Chapter 2

Molecular Pathology Methods

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Introduction

Molecular pathology is based on the principles, techniques, and tools of molecular biology as they are applied to diagnostic medicine in the clinical laboratory. These tools were developed in the research setting and perfected throughout the second half of the 20th century, long before the Human Genome Project 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 diagnostics. 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, molecular diagnostics continues to grow rapidly as in vitro diagnostic companies develop new kits for the marketplace and as the insights into disease gained by the progress of the Human Genome Project develop into laboratory tests.

Molecular pathology is a natural extension of anatomic and clinical pathology. As molecular research identifies the most fundamental causes and markers of disease, clinical testing is moving to the nucleus and its genetic material. Underlying mutations responsible for genetic diseases, including cancers, are being discovered and used in molecular diagnostic 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, and deoxyribonucleic acid (DNA) sequencing. 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.

Obviously, gene products, that is, proteins and polypeptides, are molecules and could technically be classified within "molecular pathology." This chapter focuses on nucleic acid diagnostics, including investigation of deoxyribonucleic acid (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 its most nascent stages and is in extremely limited practice in molecular pathology today.

Basic Science Discoveries: The Foundation

Molecular pathology techniques are rooted in fundamental molecular biology discoveries of the 1940s to 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 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 nucleic acid hybridization, or 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 diagnostic 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, usually proteinase K, or selectively precipitated. Protein digestion is performed at about 56°C and will permanently denature many proteins but does not affect nucleic acids. This process is followed by selective 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. A variation on this theme that combines extraction and purification is the selective adsorption of nucleic acids to silica columns under chaotropic salt conditions. The isolated nucleic acid is then resuspended in a dilute salt buffer, for example, 10 mM Tris/1 or 0.1 mM EDTA pH 7.6 to 8.0 (TE buffer).

The initial lysis step is modified according to the specimen. If the specimen is fresh or frozen solid tissue, it 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 is also 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 a centrifugation step to remove erythrocytes prior to recovery of nucleic acids from leukocytes because of the inhibition of PCR by hemoglobin. This cell fractionation step generally is not performed in automated nucleic acid extraction instruments.

Organic (Phenol) Extraction

Nucleic acids have a strong 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 selective for 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, uses 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 the nucleic acid. Centrifugation allows the 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 isothyocyanate (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. The nucleic acid is purified by a series of washing steps including reducing agents such as sodium azide to further remove contaminants and inhibit remaining enzymes. The nucleic acid is 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.

RNA Versus DNA Isolation

DNA is the repository of genetic information, which is then transcribed into RNA. RNA forms ribosomes, transfer RNA (tRNA), and messenger RNA (mRNA) for protein translation, and is used for other special functions in the regulation of gene expression that have only recently been discovered. DNA is a hardy molecule present at stable cellular levels, with such well-known exceptions as the amplification and deletion of genes in tumor cells. By contrast, the level of RNA corresponding to a gene can

fluctuate dramatically within a very short time in response to changes in the cell's microenvironment and functional needs. This fluctuation results from changes in both the rate of transcription and degradation of an RNA.

DNA is relatively easy to isolate and store because deoxyribonucleases (DNases) are easily denatured by heating or inhibited by sequestration of divalent cations. 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 leads to a problem 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. The rate of degradation varies among RNA species, leading to further analytical complexity.

RNA analysis depends on successful RNA isolation and preservation. The overall techniques are similar to those described above for nucleic acid 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 quantitation of RNA 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 gloves with frequent glove changes. Addition of GITC or beta-mercaptoethanol to the RNA isolation reagents will inhibit or denature 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]

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 more accurate quantities of nucleic acid, so assessment of the yield and concentration of purified nucleic acids is useful. This 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 of EtBr correlates with the number of base pairs of DNA in which the EtBr is intercalated, which is a result of both the size 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 quantification of post-PCR DNA prior to sequencing, since the UV spectrophotometer is usually kept in the pre-PCR area (see below for PCR, sequencing, and amplicon carryover contamination). More important, the image of the EtBr-stained sample DNA can be used to assess DNA quality. High-quality, substantially intact DNA forms a single band close to the well serving as the origin of electrophoresis. In contrast, DNA degradation is apparent as a smear of EtBr-stained DNA extending downward from the well. Ethidium bromide is mutagenic and produces light background staining and is therefore being replaced by other intercalating dyes such as SYBR Green.

Electrophoresis

Electrophoresis uses an electric field 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. Conformation can be modified with denaturing conditions prior to or during electrophoresis. Nucleic acids are usually electrophoresed at a slightly alkaline pH to ionize all phosphate groups in the backbone of the molecule.

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 loaded into the wells, usually in association with a dye to track the progress of electrophoresis, and glycerol to make the samples sink to the bottom of 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 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 1000 bp. Single base pair resolution can be achieved, allowing polyacrylamide gels to be used for sequencing under denaturing conditions (see below). 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, then pouring the solution into a horizontal casting tray. Multiple gel combs can be used to form rows of wells. After cooling and polymerization, the gel is loaded in a horizontal electrophoresis apparatus and covered with buffer in a single chamber. Wells are loaded and electrophoresis performed as described above. Agarose gels have a large pore size. Agarose gels with a concentration of 1% are used to separate DNA fragments of 1 to 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. Capillary electrophoresis 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 0.5 hour or less, and if eight or 16 capillaries are run simultaneously, the process reduces the time from standard electrophoresis, which requires 3 to 4 hours. This is a significant time saving in the clinical laboratory for applications such as 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.

In CE, electrophoretic separation takes place in a capillary tube ranging in length from 25 to 100 cm and approximately 50 to 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.

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 the injection of the sample. Sample injection in CE is frequently performed by electrokinetic injection. In electrokinetic injection, the capillary and electrode are moved into the sample well. The sample enters the capillary when a voltage of 2 to 5 kV is applied

for approximately 5 to 15 seconds. 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 during migration through the capillary and are detected through the window at the far end of the capillary.

In the clinical molecular laboratory, DNA sequencing and DNA fragment sizing 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 postreaction 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 postreaction 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 florescence, 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 found in 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 to 8 bp long). Bacteria use REs 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. Fractionated using agarose gel electrophoresis. Restriction endonuclease digestion is commonly used as a component of clinical molecular tests.

Specific Methods

DNA Sequencing

Prior to the performance of a sequencing reaction, the DNA template to be sequenced must be properly purified and quantitated. A sequence-specific complementary primer must be synthesized for use in the sequencing reaction. Sequencing primers must also be high-performance liquid chromatography (HPLC) purified or, at the very least, desalted. Samples must be purified following the sequencing reaction to rid the sample of salts, excess dyes, and excess primers that would compete for injection into the capillary. There are numerous protocols and commercial kits available for the postreaction purification. Ethanol precipitation is the least expensive purification method, but numerous gel-filtration and spin-column methods also can be used.

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 Human Genome Project.

The Sanger sequencing reaction uses a single DNA primer and DNA polymerase with linear amplification rather than the exponential amplification of PCR (see below). Components essential to the Sanger sequencing reaction include (1) an electrophoresis technique capable of clearly distinguishing single nucleotide length differences in DNA strands dozens or hundreds of nucleotides in length, (2) sequence-specific complementary primers, with one primer used in the forward reaction and the other used in the reverse reaction for each DNA template strand, and (3) the addition of small proportions of dideoxynucleoside triphosphates (ddNTPs) in addition to the conventional deoxyribonucleoside triphosphates (dNTPs) used in the polymerase reaction. Dideoxynucleotides differ from deoxynucleotides by having a hydrogen atom attached to the 3′ carbon rather than an OH group, which is present on the deoxynucleotide. Because the ddNTPs lack a 3′-hydroxyl group, elongation of the newly polymerized DNA chain cannot occur once a ddNTP has been incorporated (arabinonucleosides can also 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, for each template four reactions were performed with the addition of a single inhibitor to each, either 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. These 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.

Today, sequencing is very similar except that radioactive labeling has largely been replaced by fluorescent labeling. Two major categories of fluorescent labeling exist. In dye-primer labeling, the sequencing primer is labeled, and the sequencing reaction requires 4 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. This enables the entire sequencing reaction to be performed in a single tube. Dye-terminator labeling has therefore taken precedence in sequencing. Dye-primer labeling is frequently 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. Therefore, the introduction of CE was a welcome change that facilitated the incorporation of sequencing and fragment analysis into the clinical laboratory.[8] The sequencing reaction products must be purified before injection into the CE unit to remove excess salts, dyes, and unincorporated primers that would compete for injection into the capillary. Purification is accomplished by ethanol precipitation or a chaotropic salt–silica column technique. After the postreaction purification step, samples are then 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 florescence, 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. Congenital adrenal hyperplasia mutation analysis
2. Retinoblastoma mutation analysis
3. *BRCA1* mutation analysis
4. HIV genotyping to monitor drug resistance and sensitivity
5. 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 still used today, though it is being replaced by amplification methods. The implementation of Southern blot 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 relatively labor-intensive, time-consuming clinical laboratory method.[9] High-quality DNA is isolated from a patient specimen, subjected to RE digestion, and then fractionated by gel electrophoresis. “Blotting” is the transfer of fractionated DNA from the gel to a solid support such as a nylon membrane. The DNA is then hybridized to a small piece of complementary DNA labeled in a variety of ways and called a probe. This detection step allows the gene of interest to stand out from the vast background of DNA present in the sample. If the pattern of banding visualized on the membrane is different from the normal pattern, this may be indicative of a mutation.

Because no amplification of target DNA occurs, Southern blot analysis requires a large mass of DNA. The DNA must also be intact and of high molecular weight. Therefore, electrophoresis of the isolated DNA 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 molecular pathology 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 in the gel to the membrane may be accomplished by manual capillary transfer, automated vacuum transfer, or electrotransfer. DNA in the gel must first be “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. 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.

The blot is immersed in prehybridization buffer to prepare the DNA on the blot for hybridization with a probe. Prehybridization buffers contain 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. 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 (degree of mismatch); pH; temperature; and ionic strength of the buffer used.

DNA probes are labeled before use in hybridization assays to permit visualization of probe-target binding (in reverse hybridization assays, described below, unlabeled probes are immobilized and the target is labeled during the amplification step that precedes hybridization). Such labeling may be accomplished isotopically or nonisotopically. High-specific-activity DNA probes may be generated by in vitro biochemical reactions that synthesize new stretches of DNA from dNTPs, using the probe as a template. One of these 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 4 dNTPs in the DNA probe, is sufficient to label the entire probe for detection. The probe is then used in vast molar excess relative to target DNA in nucleic acid hybridization to drive the hybridization reaction as quickly as possible.

After hybridization, the blot is washed with buffers containing sodium chloride and detergent to remove excess probe and reduce background. Sodium chloride concentration and stringency are inversely related: the lower the sodium chloride concentration, the more stringent the wash. 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 lower-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. Hybridization with biotinylated probes is followed 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 is then applied 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, 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 pathology laboratory.

Examples of Applications of Southern Blotting

1. B- and T-cell antigen receptor gene rearrangement for leukemia and lymphoma[10]
2. Fragile X syndrome diagnosis
3. Myotonic dystrophy diagnosis

Polymerase Chain Reaction

In the mid-1980s in California, Mullis and coworkers developed a method, the polymerase chain reaction (PCR), to amplify exponentially target sequences of DNA.[11] As the name suggests, the method is a DNA polymerase–mediated chain reaction of nucleic acid amplification. Arguably, it is the single most important "invention" that has led to development of a new discipline in clinical laboratory medicine, that is, molecular pathology. Both PCR and Southern blotting are techniques used to investigate specific genomic targets. However, PCR is orders of magnitude more sensitive and much faster, permitting turnaround time of 24 hours or less. PCR lends itself to much higher test volumes than Southern blotting, a crucial point in its acceptance 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 has spread so quickly in clinical molecular laboratories.

In PCR, a unique sequence of the target nucleic acid of interest is chosen for amplification, for example, oncogene, invading pathogen DNA, genetic mutation. The inherent specificity of the ensuing reaction is provided by two short oligonucleotides, called PCR primers (see Figure 2-1). These short oligonucleotides 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

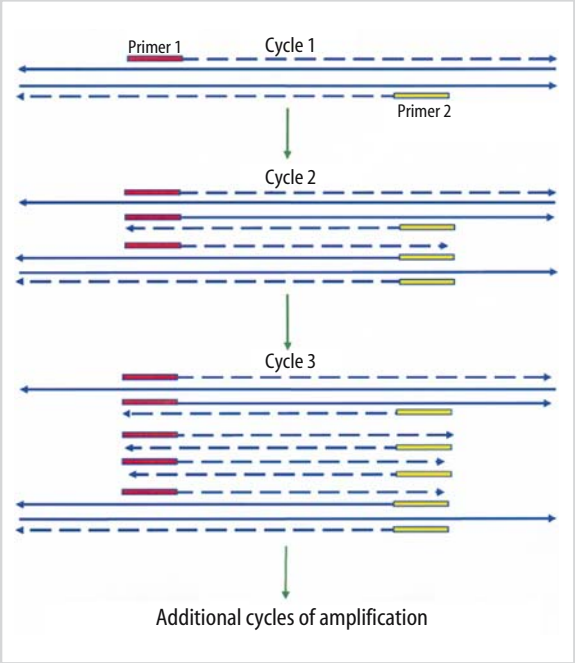

Figure 2-1. The polymerase chain reaction. (Reprinted with permission from Tsongalis GJ, Coleman WB. *Molecular Diagnostics—A Training and Study Guide*. Washington, DC: AACC Press, 2002.)

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°C to 98°C to denature the target DNA. After 10 to 60 seconds at this temperature, the temperature is reduced to about 50°C to 70°C, depending on the specific protocol, and held there for usually 10 to 60 seconds. This facilitates hybridization (annealing) between the now-denatured target and the PCR primers, and is thus called the annealing step. This hybridization event is favored over target 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 primers for DNA polymerase to bind and synthesize a new strand of DNA, using the target DNA as a template. Subsequent to the initial discovery of PCR, the opportunity for automating the cyclical nature of PCR was realized by using DNA polymerase from hot-spring living bacteria, *Thermophilus 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°C to 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 containing the reagents needed for PCR and cycle between the temperatures needed for the different steps of the PCR.[12] A single PCR tube contains template DNA (<1 ng to 1 µg), *Taq* DNA polymerase, two PCR primers (~15 to 30 nucleotides long), all four dNTPs, Mg^{2+}, and buffer to maintain an elevated pH (~8.4) optimal for *Taq*.

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 are then available to 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 identical 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. 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. These are so-called unit-length 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, often by electrophoresis with EtBr visualization.

Several factors affect the specificity and sensitivity of PCR. 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 will denature the primer from its target DNA. The temperature at which the primer melts from the target DNA varies directly with the length of the primer and the guanosine-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 T_m 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 also the use of touchdown PCR in multiplex PCR, below).

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) or glycerol, but the success 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* 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 must be verified. Simple agarose gel electrophoresis coupled with EtBr 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 therefore to be the correct target, but also is a DNA fragment that has high or perfect homology with a known probe. 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 detection of CMV. 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, as opposed to blot hybridization or sequencing, may be the assay endpoint.

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, for example, 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 washing. Subsequent 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 for clinical PCR-based detection kits for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, HCV (qualitative) and HIV.[13] (For a complete list of FDA-approved tests, go to the Resources section at http://www.amp.org/, the home page for the Association for Molecular Pathology). Subsequent generations of automated PCR instrumentation are now available that completely automate the amplification and detection process.[14] The field is moving toward real-time PCR detection (described below).

Another aspect of PCR that is attractive for the clincal molecular laboratory is the ability to use relatively crude extractions of 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.[15] Conventional PCR-based tests may be completed with turnaround times of as short as 2 to 4 hours, while real-time PCR can be completed in 30 minutes, making this technique attractive for stat 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. The term "polymorphism" is not synonymous with the term "mutation" in that

mutations are variations found less frequently in a population or occur as nongermline changes usually 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 an 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 unaffected individual. 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.

Examples of Applications of PCR-RFLP Analysis

1. Detection of sickle-cell hemoglobin (HbS)
2. Detection of the *Mnl* I restriction enzyme polymorphism created by the Factor V_{Leiden} mutation[16]

Restriction-Site Generating PCR

Some mutations create or abolish RE recognition sites and can easily be detected by PCR-RFLP. Unfortunately, most polymorphisms or mutations do not alter an 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.[17,18] The primer contains a base mismatch to the template DNA adjacent to the variable base of the mutation that creates an 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 mutation, and together they create a novel restriction site within either the mutant or wild-type amplicon. The presence or absence of the RE recognition site is determined from the pattern of digested 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 *KRAS* codon 12 mutations in colon cancer[19]
2. Identification of mutations in the *CTFR* gene in cystic fibrosis
3. Identification of mutations in the *ATM* gene in ataxia-telangectasis

Multiplex PCR

Multiplex PCR (M-PCR) is a demanding 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 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 prevent the generation of primer-dimers 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[19]
4. Identification of different bacteria in a respiratory infection[20]
5. Amplification of multide microsatellite loci for bone marrow engraftment analysis

Nested PCR

In 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.[21] 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

section below on amplicon 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.[22] 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 normal or mutant) 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 normal or mutant) or both reactions (heterozygous). Detection of the amplicon is by either gel electrophoresis or real-time PCR technology (see below). A disadvantage of AS-PCR is that unsuspected nucleotide polymorphisms or mutations 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 mutant allele in the presence of 40 copies of the normal allele. AS-PCR can be combined with M-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 alpha-1 antitrypsin deficiency mutations
3. Defection of 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.[23] 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 mutant sequence and another to the normal 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 normal or mutant 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 criticism of ASOH is the potentially ambiguous discrimination of a positive 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.[24,25] The target sequence is initially amplified using PCR and then denatured. A pair of allele-specific oligonucleotide (ASO) probes (one specific for the wild-type allele and the other specific for the mutant 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 ASO and the reporter probe. If the ASO is not a perfect match to the amplicon, the 3′ base does not anneal with the amplicon, 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.

Dynamic Allele-Specific Hybridization

Dynamic allele-specific hybridization (DASH) is a temperature-dependent, real-time variation of ASOH.[26] Dynamic allele-specific hybridization begins with amplification of polymorphic DNA regions by PCR in which one primer is biotinylated. The biotinylated product strand is bound to a streptavidin-coated microtiter plate well, and the nonbiotinylated strand is washed away. An oligonucleotide probe complementary to one allele is annealed to a bound PCR product strand, forming a DNA duplex that interacts with a double-strand-specific intercalation dye. On excitation, the dye emits fluorescence proportional to the amount of double-stranded DNA (probe-target duplex) present. The duplex is heated through a temperature range while the fluorescent signal is continually monitored. A rapid fall in fluorescent signal indicates denaturation of the DNA duplex. DNA duplexes with nonhomologous regions denature at a lower temperature than completely homologous DNA. The melting-temperature profile distinguishes homozygosity for either allele alone or a heterozygous mixture of the two.

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 David Baltimore and Howard Temin,[27] 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). Because cDNA is not subject to RNase degradation, it is far more stable than the corresponding RNA. 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, making RT-PCR more efficient. With the introduction of techniques to successfully isolate and protect RNA from ubiquitous RNases, and to synthesize cDNA by reverse transcription and the discovery of PCR, RNA analysis is now virtually as rapid and sensitive as PCR-based DNA investigation. RT-PCR is a high-volume test method for the clinical molecular laboratory due to its use in the diagnosis and quantitation of RNA virus infections, 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 PCR

Real-time 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.[28–30] 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 are dramatically increasing test menus for oncology, infectious diseases, and genetics in clinical molecular laboratories because of the wide range of readily available amplification primers and detection schemes, rapid turnaround time, the potential to eliminate the time and costs associated with electrophoresis, and the reduction in PCR contamination risk.

Real-time PCR is different from conventional PCR in several ways.

- Amplicon generation, temperature profiles, and melt curves are monitored in real time, reducing the time required for post-PCR analysis. In most cases, there is no need for postamplification processing of the PCR products. This eliminates the need for gel electrophoresis and, because the reaction tubes remain closed after PCR starts, there is decreased risk of amplicon carryover contamination within the laboratory.
- 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. This adds a check for specificity 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 EtBr, are nonsequence-specific dyes that increase in fluorescence when bound to double-stranded DNA. Intercalating dyes are often 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 since fluorescence is directly proportional to the amount of specific amplicon produced and reduces the background contributed by primer-dimers or nonspecific PCR prod-

ucts. Intercalating dye fluorescence represents all double-stranded DNA, including primer-dimers and other non-specific product 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

TaqMan 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 (~60°C). Ideally, the TaqMan probe binding site is located near one primer and the size of the amplicon is no longer than 200 to 300 bases. One negative aspect of this format 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 longer probe with a 5′ reporter dye and 3′ quencher dye. The probe forms a hairpin loop structure when not bound to target DNA, thereby juxtaposing the 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 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 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 ~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 available for the next round of amplification as well as endpoint melting-curve analysis.

Uniprimer (Amplifluor, Sunrise)

Like molecular beacon probes, the uniprimer system uses a hairpin structure in the oligonucleotide to quench fluorescence. The 3′ region of the fluorogenic oligonucleotide is identical to a nonbinding region at the 5′ end of the reverse PCR primer. This allows the fluorogenic oligonucleotide 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 oligonucleotide can be used in any PCR reaction (universal fluorogenic primer).

Scorpion

Scorpion also uses a hairpin structure in the oligonucleotide to quench fluorescence. The fluorogenic oligonucleotide is part of the reverse primer, and the nucleotides in the hairpin are complementary to the PCR amplicon sequence between the primers. The Scorpion primer 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 loop. When the primer is incorporated into double-stranded DNA, thus opening the hairpin loop, fluorescence is maximal. The

advantage of this system is lower production costs with the use of only one fluorophore.

Real-Time PCR Method

The following concepts are important for understanding the use of real-time PCR in a clinical diagnostic laboratory. When optimizing real-time PCR, the growth curve of the fluorescent signal versus the number of PCR cycles should be monitored to determine when optimal conditions have been achieved (Figure 2-2). The growth 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 (see Figure 2-1) and provides useful information about the reaction. The curve can be described by the following equation: $T_n = T_0(E)^n$, where T_n 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 growth 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 (see Figure 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 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 growth when critical components 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 are often made in conventional PCR when reaction components are limited, minor sample variations can have a relatively major effect on endpoint product. Real-time PCR focuses on the rate of amplicon accumulation (log-linear phase and cycle threshold or crossing point), not on endpoint signal. The plateau phase can be shortened by decreasing the number of cycles for a product of greater purity. Several factors contribute to the plateau phase: PCR product reannealing versus primer annealing, enzyme or dNTPs becoming limiting, and amplicon buildup with resultant reaction inhibition.

Real-time PCR utilizes the log-linear phase of the amplification curve for data analysis. This method provides a more accurate measurement than endpoint analysis. The cycle at which the curve crosses a specified threshold is called the cycle threshold (Ct), or crossing point (Cp). The Ct value can be used for qualitative or quantitative analysis. A qualitative analysis uses the defined Ct as a pass/fail measurement. A quantitative assay uses the Ct of defined standards of known template concentration to generate a standard curve. Then the Ct values for unknown samples are used to extrapolate the concentration(s) in the unknown samples from the standard curve. Some real-time instrumentation software allows determination of the Ct by a mathematical analysis of the amplification curve, rather than crossing at a set fluorescent signal threshold. Plotting the second derivative of the growth curve generates a peak that corresponds to a point near the baseline of

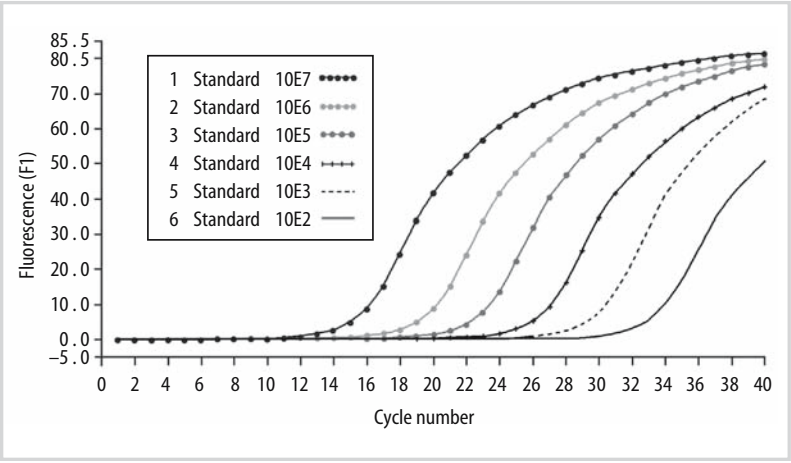

Figure 2-2. Real-time PCR curves for a 10-fold dilution series of a known standard. Data provided by Roche Molecular Diagnostics. (Used by permission. Data © 2003 Roche Diagnostics Corporation, all rights reserved.)

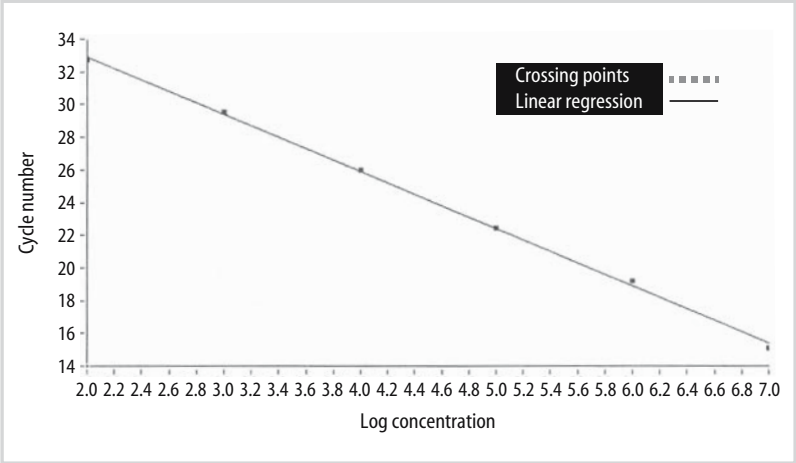

Figure 2-3. Standard curve generated by real-time PCR of a 10-fold dilution series of a known standard.

the growth curve (see Figure 2-4). The cycle at which this peak occurs is designated as the Ct or Cp. This analysis method can provide better run-to-run reproducibility than manually setting the Ct 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 followed by a guanosine (G), known as CpG dinucleotides. The CpG dinucleotides are prone to spontaneous mutations and have been selectively depleted from the mammalian genome. However, some regions of DNA have retained CpG dinucleotides and are referred to as CpG islands. The 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 transcription repression is passed on through cell division. Aberrant CpG island methylation of tumor-suppressor genes is frequent in cancer and appears to be an important mechanism of neoplastic transformation.

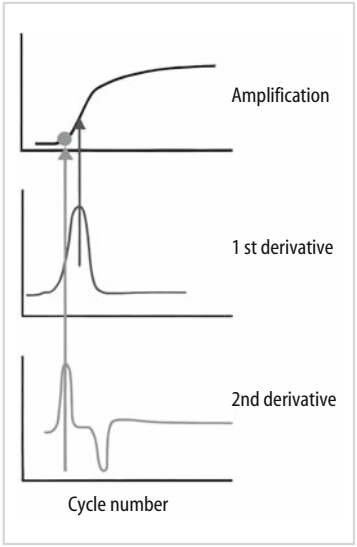

Figure 2-4. Graphical depiction of the second derivative maximum method used to identify the crossing point (Y axis is fluorescence value).

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.[31] This in vitro treatment can then be followed by one of several methods to distinguish C from U, including restriction endonuclease digestion with methylation-sensitive enzymes, sequencing, or methylation-specific PCR (MSP).[32] MSP of bisulfite-treated DNA uses primer pairs that specifically identify either methylated or unmethylated DNA. The primers are designed to hybridize to regions containing one to three CpG sites concentrated in the 3′ region of the primer to increase the specificity of amplification, 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 novel modification is the use of quantitative MSP, which 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.[33]

Examples of Applications of Methylation-Specific PCR

1. Analysis of imprinted genes
2. Clonality assessment based on X chromosome inactivation
3. Abnormal methylation in neoplasia

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 the experimental conditions. By contrast, there is an evolving interest in both research and clinical molecular pathology to identify sequence variants by scanning 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], and temperature gradient gel electrophoresis [TGGE], heteroduplex analysis [HA], single-strand conformation polymorphism [SSCP], denaturing high-performance liquid chromatography [DHPLC], and protein truncation test [PTT]) is to identify specimens with possible variant sequences, 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 is isolated and sequenced. Melting-temperature analysis in real-time PCR also can be used to identify unknown sequence variants.

Denaturing Gradient Gel Electrophoresis and Temperature Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE)[34,35] and temperature gradient gel electrophoresis (TGGE)[36,37] are similar methods for separating DNA fragments with similar lengths but with different sequences according to their mobilities under a linear gradient of increasingly denaturing conditions. The gradient is created in DGGE with a mixture of urea and formamide, and in TGGE with a combination of water baths and a cooling plate under the gel. Both DGGE and TGGE take advantage of 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 G and C together, as opposed to the two hydrogen bonds between A and T. 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.

In DGGE and TGGE, the denaturing conditions and the time of electrophoresis should be optimized such that normal sequences migrate to an intermediate position in the gel by the end of electrophoresis. This allows 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 normal sequence in all lanes to find the optimal, narrower denaturing gradient (chemical or temperature) for later use in parallel gradient gels. Parallel gradients are then used to run samples but also to optimize the time of electrophoresis by loading the normal sequence to different lanes at different times. Double-gradient DGGE adds a sieving gradient, for example, 6% to 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 harder to melt.

Examples of Applications of DGGE or TGGE

1. *APC* gene mutation analysis in familial adenomatous polyposis[38]
2. *CTFR* gene mutation analysis in cystic fibrosis[39]
3. *TCRG* gene rearrangements in lymphoma[40]

Heteroduplex Analysis

Heteroduplex formation results when wild-type and mutant alleles are coamplified, denatured, and allowed to reanneal in a post-PCR annealing step, usually heating and cooling.[41] 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 in electrophoresis than the fully annealed homoduplexes.

Two types of heteroduplex structures can be formed.[42] 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 in polyacrylamide gels. Detection of single base-pair mismatches can be enhanced in two ways. Electrophoresis can be performed with mutation detection enhancement (MDE) gels, an altered form of polyacrylamide that enhances separation of heteroduplexes. 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 (UHG).

Examples of Applications of Heteroduplex Analysis

1. *HIV* subtyping
2. *CFTR* gene mutation analysis in cystic fibrosis
3. *NF1* gene mutation analysis in neurofibromatosis type 1

Single-Strand Conformation Polymorphism

The principle of single-strand conformation polymorphism (SSCP) is the differential gel separation of ssDNA that folds into a specific secondary structure based on its sequence.[43–45] 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 wild-type sample generates two bands, one for each of the two strands of the dsDNA product. Bands of mutant ssDNA migrate to positions different from those of the wild-type ssDNA. A homozygous mutant sample generates two bands, but with different migration patterns from the two wild-type bands. If a heterozygous mutant is present, four bands are generated: two with wild-type mobility and two with mutant mobility. Three also can be observed bands in heterozygous specimens if the mutation changes the conformation of only one strand but not the other. The bands with altered mobility can be isolated from wild-type bands in the gel, allowing even rare somatic mutations in tumors to be sequenced.

Temperature, ionic environment, and pH affect the conformation and therefore must be held constant throughout the SSCP run. Accurate temperature control during SSCP increases reliability and is an easily modifiable parameter in repeatable, nonisotopic experiments 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 wild type. Another disadvantage is that multiple experimental 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 fragment length increases. This can be overcome by multiplexing differently sized fragments onto a single gel lane and by restriction enzyme digestion prior to electrophoresis. When restriction endonucleases are used, the procedure is referred to as restriction endonuclease fingerprinting–single-strand conformation polymorphism (REF-SSCP). Additionally, SSCP is relatively less sensitive for detecting G to C mutations. The 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 (DOVAM-SSCP). RNA is more stable and adopts more conformational structures than does ssDNA, allowing enhanced detection using rSSCP. RNA-SSCP is not widely used because of the relative difficulty in producing 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. Bidirectional ddF is an advancement of ddF whereby the dideoxy Sanger termination reaction is performed with two opposing primers in the same tube. SSCP detection of virtually all mutations is a recently described modification in which SSCP is performed under different conditions with different buffers and gel matrices that result in overall increased sensitivity for mutation identification.

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 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 in Wilson disease
3. Mutation analysis in *BRCA1* (in familial breast cancer)
4. Pathogen identification[46]

Denaturing High-Performance Liquid Chromatography

Denaturing high-performance liquid chromatography (DHPLC) is an ion-paired, reversed-phase, liquid chromatography method used to identify mutations, including SNPs and small insertions or deletions, through its ability to separate heteroduplex DNA from homoduplex DNA[47]. DHPLC is conceptually similar to heteroduplex analysis (HA; see above). Conventional HA makes use of a gel matrix to separate homo- and heteroduplex species in a nondenaturing environment, whereas DHPLC uses partially

denaturing conditions in a liquid chromatography column to exaggerate the separation between the two species.

The gene to be studied is first amplified using PCR. High-fidelity PCR is used to prevent the production of PCR artifacts (pseudoalleles) that could produce false-positive results. Optimal amplicon length is between 100 and 500 bp. Purifying the PCR product is usually 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. 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 normal and patient samples are mixed in equal proportions before heating and cooling to produce heteroduplex DNA and distinguish from homozygous wild-type alleles. The addition of normal 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 (TEAA). Because the stability of the binding depends on the temperature, the column is optimally held at the melting temperature of the PCR fragment. The melting temperature can be calculated using a variety of proprietary or free software programs. The DNA is next eluted using acetonitrile, an organic solution that facilitates the subsequent separation of the DNA from the column matrix, and DNA absorbancy is measured at 260 µm. 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 carried out 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 mutations, 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[48]
2. *BRCA1* and *BRCA2* mutation analysis[49]

Protein Truncation Test

The protein truncation test (PTT) is used to identify mutations that result in premature termination of protein translation. PTT detects mutations at the protein rather than the genomic DNA or RNA levels. Initially developed for Duchenne muscular dystrophy (DMD) testing, PTT now is used more widely,[50] since protein-truncating mutations are associated with multiple types of hereditary cancer syndromes, including breast, ovarian, and colon.

For 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 indicated by the visualization of a lower-molecular-weight protein band than for the wild-type 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[51]
3. *APC* mutations in colorectal cancer[52]

Other Forms of In Vitro Nucleic Acid Amplification

PCR is widely used in the clinical laboratory. The proprietary nature of PCR prompted other in vitro diagnostic companies to develop alternative methods of in vitro 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)[53,54] 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 demands a priori knowledge of the sequence of the DNA target. After denaturation and subsequent reaction cooling, the four probes present 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. In practice, amplification is less than exponential, but sufficient to achieve target DNA identification by various detection methods.

There is a tendency for target-independent blunt-end ligation of the probes in the reaction to occur in LCR, which can cause unacceptably high levels of background signal, limiting the assay's sensitivity and specificity. 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. This gap is then biochemically "filled" in vitro, thus providing a suitable substrate for DNA ligase, which then performs target-dependent ligation.

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 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 (Figure 2-5). 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 activity of the enzyme RNase H inherent in reverse transcriptase. The other primer in the reaction mixture hybridizes to the DNA

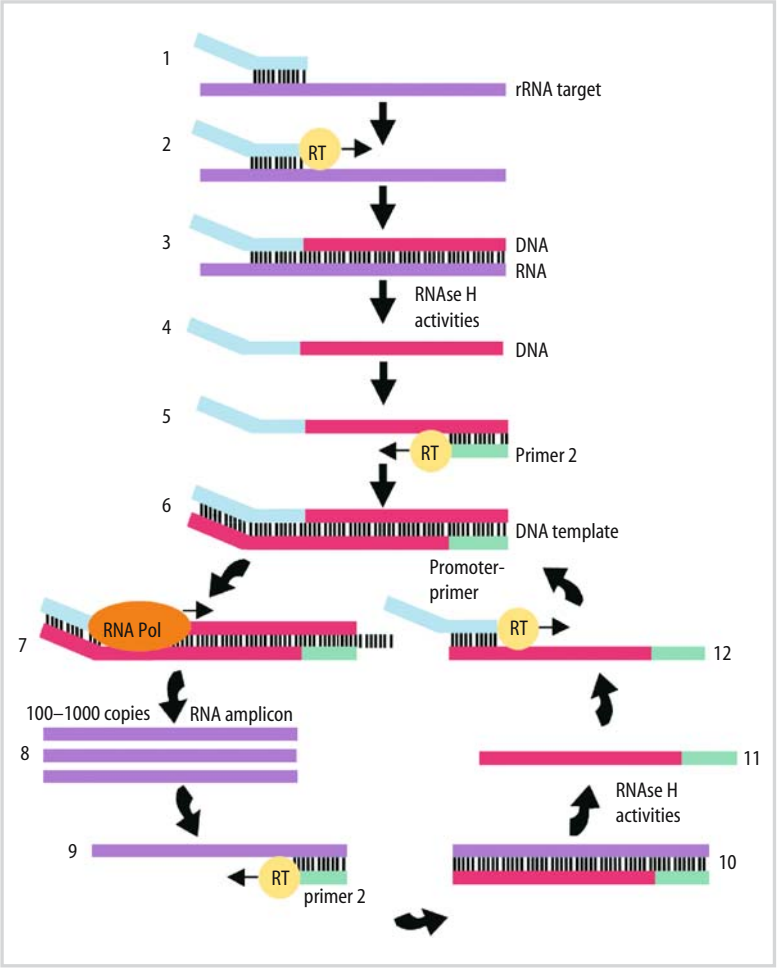

Figure 2-5. Transcription-mediated amplification cycle (TMA):
Step 1. Promoter-primer binds to rRNA target.
Step 2. Reverse transcriptase (RT) creates DNA copy of rRNA target.
Step 3. RNA:DNA duplex.
Step 4. RNAse H activities of RT degrades the rRNA.
Step 5. Primer 2 binds to the DNA and RT creates a new DNA copy.
Step 6. Double-stranded DNA template with a promoter sequence.
Step 7. RNA polymerase (RNA Pol) initiates transcription of RNA from DNA template.
Step 8. 100 to 1000 copies of RNA amplicon are produced.
Step 9. Primer 2 binds to each RNA amplicon and RT creates a DNA copy.
Step 10. RNA:DNA duplex.
Step 11. RNAse H activities of RT degrades the rRNA.
Step 12. Promoter-primer binds to the newly synthesized DNA. RT creates a double-stranded DNA and the autocatalytic cycle repeats, resulting in a billion-fold amplification.
(Reprinted with permission from Gen-Probe.)

copy, and a new DNA strand is synthesized from the end of the primer by RT, 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 to 10^3 copies of RNA amplicon, with the potential for 10^8- to 10^9-fold amplification in less than 1 hour. The process is autocatalytic and isothermal. Acridinium ester–labeled DNA probes are added on completion of the reaction to initiate detection and quantitation based on chemiluminescence.

Examples of Applications of TMA

1. *Chlamydia trachomatis* detection[55]
2. *Neisseria gonorrhoeae* detection
3. HCV detection (qualitative)[56]

Strand Displacement Amplification

Strand displacement amplification (SDA) is an isothermal in vitro nucleic acid amplification technique.[57] Hemimodified DNA is polymerized by using three conventional dNTPs and one containing a 5′-[alpha-thio]triphosphate. The primer(s) is designed with an RE recognition site in the 5′ 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′-[alpha-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. 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,[58] which may become a clinically useful method to combine amplification of low copy number targets with the multiple features of microarray in a format that can be automated.

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 Figure 2-6), and the coordinated activities of three

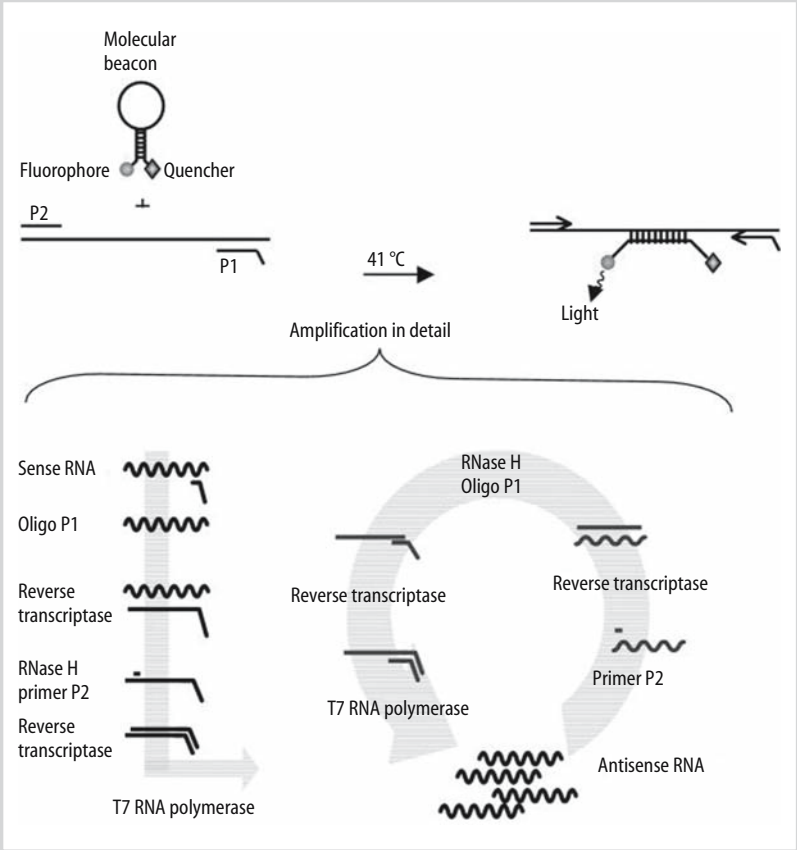

Figure 2-6. Schematic of NASBA reaction. Two primers are used: P1 (antisense) and P2 (sense). The P1 overhang is a promoter sequence for T7 RNA polymerase. A molecular beacon (with fluorophore and quencher, by definition) serving as probe with reporter molecules coupled to NASBA generates a real-time detection system.

enzymes, avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase.[59] 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 uses the second primer to synthesize dsDNA. The T7 RNA polymerase uses the dsDNA to synthesize multiple antisense RNA transcripts, and the cycle is repeated. Generally, amplification is approximately 10^{12}-fold in 1 to 2 hours.

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. It is therefore possible to detect RNA in a genomic DNA background without getting false-positive results. Specific DNA amplification using NASBA may be done by introducing a denaturation step before amplification.

Quantitative detection of target nucleic acids is achieved by use of an internal calibrator added at 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. Quantitation 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 techniques are used. By contrast, signal amplification methods (see below) do not generate vast quantities of amplicon and so 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 must 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 ultraviolet techniques to destroy amplicons or make them unsuitable for amplification. The physical barriers include large-scale separation of nucleic acid isolation, PCR setup, thermal cycling, and post-PCR analysis in separate areas of the laboratory (different rooms). 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 PCR steps. Small-scale physical separation techniques include the use of barrier pipette tips, frequent glove changes, designated lab coats that do not leave the pre- or post-PCR areas of the laboratory, and PCR tube openers or careful, slow opening of tubes to prevent aerosolization of contents. Real-time PCR reduces the chances of amplicon contamination since the PCR product can be detected and quantified without opening the real-time PCR reaction vessel after PCR.

Chemical techniques include thorough cleansing with bleach of work areas and instruments before and after use. Ultraviolet lights are frequently placed in hoods and work areas. Ultraviolet 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 amplicons before PCR proceeds.[62] So-called UNG sterilization may therefore be performed prior to PCR to rid the reaction of any amplicon contaminants that may be present.

Signal Amplification Methods

Branched DNA Method

The branched DNA method (bDNA)[63,64] is carried out in microwells and 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 assay, the capture extenders hybridize to multiple sequences in the *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 that hybridize to different, conserved sequences on the target RNA or DNA. In the HIV bDNA assay, more than 80 target probes covering a large portion of the 3000 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 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 chemiluminescence. The luminescent 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. A schematic of this technology is shown in Figure 2-7.

Examples of Applications of bDNA method

1. HIV quantitation[65,66]
2. Hepatitis B virus (HBV) quantitation[67–70]
3. HCV quantitation[71,72]

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 then is added. 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.[73] 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
2. CMV quantitation[74]
3. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* detection[75]
4. HBV quantitation[76]
5. HSV detection[77]

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) uses fluorescently tagged DNA or RNA probes to identify genomic

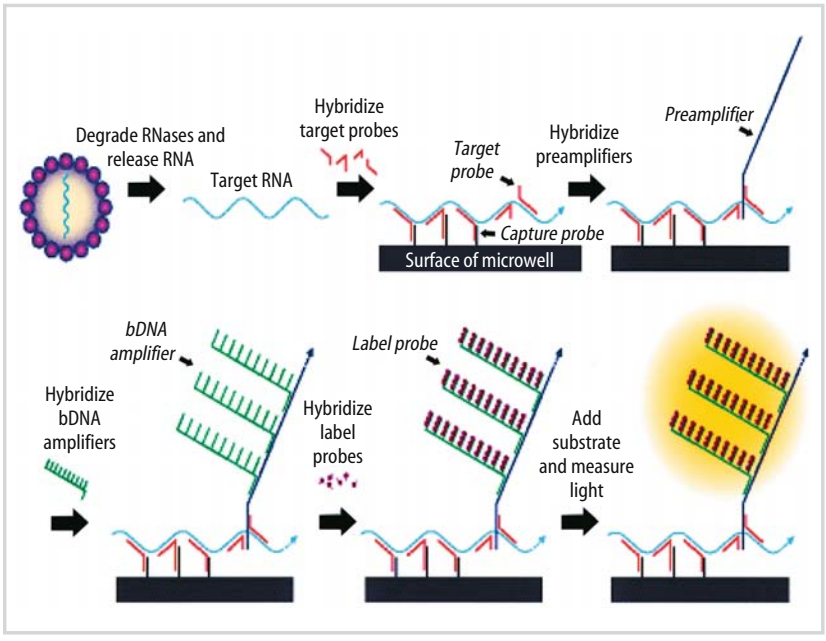

Figure 2-7. Diagram of branched DNA (bDNA) method. (Reprinted with permission of Bayer Healthcare.)

sequences of interest.[78,79] The major advantages of FISH are the ability to utilize FFPE tissue sections, allowing 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 similar to Southern blot in that sequential steps of denaturation, hybridization, and washing are involved. Slides are prepared in the cytogenetic or histology laboratory. 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 without binding to nonspecific sites. Excess nonspecifically bound probe is washed away, and the pattern of fluorescence is read by 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 the selection of the probe. 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 they generate. 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 alpha-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 normal allele shows the fusion signal and the rearranged allele shows two split signals. This is the technique used for the *MLL* gene, which is rearranged to over 30 different partner genes in various types of leukemia.

Spectral karyotyping (SKY) and multiplex FISH (M-FISH) are relatively new advancements of conventional FISH that utilize multiple fluorochromes, specialized optics, and image analysis that can simultaneously identify all chromosomes.[80] Comparative genomic hybridization (CGH), also called "copy number karyotyping," is a variation of FISH that detects relative gains or losses of the genome.[81] This method compares the ratios of patient specimen DNA, labeled with one fluorochrome, to that of normal DNA, labeled with a different fluorochrome when hybridized to control chromosomes. CGH is used predominantly in research to identify possible pathways involved in tumor progression, recurrence, or metastasis. 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[82]

DNA Arrays and Chips

Arrays are a relatively recent phenomenon in the field of molecular pathology.[83] The term "array" is jargon for an orderly collection of molecules on solid supports ranging from nylon membranes to printed circuit board 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.

Though most do not think of line probe assays as belonging in a section describing DNA chips, they are indeed arrays. Line probe assays are being used to determine mutations in the HIV genome, to type HCV, and to detect *CFTR* mutations for cystic fibrosis.[84] The method is based on classic nucleic acid hybridization using nylon or nitrocellulose strips as a solid support matrix for hybridization and detection. Generally, reverse hybridization is employed. Oligonucleotide probes specific for mutations or polymorphisms of interest are bound to the membrane strip in a parallel line format. Patient specimen DNA is amplified with biotinylated primers, thereby labeling the amplicon, which is hybridized to probes on the membrane. After hybridization, alkaline phosphatase-labeled streptavidin is added and binds to the biotin of the bound amplicons. Detection is colorimetric following incubation with a specific chromogen, generating a purple-

brown precipitate. The resulting colored lines are read as positive results, hence the name of the assay.

DNA chips, such as those produced by Affymetrix, Nanogen, Clinical Micro Sensors, and other companies, may be thought of as miniaturized ASOH platforms with multiple oligonucleotide probe assays. In one model, the oligonucleotides are synthesized directly onto a solid support, for example, glass or silicon, that is, the "chip." Some companies' chips can hold thousands of unique sequences. The sample DNA is labeled with fluorescent dye, denatured, and hybridized with the oligonucleotides on the chip. The chip is then scanned and the fluorescence patterns are measured with the aid of computer analysis to determine the presence of mutant or wild-type sequences. Gene expression chips use probes that bind to many different cDNA species (up to 33,000 in one array at the time of this writing) to quantitate the level of each cDNA isolated in a sample. Gene expression microarrays with large densities are appropriate for analysis of differential patterns of gene expression between normal and diseased tissues.

Clinical molecular laboratory diagnostic applications for DNA chips currently are limited to those with clinically appropriate density, in the range of 10 to 200. Utility for these platforms is found in SNP and mutation detection[85] as well as pathogen identification. Chips with densities in the thousands have limited, if any, use in clinical laboratories at this time. So much data are generated that more analysis needs to be done to winnow down the numbers to a relevant few genes or transcripts that can be used in clinical assays. This work is progressing well. Furthermore, the informatics software currently being used to analyze these data requires simplification and modification so that useful algorithms for data analysis can be employed for disease diagnosis or prognosis. While these are certainly considerable challenges, it should be noted that DNA array chip technology benefits enormously from robotic manufacturing techniques and rapidly advancing computer software. This has greatly decreased the costs per array feature of DNA chips, giving DNA microarrays enormous potential in the clinical molecular laboratory of the near future.

Examples of Applications of Arrays

1. Line probe assay for HCV genotyping
2. DNA chips for detection of polymorphisms in cytochrome P450 genes for pharmacogenetics

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