Conventional DNA sequencing with polyacrylamide gel electrophoresis (whether using manual or automated sequence detection) is time-consuming and labor-intensive. The introduction of CE facilitated the use of sequencing and fragment analysis by the clinical laboratory [8]. The sequencing reaction products are purified by ethanol precipitation or a chaotropic salt-silica column technique before injection into the CE unit to remove excess salts, dyes, and unincorporated primers that would compete for injection into the capillary. Numerous protocols and commercial kits are available for the post-reaction purification. After the post-reaction purification step, samples are resuspended in a sample loading solution containing high-quality deionized formamide to denature the DNA. Formamide also provides a very stable environment for fluorescent dyes. The fragments are injected into the capillary, detected by laser-induced fluorescence, and rendered into sequence by the analysis software. An electropherogram of the DNA sequence is generated by the detection software by correlating the fluorescent intensity of each dye wavelength corresponding to a specific ddNTP as a function of migration time.

Examples of Applications of DNA Sequencing

1. *CFTR* mutation analysis for cystic fibrosis
2. *BRCA1* mutation analysis for breast/ovarian cancer
3. *CEBPA* mutation analysis for acute myeloid leukemia (AML)
4. High-resolution human leukocyte antigen (HLA) typing for allogeneic bone marrow transplantation

Southern Blot

The Southern blot was developed by E.M. Southern in 1975 and was the first molecular biology tool to have a major impact on clinical molecular pathology. The Southern blot is in limited use, having been largely replaced by amplification methods. Development of the Southern blotting was based on prior knowledge of nucleic acid isolation, gel electrophoresis, RE digestion, and nucleic acid probe labeling for detection of DNA sequences of interest.

The Southern blot is a labor-intensive, time-consuming clinical laboratory method [9]. High-quality DNA is isolated from a patient specimen, subjected to RE digestion, and then separated by size (fractionated) by agarose gel electrophoresis. "Blotting" is the transfer of the fractionated DNA from the gel to a solid support such as a nylon membrane. A small piece of DNA complementary to the sequence of interest for the test being performed is labeled in one of a variety of ways and called a probe. The probe is hybridized to the fractionated DNA on the membrane, and then the location of the probe (and the DNA fragments relevant to the test) is detected using the probe label. This detection step allows the gene of interest to stand out from the vast background of DNA present in the sample. If the banding pattern visualized on the membrane is different from the normal pattern, this may be indicative of a mutation in the targeted sequence. As examples, a more intense probe signal indicates sequence amplification, lack of a signal indicates sequence deletion, and a shift in the band size indicates a change in a RE recognition site.

Because no amplification of target DNA occurs, Southern blot analysis requires a large mass of DNA. Because the banding pattern depends on the specific cuts made by the RE and not just random DNA breaks, the DNA must be largely intact and of high molecular weight. Therefore, electrophoresis of the isolated DNA to be used for a Southern blot test prior to analysis is important for assessing the integrity of the DNA, since only a small degree of DNA degradation is tolerable. Degraded DNA may produce false-negative results if a signal from high-molecular-weight DNA is expected, while false-positive results may occur if partially degraded DNA results in unusually sized bands. Fortunately, most tests in the clinical molecular laboratory today are based on PCR, which is less affected by DNA degradation. Polymorphisms within RE recognition sites also change banding patterns, a principle used to advantage in other molecular tests.

The physical movement of the DNA from the gel to the membrane may be accomplished by manual capillary transfer, automated vacuum transfer, or electrotransfer. DNA in the gel first is "conditioned": depurination with dilute HCl and subsequent denaturation with NaOH. Dilute and brief acid treatment causes hydrolysis of the DNA phosphodiester backbone to occur spontaneously at the sites of depurination. This acid-induced fragmentation facilitates efficient transfer of the highest-molecular-weight DNA species from the gel to the membrane, but does not alter the original size fractionation achieved by RE digestion and electrophoresis. Alkali treatment denatures double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA), essential for subsequent nucleic acid hybridization with a labeled ssDNA probe. The DNA is permanently fixed to the membrane by thoroughly drying the blot in an oven or by exposing the blot to a precise amount of UV irradiation.

DNA probes are labeled before use in hybridization assays to permit visualization of probe–target binding. Note that in reverse hybridization assays, described below, unlabeled probes are immobilized and the target is labeled during the amplification step that precedes hybridization. Probe labels may be isotopic or nonisotopic. High-specific-activity DNA probes may be generated by in vitro biochemical reactions that synthesize new DNA from dNTPs, using the probe as a template. One type of the dNTPs is labeled with a

reporter molecule such as ^{32}P, biotin, or digoxigenin. When incorporated into the newly synthesized DNA, the labeled dNTP, even though it is only one of the four dNTPs in the DNA probe, is sufficient to label the entire probe for detection. The probe is used in vast molar excess relative to the target DNA in nucleic acid hybridization to drive the hybridization reaction to occur as quickly as possible.

The blot is immersed in prehybridization buffer to prepare the DNA on the blot for hybridization with a probe. Prehybridization buffer contains blocking agents included to minimize unwanted nonspecific DNA probe binding that would otherwise contribute to high background on the final image of the Southern blot used to view the results and make diagnostic conclusions. The prehybridization step equilibrates the membrane and blocks sites on the nylon membrane without DNA to prevent the probe from binding nonspecifically and increasing background nonspecific signal. A large volume of blocking agent is therefore advantageous. Addition of the labeled probe to the blot begins the hybridization phase of the Southern blot process. A small volume of buffer is used to facilitate probe and target specifically finding each other, thereby promoting hybridization. Hybridization takes several hours to overnight at an appropriate temperature determined by multiple variables: concentrations of the two species; time permitted for hybridization; complexities of the nucleic acids involved; length of the probe and its target and their complementarity to each other (or degree of mismatch); pH; temperature; and ionic strength of the buffer used.

After hybridization, the blot is washed with buffers containing sodium chloride and detergent to remove excess probe and reduce background nonspecific hybridization of the probe. Sodium chloride concentration and stringency are inversely related: the lower the sodium chloride concentration, the more stringent the wash condition. Increasingly stringent washes remove more nonspecifically bound probe. The temperature of the wash buffer and stringency are directly related: high-temperature washes are more stringent than low-temperature washes and further contribute to hybridization specificity. When appropriately stringent washing of the blot is complete, only the specific hybrids of interest should remain. Visualization of these specific hybrids, which appear as bands, is achieved by autoradiography for radioactive probes or by luminography for chemiluminescent probes. Biotinylated probes are visualized by chemical reactions, resulting in insoluble colored precipitates at the site of hybridization on the blot itself that serve as the endpoint (this is also the detection scheme used in the line probe assay; see below). Simple visual inspection of the blot or the radiograph is used for both isotopic and nonisotopic Southern blots to determine the position where the labeled probe hybridized to its target patient DNA. That position, relative to detection of appropriate controls and the distance of migration from the gel wells, allows interpretation.

Northern blotting is an extension of Southern blotting that uses RNA instead of DNA as the target of investigation. Northern blotting is as labor intensive as Southern blotting but even more problematic due to the highly labile nature of RNA. While northern blotting has been very useful in the research setting to demonstrate the selective expression of genes in various organs, tissues, or cells, it has not become a routine tool in the clinical molecular laboratory.

Examples of Applications of Southern Blotting
1. Fragile X syndrome diagnosis
2. Myotonic dystrophy diagnosis

Polymerase Chain Reaction

In the mid-1980s, Mullis and coworkers developed a method, the polymerase chain reaction (PCR), to amplify target sequences of DNA exponentially [10]. As the name suggests, the method is a DNA polymerase-mediated cyclical reaction resulting in amplification of specific nucleic acid sequences. Arguably, PCR is the single most important "invention" leading to the development of a new discipline in clinical laboratory medicine, that is, molecular pathology. Both PCR and the Southern blot are techniques used to investigate specific genomic targets. However, PCR is orders of magnitude more sensitive and rapid, permitting turnaround times from specimen receipt to report generation of 24 h or less. PCR lends itself to much higher test volumes than Southern blotting, a crucial point in its adoption in the clinical laboratory setting. Opportunity for high test volumes, excellent specificity and sensitivity, and the rapid turnaround times of PCR are the principal reasons this technology is used so widely in clinical molecular laboratories.

In PCR, a unique sequence of the nucleic acid of interest, e.g., oncogene, invading pathogen DNA, gene mutation, is chosen as the target for amplification. The inherent specificity of the ensuing reaction is provided by two short oligonucleotides (see Fig. 2.1) that serve as primers for DNA polymerase-mediated DNA synthesis using denatured target DNA as a template. The two primers are complementary to opposite strands and opposite ends of the targeted DNA template region. Usually the primers bracket the area of interest, but one type of PCR (allele-specific PCR; see below) uses primers that overlap the area of interest. Successful PCR depends on temperature cycling, and in the first step of PCR the reaction temperature is raised to 95–98 °C to denature the target DNA, and thus is called the denaturation step. After 10–60 s at this high temperature, the temperature is reduced

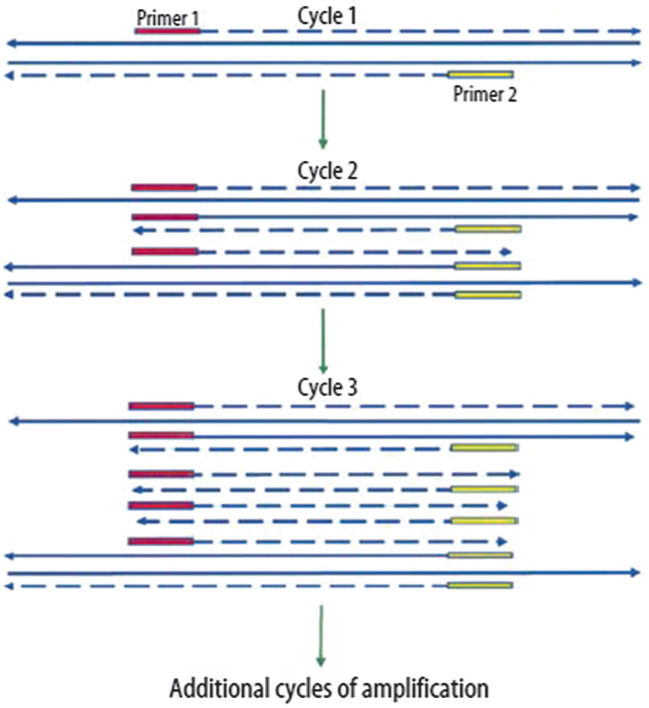

Figure 2.1 The polymerase chain reaction

to 50–70 °C, depending on the specific protocol, and held there for usually 10–60 s. This facilitates hybridization (annealing) between the denatured target DNA and the PCR primers, and is called the annealing step. This hybridization event is favored over target DNA reannealing because the PCR primers are small and present in vast molar excess, and move more rapidly in solution than larger DNA molecules.

The hybridized PCR primers form local areas of double strandedness with the template DNA, thereby serving as sites for DNA polymerase to bind and synthesize a new strand of DNA, using the target DNA as a template and dNTPS present in the reaction solution. Subsequent to the initial discovery of PCR, the opportunity for automating the temperature cycling was realized by using DNA polymerase from hot-spring living bacteria, *Thermus aquaticus* (hence the term "*Taq* polymerase"). *T. aquaticus* thrives at very high temperatures, and so its proteins do not denature at the high temperatures needed to denature DNA in the first step of PCR. Catalysis by *Taq* polymerase of a new strand of DNA proceeds at a temperature intermediate to the near-boiling temperature used for denaturation and the relatively lower temperature used for annealing. DNA polymerization occurs during this extension step, typically at 65–75 °C. Taken together, these three steps (denaturation, annealing, and extension) define one PCR cycle.

Temperature cycling is automated through the use of an instrument called a thermal cycler. Thermal cyclers hold small capped tubes (or 96- or 384-well microtiter plates for larger volume testing) containing the reagents needed for PCR, and cycle among the temperatures needed for the different steps of the PCR [11]. A single PCR tube contains template DNA (<1 ng to 1 μg), *Taq* DNA polymerase, two PCR primers (15–30 nucleotides long), all four dNTPs, Mg^{2+}, and buffer to maintain an elevated pH (8.4) optimal for *Taq* polymerase activity.

The repetition of the cycles generates exponential amplification of the target DNA because each double-stranded target DNA molecule, theoretically even if there is only one, is replicated after one PCR cycle. Both the original and replicated DNA molecules can function as templates for cycle 2, in true "chain reaction" style, generating another doubling, or four copies of the original target. Cycle 3 ends with eight molecules, and doubling continues with completion of each new cycle. This doubling plateaus in later cycles since reagents, usually dNTPs, become limiting. Additionally, the enzyme may not function at 100 % efficiency, and so true exponential amplification is theoretical, although there is a true exponential phase of amplification.

Greater than one billion copies of the original target DNA region are generated after 32 cycles of PCR: 2^{32} or more than four billion, the difference owing to the fact that unit-length amplicons are not generated until the end of the second cycle of PCR. Amplicons (PCR products) are defined as replicated target molecules created by PCR. Unit-length amplicons are those whose ends are defined by the primers. During the first cycle, the primers are extended by *Taq* polymerase using template DNA. The termination of this extension is undefined and a function of how far the polymerase moves down the template during the time allotted by the temperature cycle. The enzyme, therefore, moves beyond the ends of the primer-binding site on the complementary strand. After completion of the first cycle, therefore, the newly synthesized DNA molecules are greater in length than the sequence bracketed on each strand by the primers. In the second cycle, DNA molecules are synthesized from the products of the first cycle whose ends are defined by the two primers, thus generating unit-length or specific amplicons. While all of the above is true, the practical clinical laboratory difference between one- and four billion-fold amplification is irrelevant because either number is sufficient for detection of the target, e.g., by electrophoresis with SYBR green or EtBr used as an intercalating agent for visualization.

Several factors affect PCR specificity and sensitivity. The production of specific PCR amplicons is a function of both the complementarity of the primers to the target DNA and the annealing temperature of the PCR cycle. Heating denatures the primer and target DNA. The temperature at which a primer melts from the target DNA varies directly with the length of the primer and the guanine–cytosine (GC) content of the primer, and inversely with the degree of mismatch between the primer and the target DNA. The melting temperature (T_m)

of the primer is the temperature at which 50 % of the primer is denatured from the target DNA. If the thermal cycler is programmed to reach an annealing temperature higher than the primer T_m, the efficiency of PCR is compromised and sensitivity decreased. In contrast, if the annealing temperature is substantially less than the primer T_m, the primer can bind to both complementary and noncomplementary DNA, resulting in reduced PCR specificity as nontarget DNA is amplified (and potentially decreased sensitivity as reaction components are used nonspecifically). Therefore, the ideal annealing temperature is slightly less than the Tm of both primers, and the primers should be designed to have a very similar T_m. The annealing temperature can be decreased with subsequent cycles during PCR in a process called "touchdown" PCR. This allows the initial cycles to produce specific products at high annealing temperatures, while later cycles amplify previously generated amplicons more efficiently using lower annealing temperatures, thereby increasing sensitivity (see below the use of touchdown PCR in multiplex PCR).

Taq polymerase is very sensitive to mismatches between the primer and the target DNA at the 3′ end of the primer but can withstand considerable noncomplementarity at the 5′ end of the primer. Numerous PCR variations have been designed to take advantage of both these facts. *Taq* polymerase also requires Mg^{2+} as a cofactor for stabilization of primer annealing. Insufficient Mg^{2+} decreases PCR efficiency, while too much Mg^{2+} stabilizes nonspecific primer annealing. Primers with a high GC content may show a narrow range of tolerance for variation from ideal PCR conditions, leading to decreased amplification or nonspecific products. This may be alleviated by using PCR additives such as dimethyl sulfoxide (DMSO), betaine or glycerol, but the success and amount of these additives may need to be determined empirically for different primer pairs. Another strategy to improve specificity is the use of "hot-start" PCR, in which a crucial PCR reactant such as *Taq* is either physically or chemically sequestered from other PCR reagents until denaturation begins. This prevents the generation of nonspecific amplification products by inhibiting the activity of *Taq* at lower temperatures and until after the initial PCR denaturation step.

PCR is more sensitive than Southern blot hybridization because of the amplification of the target sequence. However, the specificity of the amplified PCR product(s) must be verified. Simple agarose gel electrophoresis coupled with intercalating agent staining may be used to observe the PCR product(s). When a clinical PCR protocol is established, such gels may be subjected the first time to blot hybridization with a specific probe complementary to the internal, non-primer sequence of the amplicon(s). This exercise proves that the PCR-generated band not only is the correct size and highly likely to be the correct target, but also is a DNA fragment that has high or perfect homology with a known probe or the correct target sequence. For example, hybridization of a particular 302 bp PCR product band detectable on an agarose gel with a defined cytomegalovirus (CMV) DNA probe confirms that the oligonucleotide primers synthesized based on the CMV sequence and used in the PCR are recognizing CMV-specific DNA and that the PCR is indeed specific for CMV sequence. An alternative method to validate the specificity of the PCR product is to sequence the PCR product. Following this one-time validation analysis, electrophoresis alone may be the assay endpoint, as opposed to blot hybridization or sequencing.

There have been significant commercial endeavors to automate or semiautomate high-volume PCR-based clinical tests. For example, denatured aliquots of completed PCRs can be added to microtiter plates with wells to which specific DNA probes are bound. In the presence of amplicon, if the patient is infected with the pathogen of interest or a specific mutation is present, the amplicons hybridize to the bound probe and are retained in the well during subsequent washing steps. Biochemical reactions are used to detect labeled moieties in the amplicons ("built in" to the PCR components), facilitating colorimetric detection of a positive patient reaction by an automated plate reader. Absence of colored product in a well indicates a negative result for that patient specimen, provided that all positive and negative controls are within tolerance limits. This scheme has gained US Food and Drug Administration (FDA) approval or clearance for clinical PCR-based detection kits for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, HCV (qualitative), and HIV [12]. (For a complete list of FDA-approved or -cleared tests, go to http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm). Subsequent generations of PCR instrumentation are available that completely automate the amplification and detection processes [13].

Another aspect of PCR that is attractive for the clinical molecular laboratory is the ability to use relatively crude DNA extractions from patient specimens rather than highly purified DNA. Cell lysis and subsequent DNA liberation accomplished by boiling or treatment with detergent may be sufficient to process a specimen in preparation for PCR [14]. Conventional PCR-based tests may be completed with turnaround times of as short as 2–4 h, while real-time PCR can be completed in 30 min, making this technique attractive for rapid clinical testing.

Examples of Applications of PCR

1. Detection of the diagnostic *BCL2–IGH* gene rearrangement in follicular lymphoma
2. Detection of *Chlamydia trachomatis* in urine

PCR Variations

PCR-Restriction Fragment Length Polymorphism Analysis

Polymorphisms are inherited differences found among the individuals in a population at a frequency >1 % of that population. The term "polymorphism" is not synonymous with the term "mutation" which is used for germline variations that are pathogenic and found less frequently in a population, or are nongermline changes in a tumor cell (somatic mutations). In the case of restriction fragment length polymorphisms (RFLP), DNA sequence differences alter RE recognition sites, manifested either as obliteration or creation of a restriction site. With obliteration of a RE site, the DNA of individuals with an RFLP exhibits a larger restriction fragment of DNA than those without the polymorphism. With creation of a new RE site, RE digestion results in two smaller fragments relative to the individual without the polymorphism. In either case, the polymorphism is detectable by creation of a new restriction fragment pattern, that is, a restriction fragment length polymorphism. In PCR-RFLP, the PCR products are digested by one or a combination of REs and electrophoresed to detect polymorphisms or mutations which are seen as changes in the DNA fragment sizes reflected by changes in the band pattern on the gel (or chromatogram).

Examples of Applications of PCR-RFLP Analysis

1. Detection of sickle-cell hemoglobin (HbS) gene mutation
2. Detection of the *Mnl*I restriction enzyme polymorphism created by the Factor V Leiden mutation [15]

Restriction-Site Generating PCR

Some DNA sequence variants create or abolish RE recognition sites and can easily be detected by PCR-RFLP. Unfortunately, most variants do not alter a RE recognition site. In restriction-site generating PCR (RG-PCR) (and a related research technique called PCR-mediated site-directed mutagenesis [PSDM]), an artificial RE recognition site is generated during PCR using a specially designed PCR primer [16, 17]. The primer contains a base mismatch to the template DNA adjacent to the variable base of the variant that creates a RE recognition site in the PCR product. The mismatched base in the primer is located near or at the 3′ end of the primer, which is near or adjacent to the variable base of the variant, and together they create a novel restriction site within either the variant or non-variant amplicon. The presence or absence of the RE recognition site is determined from the pattern of digested PCR product fragments by gel electrophoresis. Not all sequences are amenable to the generation of a restriction site, and the amplification efficiency is often decreased due to destabilization of the primer with the mismatch.

Examples of Applications of RG-PCR

1. Identification of mutations in the *CTFR* gene in cystic fibrosis
2. Identification of mutations in the *ATM* gene in ataxia–telangiectasis

Multiplex PCR

Multiplex PCR is a technique used for amplification of several discrete genetic loci with multiple PCR primer pairs in a single reaction. Multiplex PCR simultaneously answers several related questions about a specimen without the need for multiple individual PCR reactions. Multiplex PCR is commonly used for verification that amplifiable nucleic acid is present in the sample, for example, amplification of a housekeeping gene in addition to the gene sequence(s) of interest, and to check for the presence of PCR inhibitors that can prevent amplification of target nucleic acid, for example, coamplification of an exogenously added internal control. Multiplex PCR often requires painstaking optimization of PCR conditions and careful design of the multiple primer pairs to match PCR efficiencies and to prevent the generation of primer-dimers (PCR products generated by the primers alone due to complementarity between primer regions) and other nonspecific PCR products that may interfere with the amplification of specific products. Touchdown PCR can be used with multiplex PCR if the primer pairs have different annealing temperatures. Concentrations of individual primer pairs may need to be optimized to account for different amplification efficiencies and competition between the primer pairs.

Examples of Applications of Multiplex PCR

1. Detection of enterovirus and herpes simplex virus (HSV) nucleic acids in cerebrospinal fluid (CSF)
2. Detection of pathogenic enteric bacteria in stool
3. Analysis of multiple *BRCA1* loci in a breast cancer patient [18]
4. Identification of different bacteria in a respiratory infection specimen [19]
5. Amplification of multiple microsatellite loci for bone marrow engraftment analysis

Single Nucleotide Extension

Another method for a multiplexed assay is single nucleotide extension (SNE) or single base extension (SBE). In this method, either a single long-range PCR or a multiplexed PCR is used to amplify the region(s) of interest. This is followed

by a multiplexed set of extension primers of differing lengths that hybridize one base upstream to the variant(s) of interest. A second, linear amplification, similar to Sanger sequencing, adds the next nucleotide (at the variant position) using ddNTPs, with each type labeled with a different fluorophore. The products are separated by CE or mass spectrometry, and the specific fluorescent signal of the incorporated base indicates which base was added, and whether the variant is present or not. This method can be used to genotype up to approximately 20 mutations at once. SNE and SBE can be considered sequencing, but of just one base.

Examples of Applications of SNE

1. Analysis of common mutations in *GALT* for galactosemia [20]
2. Analysis of common mutations in *BTD* for biotinidase deficiency
3. Analysis of multiple mutations in the *CFTR* gene for cystic fibrosis [21]

Nested PCR

For nested PCR, two pairs of PCR primers with one set internal to the other (nested) are used to sequentially amplify a single locus. The first pair is used to amplify the locus as in any PCR assay. A dilution of the first PCR reaction then is amplified with the nested primers. Alternatively, semi-nested PCR is performed using one of the original PCR primers and one new internal primer in a second round of amplification. Both nested and semi-nested PCR generate a second PCR product that is shorter than the first one [22]. The logic behind this strategy is that if the wrong locus was amplified incorrectly or nonspecifically, the probability is very low that it would be amplified a second time by a second pair of primers. Thus, nested PCR enhances specificity while also increasing sensitivity. The problem with nested PCR is the high risk of amplicon contamination when the first-round PCR products are used to set up the second round of PCR with the nested primers (see Amplicon Carryover Contamination section below for information on PCR contamination control). For this reason, many clinical laboratories do not use nested PCR procedures.

Allele-Specific PCR

Allele-specific PCR (AS-PCR) also is referred to as amplification refractory mutation system (ARMS), PCR amplification of specific alleles (PASA) and PCR amplification with sequence-specific primers (PCR-SSP). AS-PCR is based on the principle that a 3′ mismatch between a PCR primer and the template DNA prevents PCR amplification [23]. AS-PCR is especially useful for detection of single nucleotide polymorphisms (SNPs) or mutations. For AS-PCR, target DNA is amplified in two separate and simultaneous reactions. Each reaction contains an allele-specific primer (either non-variant or variant) and a second primer common to both reactions. PCR is performed under stringent conditions, to prevent PCR amplification if a mismatch is present. Genotype is based on amplification in either one of the reactions alone (homozygous non-variant or variant) or both reactions (heterozygous). Detection of the amplicon is either by gel electrophoresis or real-time PCR technology (see below). A disadvantage of AS-PCR is that unsuspected nucleotide variants located in the DNA template at or adjacent to the 3′ binding site of the primer would prevent amplification, leading to incorrect genotyping.

AS-PCR can detect one variant allele in the presence of 40 copies of the non-variant allele. In addition, AS-PCR can be combined with multiplex PCR using multiple allele-specific primers in the same reaction tube. This technique is known as multiplex ARMS, a useful method when a single disease is caused by different mutations in one or more genes. Multiplex PCR-SSP also is commonly used in low-resolution HLA typing, in which multiple primer pairs for HLA loci are used along with control primers that amplify a housekeeping gene to verify that amplifiable DNA is present in each reaction tube.

Examples of Applications of AS-PCR

1. Detection of multiple cystic fibrosis *CFTR* mutations
2. Detection of common α-1 antitrypsin deficiency mutations
3. Detection of common phenylketonuria mutations

Allele-Specific Oligonucleotide Hybridization

Allele-specific oligonucleotide hybridization (ASOH), also known as dot-blot analysis, is used for genotyping of highly polymorphic regions of DNA. ASOH can be thought of as a variation of the Southern blot, in that patient DNA amplified by PCR is bound to a membrane and hybridized with labeled allele-specific oligonucleotide probes [24]. Reverse dot-blot analysis differs from ASOH in that unlabeled allele-specific oligonucleotide probes are spotted onto different membrane locations and hybridized with labeled PCR amplicons.

For ASOH, the PCR products are denatured and a small amount of denatured (single stranded) amplicon is spotted onto a nylon or nitrocellulose membrane. The amplicon is permanently bound to the membrane by baking under vacuum or UV cross-linking. Amplicons from different specimens can be spotted at different locations to interrogate the genotype of multiple specimens simultaneously. Duplicate membranes are made for each probe type. Each membrane is hybridized with two different labeled oligonucleotide probes (one complementary to the variant sequence and another to the non-variant sequence of the same DNA region). The membranes are washed to remove nonspecifically bound probe. Samples that hybridize strongly to only one probe indicate homozygosity for the

non-variant or variant allele; those that hybridize with both probes are heterozygous. The oligonucleotide probes are labeled and detected by radioactivity (often avoided in clinical molecular laboratories), fluorescence, colorimetry, chemiluminescence or mass spectrometry. One drawback of ASOH is the potentially ambiguous discrimination of a positive compared to a negative signal. Optimization of the assay and the use of both positive and negative controls help to define and score ASOH results.

Example of Application of ASOH
1. Low-resolution HLA typing

Oligonucleotide Ligation Assay

Oligonucleotide ligation assay (OLA) is a highly specific method for detecting well-defined alleles that differ by a single base [25, 26]. The target sequence is initially amplified using PCR and then denatured. A pair of allele-specific oligonucleotide (ASO) probes (one specific for the non-variant allele and the other specific for the variant allele), a common reporter probe (complementary to a sequence common to both alleles), and DNA ligase are added to the denatured PCR products. The ASO probes are designed to differ from one another only at the terminal 3′ base. The common reporter probe is positioned immediately adjacent to the 3′ terminal end of the ASO probes. If the ASO is complementary to the amplicon, DNA ligase can covalently join the adjacent ASO and reporter probes. If the ASO is not a perfect match to the amplicon, the 3′ base does not anneal with the template DNA, and DNA ligase cannot join the ASO and reporter probes. The ligation products are analyzed by electrophoresis. Alternatively, one of the probes can be biotinylated at the 5′ end and the other probe tagged at the 3′ end with a reporter molecule such as fluorescein or digoxigenin. If ligation occurs, the ligation product is biotinylated at one end, facilitating capture onto a streptavidin-coated microtiter plate. The opposite end contains the reporter label. Washing removes unbound label and the reporter molecule is detected.

Example of Application of OLA
1. Detection of multiple *CFTR* mutations for cystic fibrosis

High-Resolution Melting Curve Analysis

Melting curve analysis takes advantage of the principle that DNA sequences that are a perfect match will melt at a higher temperature than those that contain either a heterozygous or homozygous nucleotide variant. Typically, in genotyping by high-resolution melting curve analysis, an area of interest (approximately 50 bases, including primers) is amplified in the presence of a double-stranded DNA-intercalating fluorophore or double-stranded DNA-intercalating dye. After amplification, the temperature is decreased to the point that the DNA will reanneal. The temperature then gradually is increased while the fluorescence is monitored. Variants are identified by a change in melting curve shape as compared to a non-variant control. While the typical use of melting curve analysis is to identify single nucleotide variants of interest, it can also be used as a rapid scanning method to detect potential sequence variants in a gene of interest. Melting curve analysis can be affected by factors such as salt concentration and DNA quantity, so all samples and controls must be prepared and amplified in an identical manner to exclude this as a possible confounding factor.

Examples of Applications of Melting Curve Analysis
1. Factor V Leiden genotyping
2. *HFE*-associated hereditary hemochromatosis genotyping

Pyrosequencing

Pyrosequencing is a useful method for variant detection when analytical sensitivity (limit of detection) or quantitation is important. In pyrosequencing, amplified targets are sequenced by adding and detecting incorporation of nucleotides one at a time. First, a target region is amplified and PCR products are captured through use of a biotinylated primer, which has been included in the PCR, along with a streptavidin-coated bead. Capture of the product onto the bead via the incorporated biotin group allows purification of the specific PCR product, followed by denaturation to create a single-stranded target. A sequencing primer close to the region of interest is then annealed to the captured single-stranded DNA amplicon. Deoxynucleotides are added one at a time in the presence of four enzymes: polymerase, sulfurylase, luciferase, and apyrase. Incorporation of the nucleotide releases pyrophosphate which participates in a chain reaction with luciferin, facilitated by sulfurylase and luciferase, to generate light. The amount of light released is directly proportional to the quantity of nucleotide incorporated. Apyrase removes unincorporated nucleotides. If the complementary base is not on the strand being sequenced, then no incorporation occurs and no light is released. The next nucleotide is then added to the pyrosequencing reaction and the steps are repeated. Nucleotides may be added in cyclic fashion (ACGTACGT…) or in an order specific to the target sequence, with allowance for anticipated variants.

Compared to other methodologies, pyrosequencing is particularly useful when analytical sensitivity is of particular concern, such as in detection of somatic mutations in tumor specimens which yield both non-variant and variant DNA. Analytical sensitivity of 5 % can be achieved with pyrosequencing, as compared to approximately 20 % for Sanger sequencing and approximately 10 % for melting

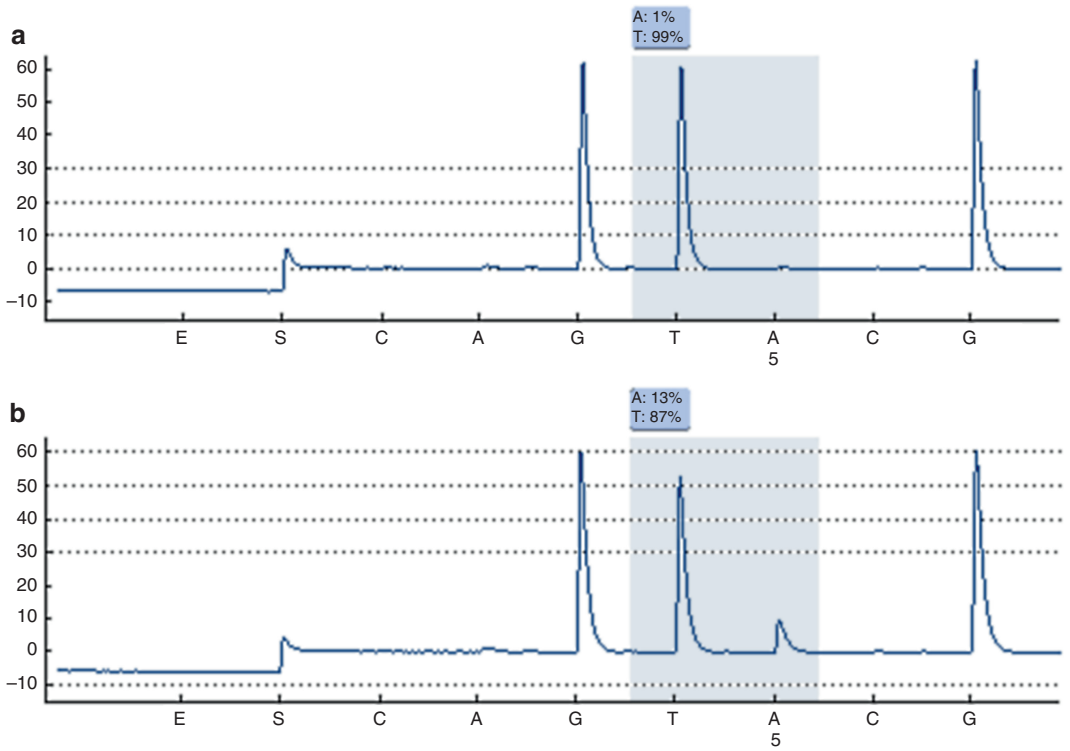

Figure 2.2 Pyrosequencing graphs for *BRAF* codon 600. Nucleotides were dispensed in the following order: CAGTACG. (**a**) Results for a non-variant sample showing the sequence GTG for codon 600. (**b**) Results for a variant sample (V600E), which has alleles with the sequence GAG, in addition to alleles harboring the normal sequence. Variant (V600E) alleles are present in this sample at 13 %.

curve analysis [27]. Quantification of mutant alleles also is provided by pyrosequencing results. Pyrosequencing is best suited for detection of variants within a targeted region. As compared to scanning methodologies, the region of interrogation may be somewhat smaller (under 100 bases, typically just multiple codons), but variants are both detected and characterized. An example of pyrosequencing results for codon 600 of *BRAF* is shown in Fig. 2.2.

Examples of Applications of Pyrosequencing
1. *KRAS* mutation detection in multiple tumor types
2. *BRAF* mutation detection in multiple tumor types
3. LINE-1 methylation

Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) may be thought of as RNA-based PCR. RT-PCR was made possible by the discovery in the early 1970s of retroviral reverse transcriptase (RT), an RNA-dependent DNA polymerase, by Baltimore and Temin [28], for which they shared the Nobel Prize in 1975. Reverse transcriptase catalyzes DNA synthesis using RNA as the template, producing a DNA strand complementary to the RNA template, called complementary DNA (cDNA). Complementary DNA is far more stable than the corresponding RNA because it is not subject to degradation by RNase. Complementary DNA can be treated like any other DNA target in subsequent PCR. Logistically, RT-PCR is trivially more time-consuming than PCR due to the extra enzymatic step of reverse transcription, but there are enzymes that combine reverse transcription and DNA polymerase activities, facilitating the use of RT-PCR in the clinical molecular laboratory. With the introduction of techniques to successfully isolate and protect RNA from ubiquitous RNases, to synthesize cDNA by reverse transcription and with the discovery of PCR, RNA analysis is virtually as rapid and sensitive as PCR-based DNA investigation. RT-PCR is a high-volume test method for the clinical molecular laboratory as used for the diagnosis and quantification of RNA viruses in human specimens, principally HIV and HCV.

Examples of Applications of RT-PCR
1. HIV and HCV viral load determinations
2. Detection of *BCR–ABL* translocation diagnostic of chronic myelogenous leukemia

Real-Time (Quantitative) PCR

Real-time (quantitative) PCR is based on the generation of a fluorescent signal by the PCR process, which is detected during PCR cycling, i.e., in real time, and reflects the amount of PCR product synthesized [29–31]. Different real-time PCR methods use alternative ways to generate a fluorescent signal during PCR. These include an intercalating dye such as SYBR Green that binds the minor groove of DNA, or an oligonucleotide used as a primer or probe and labeled with a fluorogenic dye. Instruments that combine in vitro nucleic acid amplification and real-time detection of the PCR product dramatically increased testing options for oncology, infectious diseases and genetics because of the wide range of readily available amplification primers and detection schemes, rapid turnaround time, and reduced risk of PCR amplicon contamination.

Real-time PCR is different from conventional PCR in several ways. Amplicon generation, temperature profiles and melting curves are monitored in real time, reducing the time required for post-PCR analysis. In most applications, post-amplification processing of the PCR products by gel electrophoresis or other method is eliminated. Because the reaction tubes remain closed after PCR starts, risk of amplicon carryover contamination within the laboratory is reduced. Results are more reproducible between runs since quantitation of target is based on amplification cycle threshold in the log-linear phase of amplification rather than traditional endpoint analysis in the PCR plateau phase. Real-time PCR methods have a wide dynamic range, up to 10 logs. Real-time PCR systems with intercalating dye or fluorogenic probes can be used to perform melting curve analysis, adding a check for the specificity of amplification or potentially the detection of unknown sequence variants (see below).

The simplest real-time PCR method uses intercalating dyes that insert into the stacked bases of DNA PCR products, allowing detection of amplification in real time. These dyes, for example, SYBR Green and ethidium bromide (EtBr), are nonsequence-specific dyes that increase in fluorescence when bound to double-stranded DNA. Intercalating dyes are used for melting curve analysis, qualitative and semiquantitative PCR, product discrimination and purity, and determination of primer and probe melting T_m. Intercalating dyes can be used for quantitative PCR. Results, however, are more specific and accurate with a sequence-specific probe for real time monitoring of amplicon production since fluorescence is directly proportional to the amount of specific amplicon produced which reduces the background contributed by primer-dimers or nonspecific PCR products. Intercalating dye fluorescence represents all double-stranded DNA, including primer-dimers and other nonspecific products that can be visualized with an endpoint melting curve analysis.

Most fluorogenic oligonucleotide techniques take advantage of the principle of fluorescent resonance energy transfer (FRET), in which the energy from one dye molecule (the donor) is transferred without the emission of a photon to an acceptor dye molecule when the two are in close proximity. If the acceptor is a fluorophore, a photon is emitted at a characteristic wavelength. However, if the acceptor does not emit a photon, the energy is dissipated and fluorescence from the donor is quenched. The reporter dye can be either the donor (if no FRET takes place) or the acceptor (if FRET does take place) and is defined as the one whose fluorescence correlates with the quantity of desired PCR amplicon. Several fluorogenic techniques are described below.

TaqMan

The TaqMan technique uses a short probe complementary to a non-primer internal sequence of the PCR product. The probe is labeled at the 5′ end with a reporter donor dye and at the 3′ end with an acceptor dye that quenches the reporter when the probe is intact. During the extension phase of PCR, probe bound to an amplicon is cleaved by the 5′ endonuclease activity of *Taq* polymerase, freeing the reporter dye from the quencher and resulting in fluorescence. The fluorescent signal increases proportionally to the number of amplicons generated during the log-linear phase of amplification. To ensure that hydrolysis of the probe occurs, a two-step PCR can be used with annealing and extension taking place at the same temperature (approximately 60 °C). Ideally, the TaqMan probe binding site is located near one primer and the size of the amplicon is no longer than 200–300 bases. One negative aspect of this method is that once the probe is hydrolyzed, it is unavailable for subsequent reactions or melting curve analysis, thus requiring an excess amount of probe in the reaction mix with the potential to decrease the PCR efficiency.

Molecular Beacon

A molecular beacon is a probe with a 5′ reporter dye and 3′ quencher dye, which forms a hairpin loop structure when not bound to target DNA, thereby juxtaposing the reporter and quencher dyes with quenching of fluorescence. The loop sequence is complementary to the non-primer amplicon sequence. When the loop of the molecular beacon probe hybridizes to the amplicon during the annealing step of real-time PCR, the reporter dye is separated from the quencher, resulting in fluorescence. For the molecular beacon probe to anneal to the amplicon, the amplicon-probe hybrid must be more stable than the internal base-pairing stem of the hairpin so that a fluorescent signal is generated. Generally, DABCYL is the nonfluorescent universal quencher and the other dye is a reporter fluorophore such as FAM, Cy3, TET, TAMRA, Texas Red, ROX, or Cy5.

Hybridization

Hybridization is typically a two-probe system in which one probe contains a donor dye and the other contains the reporter acceptor dye. The probes are designed to anneal to one strand of the PCR product adjacent to one another and internal to the primers. This juxtaposes the dyes, allowing FRET to occur. This probe format works well with the traditional three-step PCR with annealing at approximately 55 °C (primer specific) and extension at 72 °C, the optimal temperature for *Taq* polymerase activity. When DNA polymerase encounters the probes, they are displaced from the target strand rather than hydrolyzed and thus are available for the next round of amplification as well as endpoint melting curve analysis. In a variation of this method, a single unlabeled probe may be used in conjunction with an intercalating dye.

Uniprimer (Amplifluor, Sunrise)

Like molecular beacon probes, the uniprimer system uses a hairpin structure in the probe to quench fluorescence. The 3′ region of the fluorogenic probe is identical to a nonbinding region at the 5′ end of the reverse PCR primer. This allows the fluorogenic probe to become a primer for the newly formed amplicon by the third round of PCR. The probe is then opened in the fourth and subsequent rounds of PCR by the polymerase action of *Taq*, allowing fluorescence to occur. The advantage of this system is that the same fluorogenic probe sequence can be used in any PCR reaction (universal fluorogenic primer).

Scorpion

Scorpion also uses a hairpin structure in the probe to quench fluorescence. The fluorogenic probe is part of the reverse primer, and the nucleotides in the hairpin are complementary to the PCR amplicon sequence between the primers. The Scorpion probe unfolds and anneals to the PCR amplicon, allowing fluorescence to take place beginning in the first round of PCR.

Lux

Lux is a variation of real-time PCR that uses a single fluorophore in a primer with a hairpin loop structure. The fluorophore is quenched by the complementary structure of nucleotides in the stem of the hairpin. When the primer is incorporated into double-stranded DNA, thus opening the hairpin, fluorescence is maximal. The advantage of this system is lower production costs with the use of only one fluorophore.

The following concepts are important for understanding the use of real-time PCR in a clinical molecular laboratory. When optimizing real-time PCR, the amplification curve of the fluorescent signal vs the number of PCR cycles should be monitored to determine when optimal conditions have been achieved. The amplification curve should be sigmoidal (S shaped) with three phases: baseline (background signal or lag phase), log-linear (exponential amplification phase), and plateau. For each phase, several characteristics should be assessed. The baseline phase of the curve represents initial cycles of amplification in which accumulation of the specific signal has not yet exceeded the background signal. The fluorescent signal in this phase is from unbound probe or autofluorescing components in the reaction. The log-linear phase of the curve represents exponential amplification of the target and provides useful information about the reaction. The curve can be described by the following equation: $Tn = T_0(E)n$, where Tn is the amount of target sequence at cycle n, T_0 is the initial amount of target sequence at cycle 0, and E is the amplification efficiency of the target sequence. The crossing point represents the number of PCR cycles at which the amplification curve enters the log-linear phase. There is an inverse linear relationship between the crossing-point cycle number and the number of template copies present in a reaction.

The slope of the log-linear phase is a reflection of amplification efficiency, and the efficiency of the reaction can be determined by identifying the crossing points of known standards and plotting a line of linear regression (Fig. 2.3). The efficiency can then be determined using the following equation: $E = 10^{-1/\text{slope}}$, where E is efficiency and slope is the slope of the standard curve. Using this equation, the slope should be between -3 and -4, with -3.3 indicative of efficiency close to or at 2. The inflection point of the amplification curve is the point at which the log-linear amplification curve goes from positive to negative and begins to enter the plateau phase. If there is no inflection point, the curve may represent not amplification of DNA, but rather signal drift. Drift is characterized by gradual increase or decrease in fluorescence without amplification of product.

Plateau is defined as the phase of amplification when critical components of the PCR become rate limiting and amplicon accumulation is minimized or stops. The plateau is also the point at which incremental increase in fluorescent signal stops. As the rate of accumulation slows and enters the plateau phase, the curve levels. Since endpoint measurements often are made in conventional PCR when reaction components are limited, minor sample variations can have a relatively major effect on endpoint product production. The plateau phase can be shortened by decreasing the number of PCR cycles for reduced nonspecific amplicon production.

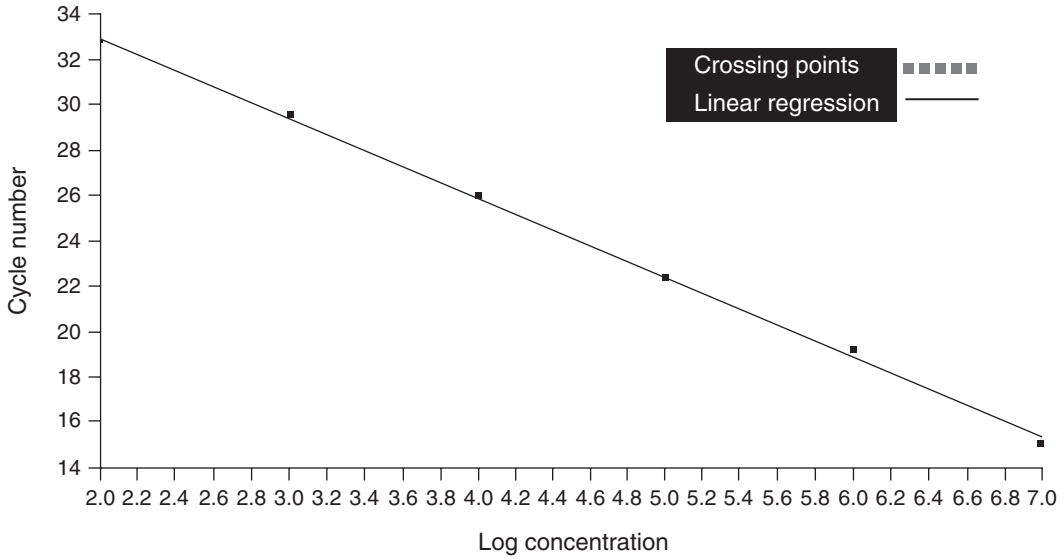

Figure 2.3 Standard curve generated from results of real-time PCR of a tenfold dilution series of a known standard

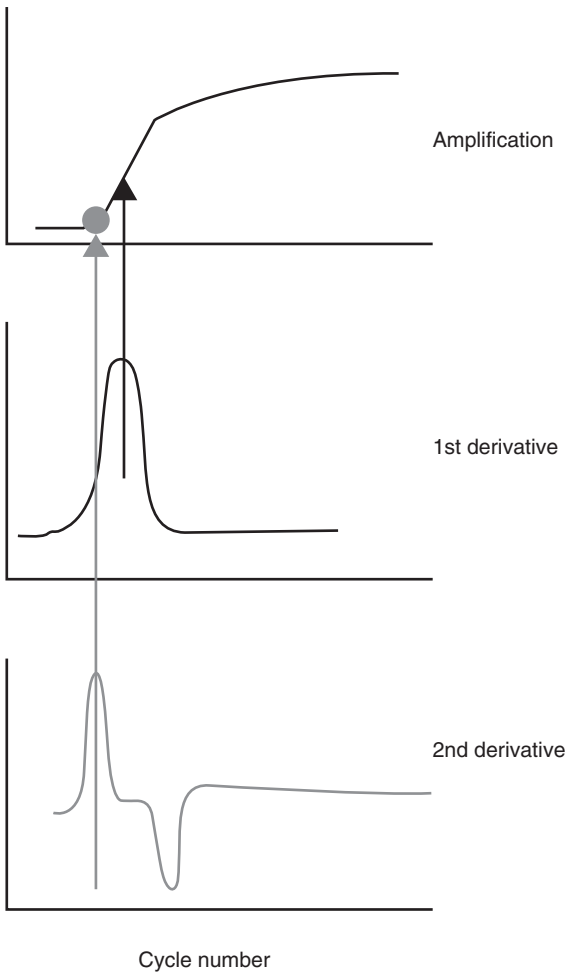

Figure 2.4 Graphical depiction of the second derivative maximum method used to identify the crossing point (*Y* axis is fluorescence value)

Several factors contribute to the plateau phase: PCR product reannealing vs primer annealing, enzyme or dNTPs becoming limiting, and amplicon buildup with resultant reaction inhibition.

In real-time PCR, the log-linear phase of the amplification curve is used for data analysis and provides a more accurate measurement than endpoint analysis. The cycle at which the curve crosses a specified threshold is called the cycle threshold (C_t), or crossing point (C_p). The C_t value can be used for qualitative or quantitative analysis. A qualitative analysis uses the defined C_t as a pass/fail measurement. A quantitative assay uses the C_t of defined standards of known template concentration to generate a standard curve. Then, the C_t values for unknown samples are used to extrapolate the concentration(s) in the unknown samples from the standard curve. Some commercial real-time PCR software allows determination of the C_t by a mathematical analysis of the amplification curve, rather than crossing at a set fluorescent signal threshold. Plotting the second derivative of the amplification curve generates a peak that corresponds to a point near the baseline of the growth curve (see Fig. 2.4). The cycle at which this peak occurs is designated as the C_t or C_p. This analysis method can provide better run-to-run reproducibility than manually setting the C_t using the primary signal.

DNA Methylation and Methylation-Specific PCR

DNA methylation is a mechanism by which the cell regulates gene expression. Methylation is an enzyme-mediated modification that adds a methyl (-CH_3) group at a selected site on

DNA or RNA. In humans, methylation occurs only at cytosine (C) bases adjacent to a guanine (G) base, known as CpG dinucleotides. CpG dinucleotides are prone to spontaneous mutation and have been selectively depleted from the mammalian genome. However, some regions of DNA contain CpG dinucleotides and are referred to as CpG islands. CpG islands are found primarily in the 5′ region of expressed genes, often in association with promoters. When the promoter CpG island is methylated, the corresponding gene is silenced and transcription does not occur. This is one method of silencing imprinted genes, as the methylation pattern and resulting transcription repression is passed on through cell divisions. Aberrant CpG island methylation of tumor-suppressor genes is frequent in cancer and appears to be an important mechanism of neoplastic transformation.

Methylated DNA can be distinguished from unmethylated DNA using sodium bisulfite treatment of DNA, which converts unmethylated C to uracil (U) but leaves methylated C intact [32]. This in vitro treatment can be assessed by one of several methods to distinguish C from U, including restriction endonuclease digestion with methylation-sensitive enzymes, sequencing, or methylation-specific PCR (MSP) [33]. In MSP of bisulfite-treated DNA, primer pairs that specifically identify either methylated or unmethylated DNA are used. The primers are designed to hybridize to regions containing one to three CpG sites concentrated in the 3′ region of the primer to increase amplification specificity, and enough non-CpG cytosines to ensure that unmodified DNA is not amplified. Gel electrophoresis is used to detect the presence or absence of the amplicon in each of the two reactions, indicating the presence of unmethylated or methylated alleles or both. A modification of quantitative MSP combines MSP with real-time PCR to distinguish the high-level CpG methylation in neoplasia from low-level methylation that can occur with aging or in nonneoplastic conditions such as metaplasia [34].

Examples of Applications of Methylation-Specific PCR
1. Analysis of imprinted genes in Prader–Willi and Angelman Syndromes
2. Clonality assessment based on X chromosome inactivation
3. Abnormal methylation in neoplasia

Mass Spectrometry

Mass spectrometry (MS) is a flexible platform for variant and target detection for clinical laboratory applications. In preparation for MS-based detection, a variety of PCR methods such as SNE can be adopted. PCR is performed to amplify the region(s) of interest, then products are enzymatically treated, diluted and/or cleaned to remove unincorporated dNTPs and reduce salts which could interfere with analysis. During MS analysis of the PCR products, samples are ionized, then separated based on their mass-to-charge ratios. The ions are detected after laser desorption with a nitrogen laser. Mass spectra of PCR products are obtained by detecting positive ions of the nucleic acids. Differences in mass due to nucleotide base changes can be detected [21]. Mass spectrometry can detect low levels of sequence variations useful for detecting mosaicism, somatic changes in a normal background and heteroplasmy in mitochondrial DNA.

Example of Application of Mass Spectrometry
1. Cystic fibrosis carrier testing

Multiplex Ligation-Dependent Probe Amplification (MLPA)

Deletions and duplications of single or multiple exons in specific genes are associated with many human diseases (reviewed in ref. 35). Although partial gene deletions or duplications account for less than 10 % of all disease-causing mutations for most hereditary conditions, some disorders can have deletion or duplication rates of 10–30 % or higher [36–44]. Multiplex Ligation-dependent Probe Amplification (MLPA, MRC Holland, Amsterdam, The Netherlands) is a semi-quantitative method used to detect abnormal copy numbers at an exon level resolution and has a high multiplexing capability [45]. The inclusion of MLPA in the clinical molecular laboratory can significantly increase the detection rate of many genetic disorders. Typically, MLPA kits contain a mixture of exon-specific probes targeted to the gene of interest and control probes that hybridize to other genomic areas.

In MLPA, DNA is denatured and incubated overnight with a mixture of probes that consist of two immediately adjacent oligonucleotides per target exon, each containing one of the PCR primer sequences (Fig. 2.5a). After hybridization, probes are ligated and the fragments are amplified by PCR using dye-tagged universal primers. Probes that are not ligated contain only one primer sequence and cannot be amplified to generate a signal. Amplification products that are typically between 130 and 480 bp in length, are separated by size using CE (Fig. 2.5a). The number of probe ligation products directly correlates to the number of target sequences in the sample. Deletions and duplications of the targeted regions are detected when the height ratios of the fluorescent peaks are lower or higher than the normal height ratio range of 0.7–1.4, respectively. An example of a large gene deletion where the deleted probes fall below the lower normal peak height ratio range of 0.7 is shown in Fig. 2.5b.

Several variations on the traditional MLPA procedure have been developed. One example is reverse transcriptase

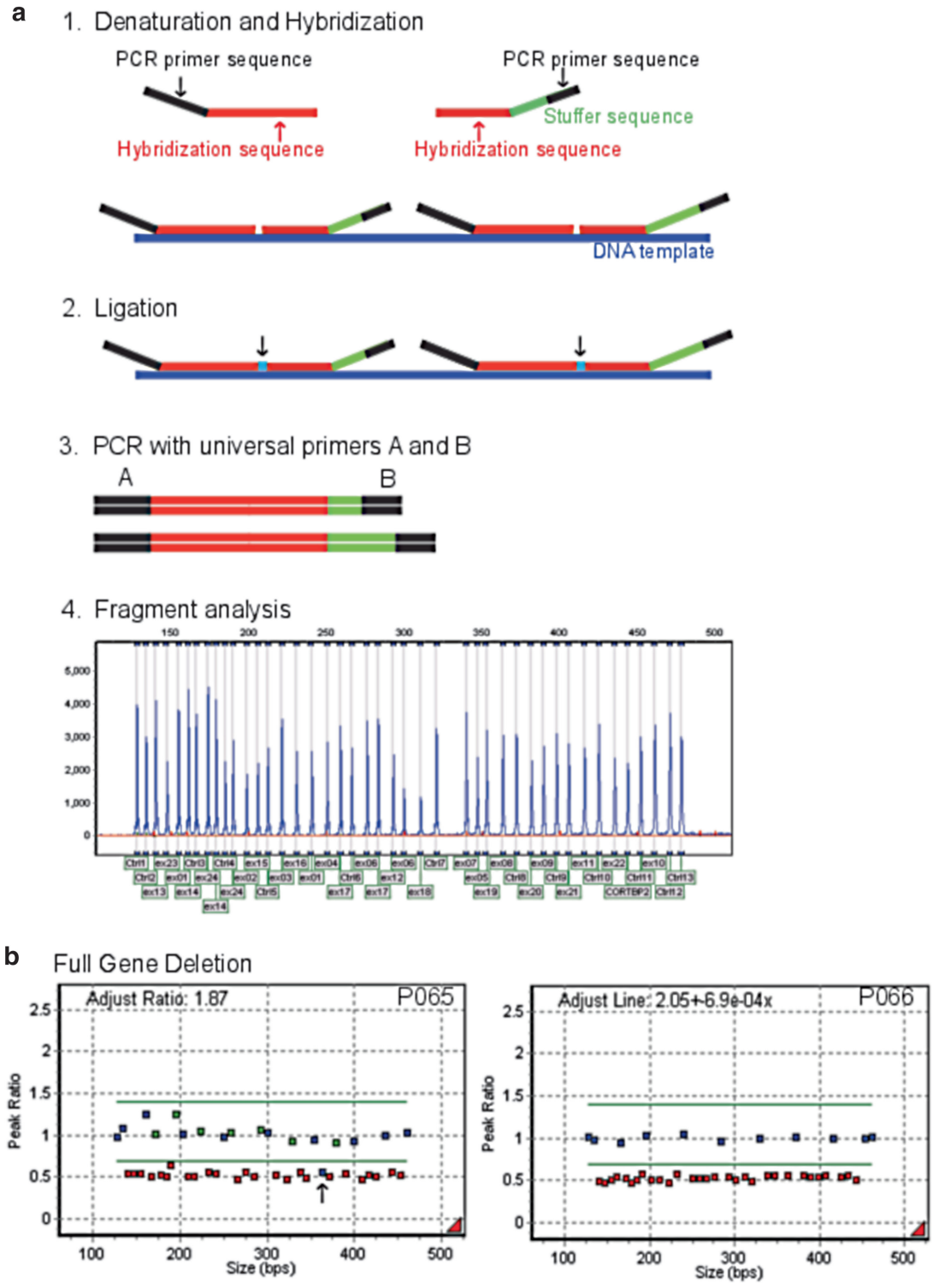

Figure 2.5 Multiplex Ligation-Dependent Probe Amplification (MLPA). In (**a**), the process of MLPA is shown. First, DNA template (*blue*) is denatured and then allowed to hybridize to exon specific probe hybridization sequences (*red*) which are targeted to the gene of interest. Adjacent probes are ligated together, and only ligated probes are amplified using universal primers A and B. Fragment analysis is used to separate the amplified fragments by size whereby the hybridization and stuffer sequences for each MLPA probe set determine the length of the amplified product. In (**b**), MLPA results of a large gene deletion are shown. Two MLPA kits (P065 and P066) were used to test for large *FBN1* gene deletions and duplications that cause Marfan syndrome. An additional control probe (*black arrow*) located 301 Kb upstream from *FBN1* exon 1 on the *DUT* (*deoxyuridine triphosphatase*) gene on chromosome 15q15-q21.1 also was deleted in this sample

MLPA (RT-MLPA) which can be used for mRNA profiling [46]. The only difference between traditional MLPA and RT-MLPA is that RT-MLPA begins with the reverse transcription of mRNA into cDNA before continuing with the typical MLPA reaction; the ligase enzyme cannot ligate probes which are bound to RNA. Methylation-Specific MLPA (MS-MLPA) is another variation that can be used to detect both copy number changes as well as the methylation status of the DNA target [47]. MS-MLPA is useful for imprinting disease testing [48–50] and the analysis of methylation aberrations in tumor samples [51, 52].

PCR Variations for Unknown Sequence Variants

Most of the techniques discussed above are used to screen for sequence variants (both mutations and polymorphisms) based on previous knowledge of the variant i.e., the sequence of the variant is either known or defined by previous scientific reports. In contrast, both research and clinical molecular pathology need methods to identify sequence variants without prior knowledge of their existence i.e., the sequence of the variant is unknown. Sequencing is the ultimate screening technique, but is costly and labor-intensive. The goal of the scanning techniques described below (denaturing gradient gel electrophoresis [DDGE], temperature gradient gel electrophoresis [TGGE], heteroduplex analysis [HA], single-strand conformation polymorphism [SSCP], denaturing high-performance liquid chromatography [DHPLC], protein truncation test [PTT], and variant screening by high-resolution melting [HRM] curve analysis) is to select DNA regions with possible variant sequences for follow up confirmation, thereby reducing costs relative to sequencing. Should an unknown variant be detected, for example by a shift in the mobility of the PCR product on a gel or capillary, the PCR product with altered mobility may be isolated and sequenced.

Denaturing Gradient Gel Electrophoresis and Temperature Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) [53, 54] and temperature gradient gel electrophoresis (TGGE) [55, 56] are similar methods for separating DNA fragments with similar lengths but different sequences and depends upon different mobilities within a linear gradient of increasingly denaturing conditions. In DGGE, the gradient is created with a mixture of urea and formamide, and in TGGE with a combination of water baths at different temperatures and a cooling plate under the gel. Both DGGE and TGGE exploit the markedly decreased mobility of partially melted dsDNA compared to either fully annealed dsDNA or ssDNA. Melting within a dsDNA fragment occurs within stretches of base pairs called melting domains. The point at which a domain begins to denature is referred to as the melting temperature (T_m), whether melting was induced by temperature or denaturing chemicals. In general, GC-rich sequences are more resistant to denaturation because of the three hydrogen bonds holding a GC pair together, as opposed to the two hydrogen bonds in an AT base pair. During electrophoresis, once a dsDNA fragment reaches the point at which the melting domain with the lowest T_m begins to denature, mobility of the fragment through the gel nearly ceases. Fragments that melt early in the gel can therefore be separated from those that melt later. Complete denaturation of the dsDNA can be prevented by adding a GC-rich region to the 5′ end of one of the primers (GC clamp), increasing the sensitivity for detection of sequence variants.

For DGGE and TGGE, the denaturing conditions and the time of electrophoresis are optimized such that non-variant sequences migrate to an intermediate position in the gel by the end of electrophoresis, allowing sequence variants creating either a higher or lower T_m to be identified. The denaturing gradient may be perpendicular or parallel to the electric field. Perpendicular gradient gels covering a broad range of denaturing conditions are loaded with the non-variant sequence in all lanes to find the optimal, narrower denaturing gradient (chemical or temperature) for later use in parallel gradient gels. Parallel gradients are used to assess patient samples but also to optimize the time of electrophoresis by loading the non-variant sequence in different lanes at different times. Double-gradient DGGE adds a sieving gradient, for example, 6–12 % polyacrylamide, colinear with the denaturing gradient in the gel matrix, further improving band resolution.

Both DGGE and TGGE work best with DNA fragments less than 500 bp in length. When GC-clamped fragments are analyzed, the sensitivity of detecting a SNP is close to 99 %. Following electrophoresis, specific bands can be isolated from the gel and sequenced. DNA fragments with a high GC content are not easily analyzed by DGGE, since all fragments are more resistant to melting.

Examples of Applications of DGGE or TGGE

1. *APC* gene mutation analysis for familial adenomatous polyposis [57]
2. *CTFR* gene mutation analysis for cystic fibrosis [58]
3. *TCRγ* gene rearrangements for lymphoma [59]

Heteroduplex Analysis

Heteroduplex formation results when non-variant and variant alleles are coamplified, denatured, and allowed to reanneal in a post-PCR annealing step [60]. Some of the strands pair with the complementary strand from the same allele and

form homoduplexes. However, some strands pair with a strand from the other allele and form heteroduplexes. Because the heteroduplexes have mismatched base pairs between strands, they form a partially open dsDNA sequence that migrates more slowly during electrophoresis than the fully annealed homoduplexes.

Two types of heteroduplex structures can be formed [61]. When the mismatch consists of one or more single-base mutations, small open areas of dsDNA called "bubble-type" heteroduplexes are formed. When the mismatch is formed by insertions or deletions between the two alleles, a pronounced bending of the dsDNA is produced and referred to as a "bulge-type" heteroduplex. Bulge-type heteroduplexes markedly affect the mobility of the dsDNA, whereas bubble-type heteroduplexes may be difficult to detect electrophoretically. Detection of single base-pair bubble-type mismatches can be enhanced in two ways. Electrophoresis can be performed with mutation detection enhancement (MDE) gels, an altered form of polyacrylamide used for improved resolution. Alternatively, the post-PCR introduction of a known sequence with a short deletion to form a bulge-type heteroduplex enhances the separation of sequences with base-pair mismatches in a process known as universal heteroduplex generation.

Examples of Applications of Heteroduplex Analysis
1. *HIV* subtyping
2. *CFTR* gene mutation analysis for cystic fibrosis
3. *NF1* gene mutation analysis for neurofibromatosis type 1

Single-Strand Conformation Polymorphism

The principle of single-strand conformation polymorphism (SSCP) is the differential gel electrophoretic separation of ssDNA that folds into a specific secondary structure based on its sequence [62–64]. For SSCP, the region of interest is amplified and the resulting amplicons are denatured using heat or a denaturation buffer, or both, prior to gel or capillary electrophoresis. Amplicons with different sequences will assume different folding conformations upon denaturation. Conformational differences reflecting sequence changes are detected as differences in electrophoretic mobility of the ssDNA in a nondenaturing polyacrylamide matrix. In general, a non-variant sample generates two bands, one for each of the two strands of the dsDNA product. Bands of variant ssDNA migrate to positions different from those of the non-variant ssDNA. A homozygous variant sample generates two bands, but with different migration patterns from the two non-variant bands. If a heterozygous variant is present, four bands are generated: two with non-variant mobility and two with variant mobility. Mutations also may change the conformation of only one strand but not the other in heterozygous specimens, resulting in three bands. DNA may be purified from the gel, allowing even rare somatic mutations in tumors to be sequenced.

Temperature, ionic environment and pH affect nucleic acid conformation and therefore must be held constant throughout the SSCP electrophoresis. Accurate temperature control during SSCP increases reliability and is an easily modifiable parameter in repeatable, nonisotopic tests that may increase sensitivity. SSCP is adversely affected if unincorporated primers are allowed to bind to the ssDNA during denaturing and cooling prior to electrophoresis, or if nonspecific bands are produced by low-fidelity PCR. In SSCP, electrophoretic mobility patterns of variant alleles can be difficult to distinguish from non-variant alleles. Another disadvantage of SSCP is that multiple test conditions are required for 100 % sensitivity for detection of all sequence variants.

SSCP is most sensitive when the DNA amplicon is less than 200 bp in length. Sensitivity decreases as the fragment length increases. This can be overcome by RE digestion of larger PCR products prior to electrophoresis. When RE digestion is used, the procedure is referred to as restriction endonuclease fingerprinting-single-strand conformation polymorphism (REF-SSCP). Additionally, SSCP is relatively less sensitive for G to C mutation detection; however, addition of glycerol enhances mutation detection in this circumstance.

Variations of SSCP include RNA-SSCP (rSSCP), dideoxy fingerprinting (ddF), bidirectional ddF (bi-ddF), and SSCP detection of virtually all mutations. RNA adopts more conformational structures than does ssDNA, allowing enhanced detection using rSSCP. RNA-SSCP is not widely used because of the relative difficulty in obtaining intact RNA for analysis. Dideoxy fingerprinting involves a dideoxy Sanger single-primer termination reaction (cycle-sequencing reaction; for additional information on the Sanger reaction, see the section on sequencing, above) followed by nondenaturing electrophoresis. A fingerprint bandshift is indicative of sequence changes. In bi-ddF, the dideoxy Sanger termination reaction is performed with two opposing primers in the same well or tube.

The detection of an altered SSCP pattern does not identify the exact sequence variation present in the analyzed DNA. Therefore, positive SSCP results require DNA sequence analysis to confirm and identify the sequence variation.

Examples of Applications of SSCP
1. Screening for mutations in the adenomatous polyposis coli (*APC*) gene
2. Mutation analysis of the *ATP7B* gene for Wilson disease
3. Mutation analysis in *BRCA1* for familial breast cancer
4. Pathogen identification [65]

Denaturing High-Performance Liquid Chromatography

Denaturing high-performance liquid chromatography (DHPLC) is an ion-paired, reversed-phase, liquid chromatography method used to identify variants, including SNPs and small insertions or deletions, by distinguishing between heteroduplex and homoduplex DNA [66]. DHPLC is conceptually similar to heteroduplex analysis (HA; see above). Conventional HA makes use of a gel matrix to separate homoduplex and heteroduplex species in a non-denaturing environment, whereas DHPLC uses partially denaturing conditions in a liquid chromatography column to exaggerate the separation between the two species.

For DHPLC, the gene to be tested is amplified using high-fidelity PCR to prevent the production of PCR artifacts (pseudoalleles) that could produce false-positive results. The optimal amplicon length is between 100 and 500 bp. PCR product purification usually is not necessary, as unincorporated primers, nucleotides, and genomic DNA do not interfere with the analysis. DHPLC requires heteroduplex formation, accomplished by heating and slow cooling of the PCR products. Therefore, for conditions in which only one variant allele type may be present (such as recessive diseases, X-linked conditions in males, or small tumor samples with loss of heterozygosity in all cells), PCR products from non-variant control samples and patient samples are mixed in equal proportions before heating and cooling to produce heteroduplex DNA and distinguish from homozygous non-variant alleles. The addition of non-variant PCR amplicons is not required when using DHPLC to test PCR products from heterozygous individuals, which naturally form heteroduplexes when denatured and slowly cooled.

The duplexes are injected into a DHPLC column, and the DNA binds to the stationary matrix. Binding is aided by triethylammonium acetate. Because the stability of the binding depends on the temperature, the column is optimally held at the T_m of the PCR fragment. The T_m can be calculated using a variety of proprietary or free software programs. The DNA is next eluted from the matrix using acetonitrile and DNA absorbency is measured at 260 nm. The linear gradient of acetonitrile established in the column allows separation of DNA fragments based on size or the presence of heteroduplexes, or both. All DNA fragments impart a characteristic profile when the absorbance is plotted against elution time. The peak of maximum absorbance is the retention time of that DNA sample at a given acetonitrile concentration. Heteroduplexes are less stable and thus have a lower affinity for the column. The concentration of acetonitrile required to separate heteroduplexes from the column is therefore lower, so heteroduplexes elute from the column earlier than homoduplexes.

The column temperature and gradient conditions can be optimized for the separation of any heteroduplex-homoduplex mixture. Some DNA fragments have more than one melting domain and the analysis may be performed at more than one temperature. One advantage of DHPLC is that reinjection of the same sample at different temperatures is possible. Other advantages include high detection rates of variants, rapid separation times per sample, a high degree of automation, and the ability to collect elution fractions and sequence each eluted fragment. Disadvantages of DHPLC include the need for expensive equipment and columns, high-fidelity PCR, and optimization of each reaction required to achieve the highest sensitivity of mutation detection.

Examples of Applications of DHPLC

1. *RET* and *CFTR* mutation detection [67]
2. *BRCA1* and *BRCA2* mutation analysis [68]

Protein Truncation Test

The protein truncation test (PTT) is used to identify mutations that result in premature termination of translation. Although initially developed for Duchenne muscular dystrophy testing, PTT has been applied widely [69], since protein-truncating mutations are associated with multiple types of hereditary cancer syndromes, including cancers of the breast, ovary, and colon.

In PTT, the gene segment of interest is amplified by PCR. The amplicons are used for in vitro transcription and translation in a coupled reaction. The resulting proteins are separated using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The presence of a premature termination codon is demonstrated by the visualization of a lower-molecular-weight protein band than for the non-truncated protein. Relatively large gene fragments (2–4 kb) can be analyzed using PTT.

Examples of Applications of PTT

1. Duchenne muscular dystrophy diagnosis
2. *BRCA1* and *BRCA2* mutation detection for breast cancer [70]
3. *APC* mutations for colorectal cancer [71]

Variant Scanning by High-Resolution Melting Curve Analysis

High-resolution melting curve analysis (described in detail above) can be used for variant scanning of many genes. In this method, a large region of a gene, e.g., an exon or any region that is ≤500 bp, is amplified and then melted in the presence of a non-variant control DNA. A deviant melt indicates a base change within the region and sequencing of that region must be performed in order to determine the clinical

significance of the detected variant. This method is useful for investigating genetic causes for suspected biochemical disorders found during newborn screening.

Examples of Applications of Variant Scanning by High-Resolution Melting Curve Analysis
1. Screening for Medium Chain Acyl-CoA Dehydrogenase (MCAD) deficiency mutations [72]
2. Screening for Ornithine Transcarbamylase (OTC) deficiency mutations

Other Nucleic Acid Amplification Methods

PCR is widely used in the clinical laboratory. The proprietary nature of PCR prompted the development of alternative methods for nucleic acid amplification. Several are described here: ligase chain reaction (LCR); transcription mediated amplification (TMA), strand displacement amplification (SDA), and nucleic acid sequence-based amplification (NASBA).

Ligase Chain Reaction

Ligase chain reaction (LCR) [73, 74] is initiated when a mixture of target DNA, thermostable DNA ligase, four oligonucleotide probes, and NAD^+ or ATP is heated to denature dsDNA (both target and complementary probes) in the reaction mixture. Two pairs of complementary probes are used, and, of necessity, their correct design requires knowledge of the sequence of the DNA target. After denaturation and subsequent reaction cooling, the four probes in the reaction mixture hybridize to their complementary sequences on each target DNA sister strand. The two probes that hybridize to one sister strand and the two probes that bind to the other sister strand are designed such that when hybridized, the 3′ hydroxyl end of the upstream probe is immediately adjacent to the 5′ phosphate end of the downstream probe. Thermostable DNA ligase enzymatically ligates the two bound probes, thus achieving a "doubling" of the mass of target DNA in the reaction. As the temperature cycling proceeds, a theoretical exponential amplification of the mass of target DNA in the original reaction occurs because the resultant ligated amplicons also serve as targets for probe hybridizations and ligations. In practice, amplification is less than exponential, but sufficient to achieve target DNA amplification and assessment by various methods.

Target-independent blunt-end ligation of the probes in the reaction can occur in LCR, which can cause unacceptably high levels of background signal, limiting the sensitivity and specificity of the method. This problem has been solved by use of gap LCR (G-LCR). In G-LCR, the probes are designed such that they cannot be ligated in a target-independent manner because they are not blunt ended. When G-LCR probes hybridize to target DNA, a gap of one or more bases exists between the probes hybridized to the same target strand. T his gap is then biochemically "filled" in the reaction, thus providing a suitable substrate for target-dependent ligation by DNA ligase.

Examples of Applications of LCR
1. *Chlamydia trachomatis* detection
2. *Neisseria gonorrhoeae* detection

Transcription-Mediated Amplification

Transcription-mediated amplification (TMA) uses RNA as the template, two primers, and two enzymes: reverse transcriptase (RT) and RNA polymerase. One primer contains a promoter sequence that binds RNA polymerase. As the amplification process begins, the promoter-containing primer hybridizes to the target RNA at a complementary site. Reverse transcriptase then synthesizes a cDNA copy of the target RNA template by extension of the 3′ end of the promoter-primer. The result is an RNA–DNA duplex. The RNA component is degraded by the RNase H activity inherent in RT. The other primer in the reaction mixture hybridizes to the DNA copy, and a new DNA strand is synthesized from the end of the primer by reverse transcriptase, generating a dsDNA molecule. The other enzyme in the mixture, RNA polymerase, binds the promoter sequence in the DNA template and initiates transcription. Each of the resulting newly synthesized RNA amplicons reenters the TMA cycle, serving as a template for a new round of replication and exponential expansion of the RNA target. Each DNA template can generate 10^2–10^3 copies of RNA amplicon, with the potential for 10^8- to 10^9-fold amplification in less than 1 h. The process is autocatalytic and isothermal. Acridinium ester-labeled DNA probes are added after completion of the reaction to initiate detection and quantitation based on chemiluminescence.

Examples of Applications of TMA
1. *Chlamydia trachomatis* detection [75]
2. *Neisseria gonorrhoeae* detection
3. HCV detection (qualitative) [76]

Strand Displacement Amplification

Strand displacement amplification (SDA) is an isothermal in vitro nucleic acid amplification technique [77]. Hemimodified DNA is polymerized by using three conventional dNTPs and one containing a 5′-[α-thio]triphosphate. The primer(s) is designed with an RE recognition site in the 5′

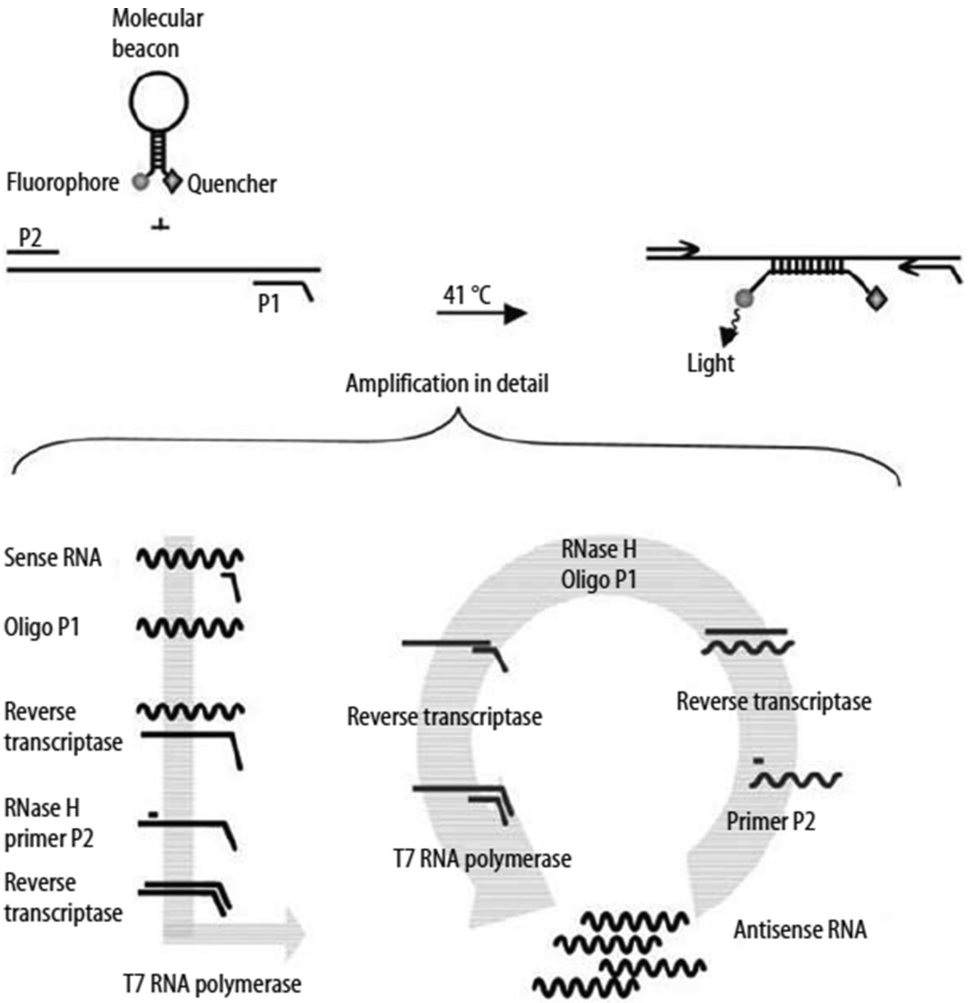

Figure 2.6 Schematic of NASBA Method. Two primers are used: P1 (antisense) and P2 (sense). The P1 overhang is a T7 RNA polymerase recognition sequence. A molecular beacon (with fluorophore and quencher, by definition) serving as probe with reporter molecules coupled to NASBA generates a real-time detection system.

overhang end. The recognition site is specific for an RE that can nick the unmodified DNA strand at a double-stranded hemiphosphorothioate recognition site, that is, when the newly synthesized strand incorporates the 5′-[α-thio]triphosphate nucleotide in the recognition sequence. DNA polymerase lacking 5′ to 3′ exonuclease activity is used to extend the 3′ end at the nick and displace the downstream strand. DNA nicking and polymerization with re-formation of the hemiphosphorothioate recognition site continuously cycle, generating complementary copies of the DNA target. Linear amplification (called target-generation SDA) occurs when a single primer is used. Exponential amplification (exponential SDA) is achieved by using two primers complementary to opposite DNA strands, with both primers containing RE recognition sites in the 5′ overhang end. Strand displacement amplification has been used in a microarray format [78].

Examples of Applications of SDA

1. *Chlamydia trachomatis* detection
2. *Neisseria gonorrhoeae* detection

Nucleic Acid Sequence-Based Amplification

Nucleic acid sequence-based amplification (NASBA) is an isothermal method for amplifying nucleic acids using two sequence-specific primers (P1, antisense, and P2, sense; see Fig. 2.6), and the coordinated activities of three enzymes: avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase [79]. A primer with a T7 RNA polymerase recognition sequence at its 5′ end is used by AMV-RT to transcribe cDNA from the RNA template. The RNA template is destroyed by RNase H. AMV-RT then extends the second primer to synthesize dsDNA. The T7 RNA polymerase binds to the recognition sequence in the dsDNA and synthesizes multiple antisense RNA transcripts, and the cycle is repeated. Generally, amplification is approximately 10^{12}-fold in 1–2 h.

In NASBA, nucleic acids serve as amplification templates only if they are single stranded and contain primer-binding regions. NASBA is performed isothermally at 41 °C, so RNA is preferentially amplified, because at this temperature genomic DNA remains double stranded and does not bind

primers, allowing RNA detection in the presence of genomic DNA without false-positive results. NASBA can be used for specific DNA amplification by introducing a DNA denaturation step before amplification.

Quantitative detection of target nucleic acids is achieved by use of an internal calibrator added during RNA isolation. The calibrator is included at a known concentration, is coamplified during the NASBA process, and is subsequently identified along with the target RNA. Quantification is based on the analysis of signals generated in real time (one color for calibrator and another for target).

Examples of Applications of NASBA
1. HIV quantitation
2. CMV detection

Amplicon Carryover Contamination

Vast numbers of target DNA copies are generated when PCR and other in vitro nucleic acid amplification methods are used. By contrast, signal amplification methods (see below) do not generate vast quantities of amplicon and do not create the potential for amplicon carryover contamination of the laboratory workspace. Amplicons from previous reactions inadvertently introduced into new amplification reactions for the same amplicon are suitable substrates for amplification. Clinical molecular laboratories therefore take precautions to prevent generation of false-positive results from amplicon carryover contamination.

Amplicon contamination and false-positive results are prevented by using physical barriers and chemical and UV light techniques to destroy amplicons or make them unsuitable for amplification. The physical barriers include performance of nucleic acid isolation, PCR setup, thermal cycling, and post-PCR analysis in separate areas of the laboratory (different rooms are ideal). Air flow is controlled such that air pressure is positive, that is, flows out of the room, in the isolation and PCR setup rooms, and is negative in the thermal cycling and post-PCR analysis rooms. Hoods are another way of providing physical separation of the different steps of amplification-based tests. Other physical separation techniques include the use of barrier pipette tips, frequent glove changes, designated laboratory coats for the pre- or post-PCR areas of the laboratory, and PCR tube openers or careful, slow opening of tubes and microtiter plates to prevent aerosolization of contents. Real-time PCR reduces the chances of amplicon contamination since the PCR product is detected and quantified without opening the real-time PCR reaction vessel.

Chemical techniques to prevent amplicon contamination include thorough cleansing with bleach of work areas and instruments before and after use. UV lights frequently are placed in hoods and work areas. UV light creates thymine dimers within amplicons, rendering the amplicons unsuitable as substrates for further amplification. The introduction of isopsoralens in PCR reactions allows DNA cross-linking of amplicons by UV light, also rendering them unsuitable for further amplification. Deoxyuridine may be used in lieu of thymidine in the reaction mixture. Use of deoxyuridine has minimal effect on amplification or product detection, but amplicons with uracil are substrates for uracil-N-glycosylase (UNG). UNG has no effect on DNA that contains only thymidine residues (new patient DNA in subsequent reactions), but digests the uracil-containing amplicons, allowing removal of contaminating amplicons before a new PCR proceeds [80]. So-called UNG sterilization is performed prior to PCR cycling to rid the reaction of any amplicon contaminants that may be present.

Signal Amplification Methods

Branched DNA Method

The branched DNA method (bDNA) [81, 82] begins with the addition of a lysis buffer to a small volume of serum, plasma, or culture supernatant containing cells or virus. The lysis reagent contains detergent to release target nucleic acid, inhibitors to prevent target degradation, and multiple capture extenders (oligonucleotides) that hybridize to specific areas of the target RNA or DNA. In the case of the HIV bDNA test, the capture extenders hybridize to multiple sequences in the viral *pol* gene. A common sequence on the capture extenders interacts with capture probes immobilized on the surface of 96-microwell plates, thereby anchoring the target nucleic acid to the plate.

Multiple target probes are added to each well that hybridize to different, conserved sequences on the target nucleic acid. In the HIV bDNA assay, more than 80 target probes covering a large portion of the 3,000 bp of the *pol* gene are used. The target probes contain key sequences that form the foundation for signal amplification, accomplished via the sequential addition of preamplifier (complementary to a region of the target probes), amplifier (complementary to a region of the preamplifier molecule), and alkaline-phosphatase-modified label probes (complementary to portions of the amplifier molecule).

Preamplifier, amplifier and label probes, as well as the preamplifier region of the binding probes, contain the non-natural nucleotides 5-methyl-2′-deoxyisocytidine ($iso^{Me}C$) and 2′-deoxyisoguanosine (isoG). These isomers of natural

bases can participate in Watson–Crick base pairing with each other but not with cytosine or guanine residues in probes or in natural DNA or RNA sequences. Incorporation of non-natural bases into the synthetic probe molecules increases the specificity of hybridization by decreasing nonspecific probe interactions, and increases the sensitivity of the assay since higher concentrations of probes can be used.

The series of probes results in formation of large hybridization complexes on the target RNA or DNA. For example, if each hybridization step was 100 % efficient in the HIV bDNA assay, each target molecule would be labeled with more than 10,000 alkaline phosphatase molecules. Addition of dioxetane substrate for the alkaline phosphatase results in steady-state chemiluminescent signal. The signal is proportional to the amount of target RNA or DNA present in the sample. The amount of target RNA or DNA in a specimen may be calculated by interpolation from a standard curve generated by signals produced from calibrators that contain known concentrations of the specific viral, bacterial, or cellular RNA or DNA.

Examples of Applications of bDNA method
1. HIV quantitation [83, 84]
2. Hepatitis B virus (HBV) quantitation [85–88]
3. HCV quantitation [89, 90]

Hybrid Capture

Hybrid capture (HC) is a signal amplification system based on antibody binding of RNA–DNA hybrids. The first step of HC is the hybridization of unlabeled RNA probes with denatured target DNA. The resulting RNA–DNA hybrids are captured to the surface of microplate wells by an immobilized antibody that recognizes RNA–DNA hybrids. A second anti-RNA–DNA monoclonal antibody conjugated to alkaline phosphatase is added next. Many secondary antibodies bind to each RNA–DNA hybrid. Dioxetane-based substrate is added, which is cleaved by the alkaline phosphatase, producing a chemiluminescent signal. The signal is measured using a microplate luminometer and is proportional to the amount of target DNA in the specimen [91]. The sensitivity of HC can be increased by using a precipitation buffer and high-speed centrifugation.

Examples of Applications of HC
1. Human papilloma virus detection [92]
2. CMV quantitation [93]
3. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* detection [94]
4. HBV quantitation [95]

Fluorescence In Situ Hybridization

In fluorescence in situ hybridization (FISH), fluorescently tagged DNA or RNA probes are used to identify genomic sequences of interest [96, 97]. The major advantages of FISH are the utility for testing of FFPE tissue sections, correlation of probe hybridization with tissue morphology, and the increased resolution provided by FISH for identification of specific abnormalities when partnered with conventional cytogenetics. The number and location of the fluorescent signal(s) can identify chromosomal abnormalities including gene amplification, gene deletion or structural rearrangements such as translocations.

FISH is performed by sequential steps of denaturation, hybridization, and washing. Slides are prepared in the cytogenetic or histology laboratory. Fluorescently labeled probe(s) are then applied to the slide along with a nuclear counterstain and reagents to enhance denaturation and reduce background. The slides are sealed and incubated (usually overnight) in a humid environment at high temperature. These conditions denature the probe and patient DNA, allowing hybridization to occur between the probe and its complementary DNA sequence in the specimen without binding to nonspecific sites. Excess nonspecifically bound probe is washed away, and the pattern of fluorescence is observed with fluorescence microscopy. The fluorescent signal(s) can be enhanced by the use of a digital imaging system and computer software.

The specificity of FISH is largely based on probe selection. Ideally, the probe is complementary to the gene of interest; however, if the disease gene is unknown, satellite probes that identify a chromosomal region linked to the disease may be used. Labeled bacterial or yeast artificial chromosomes (BAC or YAC, respectively) are typically used as FISH probes, but short oligonucleotides also can be used with signal amplification techniques. Probes that identify individual whole chromosomes or chromosomal arms are often called "painting probes" due to the colorful patterns generated. Other probes that hybridize to a specific gene can be used for the detection of deletions or duplications and are called single-copy gene probes. Probes that hybridize to the α-satellite regions near centromeres are used in clinical cytogenetics to identify and count individual chromosomes. Probes that hybridize to the subtelomeric portions of chromosomes are used to identify cryptic telomeric abnormalities such as translocations.

Dual-color FISH (dFISH) employs two probes with different fluorescence wavelengths to identify structural chromosomal rearrangements. Each probe generates a characteristic color by itself (split signal) and a third color when the two probes are juxtaposed (fusion signal). A fusion

signal indicating chromosomal rearrangement is used to identify disease-causing mutations that predictably involve only two partner genes, for example, *BCR* and *ABL*. However, when a particular gene with multiple potential translocation partners is tested, it is more efficient to have both probes bind to the 5′ and 3′ ends of the particular gene such that the non-rearranged allele shows the fusion signal and the rearranged allele shows two split signals (so-called "break apart" probes). This is the technique used for *MLL* gene detection, which is rearranged with over 30 different partner genes in various types of leukemia.

Spectral karyotyping (SKY) and multiplex FISH (M-FISH) are modifications of conventional FISH that utilize multiple fluorochromes, specialized optics, and image analysis that can simultaneously identify all chromosomes [98]. Array-based comparative genomic hybridization (aCGH, described below), also called "copy number karyotyping," is a variation of FISH that detects relative gains or losses of the genome [99]. Array-based CGH is used to compare the ratios of patient specimen DNA, labeled with one fluorochrome, to that of control non-variant DNA, labeled with a different fluorochrome when hybridized to control chromosomes. Colorimetric probes are used in chromogenic in situ hybridization (CISH), which has the advantage that the signal does not fade with time or require fluorescence microscopy for analysis.

Examples of Applications of FISH

1. Detection of *BCR–ABL* in chronic myelogenous leukemia
2. Detection of *HER2* gene amplification for breast cancer diagnosis and prognosis
3. Detection of *MYCN* amplification in neuroblastoma [100]

DNA Arrays

In DNA arrays, the word "array" is jargon for an orderly distribution of molecules on solid supports ranging from nylon membranes to printed circuit boards to glass slides to silicon surfaces. There are macroscopic arrays, for example, reverse line blots on nylon membranes, and microarrays, for example, DNA chips. Synonyms for microarrays include gene chip, DNA chip, genome chip, biochip, gene array, DNA array, and DNA microarray. Forms of arrays currently utilized in the clinical molecular laboratory include (but are not limited to): aCGH, SNP arrays, and expression arrays.

Comparative Genomic Hybridization Arrays

First described by Kallioniemi in 1992, array-based comparative genomic hybridization (aCGH) is a technique developed for genome-wide characterization of copy number changes [101]. In its original form, differentially labeled test and reference genomic DNA were co-hybridized to normal metaphase chromosomes. The relative fluorescence ratio along the length of the chromosomes represented the relative DNA copy number. Array CGH (aCGH) is an advancement of CGH technology that uses clones, most commonly BACs or oligonucleotides, arrayed along a physical surface as the hybridization target, rather than metaphase chromosomes. The arrayed surfaces are commonly called microarrays, and the surface may hold millions of unique sequences as the hybridization targets. Much like the original CGH process, test and reference genomic DNAs are labeled with different fluorescent dyes, denatured, and then hybridized with the arrayed clones on the surface of the chip. The relative fluorescence ratios of the reference and test signals at the arrayed DNA elements provide a locus by locus measure of DNA copy number variation. In aCGH, the targets can be nonoverlapping clones evenly spaced across the genome, overlapping clones densely packed only in areas of interest, or a combination of the two approaches.

The uses of aCGH include gene discovery, cancer classification, and diagnostic assays. Analysis of DNA from mantle cell lymphomas by aCGH revealed homozygous deletions at 2q13 in 3 of the 29 samples leading to the discovery of the tumor suppressor gene BIM [102]. Since aCGH has a much higher resolution than conventional karyotyping, its use has led to the recognition of new microdeletion and microduplication syndromes such as that at 17q21.31 [103, 104]. Such discoveries are indicative of the role of aCGH in gene discovery and the development diagnostic tests.

The patterns of copy number variations detected by aCGH have led to diagnostically significant subgroup classifications of cancers. One example is that of differentiating diffuse large B-cell lymphoma types into subtypes based on copy number patterns [105]. The ABC subtype is genomically characterized by gain of 3q, 18q, and 19q and loss of 6q and 9p21, whereas the GCB subtype is genomically characterized by gain of 1q, 2p, 7q, and 12q. The subtypes have disparate biology and clinical outcomes.

As the number of unique targets on an array increases, so does the resolution. In 2005, a targeted array capable of detecting exonic level copy number variations was used to screen 162 exons of 5 genes [106]. In 2008, three groups showed the utility of CGH to detect exonic level copy number variation in the dystrophin gene [107–109]. The ability of custom-designed aCGH to detect exon copy number changes provides new opportunities for research and diagnosis.

Within the past decade, aCGH has emerged as a diagnostic tool in the clinical laboratory. Occasionally referred to as molecular karyotyping, the International Standard Cytogenomic Array Consortium recommended in 2010 genome-wide aCGH as the first-tier clinical diagnostic test for individuals with multiple congenital anomalies and

developmental delays [110]. As a result, targeted arrays are increasingly being used in the clinical laboratory for both cancer and congenital conditions.

SNP Arrays

The basic principle of the SNP array is the same as the DNA microarray. DNA is fluorescently labeled, hybridized with arrayed DNA probes, imaged, and interpreted. The difference is that for SNP arrays, the target probes are designed around single-nucleotide polymorphisms (SNPs) distributed throughout the genome. Signal intensity following hybridization is dependent on both the amount of target DNA as well as the affinity to the target. Thus, interrogating the SNP targets provides both copy number and SNP allelic information. The resolution of SNP arrays is limited by the distribution of SNPs in the human genome. The highest density SNP chips now have over four million SNP targets (Illumina Human Omnio5-Quad Bead Chip). Applications of SNP arrays include genome-wide association studies (GWAS), determination of heterozygosity, and molecular karyotyping of clinical samples.

Expression Arrays

Gene expression arrays are a powerful tool for comparing complex RNA populations. RNA is isolated from a test sample, converted to fluorescently labeled cDNA, and hybridized to a microarray. The fluorescence at each DNA element is then quantified, providing a measure of the abundance of the RNA molecules in the test sample. Detection limits are as low as 1–10 copies of mRNA per cell depending on the technology and cell type. As a means of candidate gene discovery, genome-wide expression microarrays with large densities are appropriate for analysis of differential patterns of gene expression between normal and diseased tissues while high-density, targeted expression arrays can identify novel exon skipping events and alternative splicing events [111, 112]. Expression arrays have gained acceptance in the clinical laboratory as a means of defining clinical subtypes of cancer [113]. Such arrays have demonstrated gene expression patterns which have been correlated with clinical outcomes and therapy response. Currently, five gene expression arrays are offered clinically for prognostic testing for breast cancer: Mammaprint, MapQuant Dx, OncotypeDx, PAM50 Breast Cancer Intrinsic Subtype Classifier, and Theros Breast Cancer Index.

Massively Parallel Sequencing

Massively parallel sequencing, also known as next-generation sequencing (NGS), has been widely adopted by the basic research community [114, 115]. Until recently, the use of NGS for clinical diagnostics was hindered by the relatively high cost of instruments, assay development and sequencing, as well as the bioinformatics infrastructure and expertise required for analysis. Since its introduction, advances in NGS platforms and sequencing chemistries have improved workflow, base qualities, read lengths, and total bases of sequence per experiment, all while reducing the cost per base sequenced [116]. Advances in enrichment and capture technologies have enabled the development of cost effective gene panels or exome sequencing for inherited disorders. NGS assays developed or under development include gene panels for autism, cardiomyopathies, X-linked mental retardation, the RAS pathway, among others, as well as the introduction of exome and genome sequencing in laboratories accredited by CMS under the Clinical Laboratory Improvement Act of 1988 (CLIA) [117–119]. The use of NGS for inherited disorders will supplant sequencing strategies such as Sanger sequencing or array-based genotyping when it is cost-effective and diagnostically viable. At this time bioinformatic analysis is still cumbersome and Sanger verification of putative variants of significance remains commonplace. However, improvements and standardization of bioinformatic analysis should ultimately reduce confirmatory Sanger verification and permit a high degree of certainty and reproducibility when detecting and reporting variants. (See Chap. 62 for a full discussion of NGS.)

Next Generation Sequencing Methods

The principles of NGS sequencing methodologies include sequencing by synthesis and sequencing by ligation. All platforms require the incorporation of adapters to target DNA and subsequent PCR-based generation of clonally amplified and clustered DNA. The two strategies primarily used for colony/cluster generation include in-solution emulsion PCR (emPCR) and surface bridge amplification. Once the DNA has been clonally enriched, sequencing chemistries differ depending upon platform [114, 115]. Illumina sequencing takes advantage of reversible dye-labeled terminators. Each dye terminator representing A, T, C, or G is sequentially introduced to the DNA molecule being synthesized and the surface of the flow cell is imaged. Once the complementary base has been incorporated into the extending DNA molecule, no further extension can occur until the dye is cleaved, resulting in an extendable deoxyribonucleotide triphosphate (dNTP), such that a single base is added during each cycle [114]. Technology from Roche relies upon the sequential introduction of the different dNTP bases, except the method of detection is pyrosequencing. The incorporation of a given base to the DNA being synthesized results in the release of a pyrophosphate and subsequent luciferase luminescence [120]. Since the reaction uses dNTP, single base extension does not always result, as homopolymer extension may occur during a single cycle. Technology from Life Technologies does not rely on DNA polymerase exten-

sion, rather sequencing by ligation is used. The process involves the use of multiple primers offset by one base at the 3′ end of the adapter. Fluorescently labeled interrogation probes representing two adjacent nucleotides are ligated to the primer. Once the ligation reaction has occurred and imaging is completed, the dye is cleaved off the interrogation probe, and a subsequent ligation can be performed [121]. Other methods of polymerase-mediated NGS include measuring the charge associated with nucleotide extension and the visual detection of dye-labeled nucleotide extension.

High throughput NGS platforms have made sequencing of an individual human genome in a reasonable timeframe a reality. Genome sequencing for clinical purposes is not commonplace at this time and is pursued mostly for gene discovery. Paradoxically genome sequencing is the simplest application of NGS and the most bioinformatically intensive. Because of the ease of set up, lack of need for specific assay design, and the costs associated with target-specific enrichment, genome sequencing will ultimately be performed for clinical purposes in the future [122]. However, a number of bioinformatic, regulatory, and ethical issues will need to be addressed before genome sequencing for clinical purposes will be commonplace.

Exome sequencing is a method that holds significant promise for clinical molecular laboratories. Exome sequencing can be used for gene discovery and in certain instances can be used for gene panel or pathway analysis. Because the human exome is roughly 1.5 % of the human genome, bioinformatic analysis is not as daunting as genome analysis, yielding roughly 30,000–50,000 variants [123]. Exomes from different patients can be differentially labeled using unique short sequence tags, multiplexed and sequenced in the same sequencing run, which reduces sequencing costs.

While high-throughput NGS platforms can be used to sequence genomes or multiple exomes in a single test, these instruments may not always be optimal for NGS of smaller gene panels. The use of barcodes or indexes enables pooling of multiple samples to leverage sequencing throughput, but the time required for sequencing can take days. Recently, a number of platforms have been introduced with lower throughput and faster turnaround times that facilitate the sequencing of smaller gene panels or exomes. This advancement has permitted the introduction of time-sensitive NGS-based clinical tests that are less expensive than Sanger sequencing tests, with a similar turnaround time.

Bioinformatics and Data Analysis

A typical NGS test may yield from 1 to 600 gigabytes (Gb) of sequence data. Conventional analysis pipelines and software programs developed for Sanger sequencing are totally inadequate for analysis of the large volumes of NGS data. A number of commercial and freeware software applications are available that align and perform variant calling. The steps of data analysis involve assessing the quality of the sequencing data, aligning the sequence fastq files to a reference sequence, determining the differences between the reference sequence and the patient's sequence, and collecting the data [124]. These sequence variants are bioinformatically processed to ensure the quality and accuracy of the alignment. Once the data have been processed and filtered, annotation of the variants is performed, and subsequent post-annotation filtering allows for the selection of a subset of variants of interest [116, 123, 125]. The process from raw sequence data to final variant reporting is bioinformatically intensive.

Development of bioinformatics and statistical workflows for NGS are not universal; different purposes for NGS testing require different metrics for alignment and data reporting. Public and private databases are used for annotation purposes to help determine what variants should be examined further. It is important that internal databases be developed that track areas of reproducible misalignments, miscalls, and variant frequencies to supplement public, nonclinical, and sometimes inaccurate, research-derived databases. Because of the vast amount of data associated with one test, it is not feasible to manually verify data for each variant. Tests may yield from a few to over three million variants. A paradigm shift is required for clinical laboratorians and clinicians who are accustomed to manually analyzing all variants that are reported. While graphical user interfaces are available to examine variants (should one wish to do so) clinical laboratorians and clinicians will have to rely upon the tools and pipelines developed by bioinformaticians. It is extremely important that all stakeholders are involved in pipeline development and validation to ensure that everyone involved with test interpretation understands the process and that the data reporting is accurate and useful.

Clinical Applications of Next-Generation Sequencing

NGS technologies permit sequencing of entire individual patient genomes. As the cost of NGS decreases, genome sequencing is likely to become increasingly popular as a strategy for identifying the genetic basis of diseases as it offers the potential of finding mutations in the noncoding regions, such as regulatory elements or noncoding RNAs, and the ability to detect large gene rearrangements [126, 127]. However, at present, the expense of NGS reagents and the high demand in bioinformatics makes the targeting of a subset of genome more appealing at the present time. The subset of a genome often focuses on a set of genes at different chromosomal loci which are important in a specific disease [119]. Two examples are provided by an X-Linked intellectual disability 92-gene NGS panel and a cardiomyopathy panel of 46 genes [117, 118]. This use of NGS is the

logical extension of Sanger sequencing of a panel of selected genes associated with a particular phenotype.

Exome sequencing uses a targeted selection approach to capture the majority of the protein-coding exons of genes, microRNAs and noncoding RNAs in the human genome, followed by NGS sequencing. Because the exome represents only approximately 1.5 % of the genome and contains 85 % of known disease causing mutations, exome sequencing is considered the most cost-effective method for identifying the causative mutation in rare diseases [126]. However, with a massive amount of variants detected by exome sequencing, data analysis is complicated, especially when putative causative mutations such as missense mutations are identified. Family history and pedigree segregation studies are useful to determine the causality of the mutations [128].

Conclusion

The molecular methods used in the clinical laboratory will continue to evolve and develop as researchers and instrument manufacturers develop new methods that improve on the sensitivity, specificity, cost and speed of current methods. Drivers for adoption of new technologies in the clinical laboratory include but are not limited to reduced technologist hands-on time, reduced cost, shortened turnaround time, interfacing of results to information systems to reduce human transcription errors, and improvements in the detection of analytes with clinical significance. Molecular pathology will continue to be at the leading edge of methods development, resulting in a constant stream of new test and method validations with all the required steps new tests require from a regulatory compliance perspective. While this is challenging for the molecular pathology laboratory leadership and staff, this innovation also is one of the exciting aspects of molecular pathology practice.

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