Chapter 2
Clinical Molecular Diagnostic Techniques: A Brief Review

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Abstract The identification and characterization of the genetic basis of disease is often fundamental to diagnosis. Detection of pathogenic mutations in a DNA sample can lead to a diagnosis, possible prognosis, and prospective therapy treatments. Over the years, a variety of molecular biology techniques have been utilized in clinical diagnostic laboratories in the analysis of patient samples. The recent development of next-generation sequencing (NGS) techniques has revolutionized the field of clinical molecular diagnostics. In this chapter, we review the development of molecular diagnostic approaches and some of the most commonly used assays prior to the NGS era. Although PCR-based methods are the most commonly used assays in molecular diagnostics today, a number of caveats must be taken into consideration and are also discussed.

1 Introduction

Genetics and the study of the human genome have become an integral part of medicine and public health. Determining the full molecular characteristics of genetic disorders provides additional information in the diagnosis of a patient. In addition, identification of familial mutations leads to appropriate genetic counseling for families and possible prenatal diagnosis or preimplantation genetic diagnosis (PGD) for future pregnancies. The field of clinical molecular diagnostics has grown considerably in the last couple of decades, benefitting from advancements in

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human genetics basic research and technologies. In the early years, research laboratories primarily developed the techniques used to analyze genetic mutations. Many of these assays were then implemented into the clinical molecular diagnostic repertoire. The earliest assays were generally targeted to common disorders such as hemoglobinopathies and cystic fibrosis. These early molecular diagnosis methodologies often involved indirect mutation detection through haplotype and linkage analyses, which are extremely laborious, required large amounts of patient DNA, generally required extensive knowledge of the genomic region in question, and did not always result in an easily interpretable result. Nevertheless, they provided a foundation for molecular diagnostics as we know it today, and some of these techniques are still currently in use.

The discovery of polymerase chain reaction (PCR) essentially revolutionized molecular diagnostics. First described by Mullis et al. in 1986 [1], PCR provided the ability to produce many copies of a target DNA region, allowing for faster analysis and direct mutation identification. Assays that were in use prior to the discovery of PCR were quickly modified to incorporate the use of PCR-amplified DNA rather than genomic DNA. These allele-specific detection assays rapidly developed into high-throughput systems to analyze patient samples on a larger scale. The implementation of PCR-based assays also provided laboratories with a means to analyze rare disorders in addition to common ones. Today, with resources such as the 1000 Genomes Project, a detailed catalogue of human genetic variation, diagnostic molecular laboratories have access to the sequence of all human genes and a continuously growing database of human variation. While next-generation sequencing (NGS) technologies are becoming more and more popular, automated Sanger sequence analysis appears to presently be the most common technique for analysis of many genetic disorders in clinical molecular diagnostic laboratories. However, the assay choice often depends on the gene or alleles of interest and the volume of patients to be screened. In general, most current molecular diagnostic assays are either targeted to specific alleles or analyze particular genes or groups of genes if there is no specific allele of interest. Here we describe some of the more common molecular techniques used in the analysis of both known and unknown mutations (Table 2.1) and discuss possible pitfalls of conventional PCR-based methodologies.

2 Targeted Analyses

Allele-specific mutation detection methods were the first assays implemented in clinical diagnostic laboratories. The initial techniques were developed in the early 1980s and some are still in regular use in clinical laboratories today. These assays are attractive in their ease of use and most are easily convertible to high-throughput applications. However, they can only be used to detect known mutations and polymorphisms and therefore need to be combined with additional assays if full comprehensive mutation detection is required.

Table 2.1 Select techniques used in a variety of clinical molecular diagnostic laboratories

Select techniques used in clinical diagnostic laboratories
Targeted mutation analysis
Southern/restriction fragment length polymorphism (RFLP)
Allele-specific oligonucleotide (ASO)
Allele refractory mutation system (ARMS)
Oligonucleotide ligation assay (OLA)
Pyrosequencing
Real-time PCR
Sanger sequence analysis (if mutation is know)
Detection of unknown mutations
Gradient gel electrophoresis (GGE)/denaturing (DGGE) and temperature (TGGE)
Single-strand conformation polymorphism (SSCP)
Heteroduplex analyses (HDA)
Denaturing high-performance liquid chromatography (DHPLC)
Protein truncation test (PTT)
Sanger sequence analysis
Detection of copy number variations
Southern blot
Multiplex ligation-dependent probe amplification (MLPA)
Array comparative genomic hybridization (aCGH)
Single-nucleotide polymorphism (SNP) arrays

2.1 Restriction Fragment Length Polymorphism

One of the first techniques utilized in clinical molecular diagnostics was the detection of genomic changes using Southern blotting and restriction fragment length polymorphism (RFLP). The Southern blot transfer hybridization assay was developed in 1975 [2]. Around that same time, cDNA synthesis and cloning provided the ability to determine the primary sequence of a number of genes [3]. Some of the first studies using cloned human cDNA were to identify the nucleotide sequences of the human alpha, beta, and gamma globin genes [4]. When combined with RFLP, the availability of the sequence of these genes provided a means to map normal and mutant genomic DNA. For example, genetic variations in a restriction enzyme site close to the beta-globin structural gene were identified only in people of African origin [5]. These polymorphic sites were then used in the diagnosis of sickle-cell anemia. These early studies set the stage for the use of RFLP and Southern blotting in diagnostic tests such as linkage analysis and prenatal diagnosis. Disorders such as the thalassemias, cystic fibrosis, and phenylketonuria were among the first to be

described [6, 7]. However, in the early days to detect mutations using RFLP was laborious. To identify a disease causing mutation in a gene meant first cloning the gene in question to create probes for Southern blot analysis. Genomic DNA was then digested with a variety of restriction enzymes and probed for products that were polymorphic in size. If carrier parents of an affected proband could be identified, the polymorphic fragment sizes could then be used for prenatal diagnosis. It was also possible to further analyze families that were non-informative by single enzyme digestion using a combination of restriction enzymes to determine their haplotype and possible carrier status. This technique was used to identify different forms of beta-thalassemia by taking advantage of the fact that specific mutations were generally found on a particular haplotype background [8]. This analysis method avoided the repeated isolation of the same mutation by selecting genes based on their associated haplotype.

Since RFLP was already a mainstay in molecular analysis when PCR was developed, PCR amplification of a region of DNA followed by RFLP quickly became a widely used approach. In this case, the mutation of interest was known and an enzyme that cuts at the mutation site was used. Patients that were carriers could be distinguished from those that were either homozygous wild type or homozygous mutant by the banding pattern of the PCR products on a gel. Some of the first applications of PCR-based RFLP analysis were in the characterization of sickle-cell anemia alleles [9].

2.2 Allele-Specific Oligonucleotide Hybridization

Allele-specific oligonucleotide (ASO) hybridization, or dot blot analysis, was also an early approach to detect specific mutations in particular disorders. This assay is based on the principle that when probing a region of DNA, even a single-base-pair change between a target region and the probe can destabilize the hybrid. In general, two synthetically created probes are designed to the region of interest, one complementary to the wild-type allele, one complementary to the mutant allele. The digested DNA is separated by gel electrophoresis and immobilized on a membrane. It is then hybridized with radioactively labeled probes. If both probes react, then the individual is heterozygous for the mutation of interest, if only one probe reacts then that individual is homozygous for either the wild-type or mutant allele. This technique was used in the early 1980s in the detection of the sickle-cell allele [10] and prenatal diagnosis of β-thalassemia [11].

ASO was also a technique that benefitted from the use of PCR. Instead of probing non-amplified genomic or cloned DNA, regions of interest could be PCR amplified first and then probed. The addition of PCR to ASO allowed for a more rapid detection of mutations [12] and became one of the more widely used techniques to study targeted alleles in the mid-1980s. Early on, the ASO probes were labeled with radioactivity; however, subsequent protocols used probes that were conjugated to biotin. This allowed for detection using streptavidin conjugated to horseradish

peroxidase (HRP) and colorimetric or chemiluminescent detection without the use of radioactivity. The original method of ASO is generally known as forward ASO, where patient DNA is immobilized on a membrane and hybridized with probes targeted to a specific allele. This technique is most useful when a large number of patient samples are to be screened for a small number of mutations. However, each oligonucleotide probe must be labeled separately and as the number of mutations in the screen increases, the assay becomes more complex. Reverse ASO was developed as a solution to this problem. In reverse ASO, also known as a reverse dot blot, the probes are immobilized onto the membrane and the PCR-amplified patient DNA sample is hybridized to the membrane. This allows for multiple mutations in a variety of genes to be assayed simultaneously in a single patient sample.

2.3 Amplification Refractory Mutation System

Amplification refractory mutation system (ARMS) is a PCR-based method developed in the late 1980s also for the analysis of known point mutations. ARMS is based on the fact that DNA amplification is inefficient if there is a mismatch between the template DNA and the 3′ terminal nucleotide of a PCR primer [13]. A primer with a 3′ terminal nucleotide that is complementary to the wild-type allele will not have efficient extension when a mutation is present and vice versa. Therefore, one can differentiate between two alleles by simple PCR amplification. The design and optimization of ARMS assays is primarily a function of the alleles of interest and the nucleotides surrounding them. Often, incorporating additional mismatched nucleotides near the target allele can enhance the reaction [13]. Multiple sets of primer pairs can be used simultaneous in a single tube allowing for the analysis of many mutations at one time. This particular technique has been used to identify patients that carry known mutations in many disorders such as cystic fibrosis and phenylketonuria and to determine heteroplasmy levels of mitochondrial mutations [14].

2.4 Oligonucleotide Ligation Assay

The oligonucleotide ligation assay (OLA) combines PCR with ligation in one reaction at a target allele site. After PCR amplification around the target region is performed, three oligonucleotides are added to the reaction. One, generally known as the reporter, is a common probe that is complementary to the target DNA sequence immediately 3′ to the allele of interest. The other two "capture" probes are complementary to the target DNA sequence immediately 5′ to the target allele and differ only in their final 3′ terminal nucleotide which is the target allele. Only if there is a perfect match between the capture probe and the target allele can ligation between the capture probe and the reporter probe occur. A number of different detection methods for OLA have been developed including detecting different lengths of

ligated products for the two target alleles and alternate labels on the capture probes such as fluorescence or biotin [15]. While optimization of the assay is often required, the detection methods of OLA allow for rapid and sensitive detection of alleles in a high-throughput capacity at a decreased expense. The OLA has been utilized in the detection of mutations in a number of metabolic disorders, cystic fibrosis, and pharmacogenetics [16–18].

2.5 Pyrosequencing

Pyrosequencing is a DNA sequencing technology based on real-time detection of DNA synthesis monitored by luminescence. First described in 1985 as an enzymatic method for continuous monitoring of DNA polymerase activity [19] and modified in subsequent years to optimize the reaction [20–22], the assay is based on a reaction in which each sequential nucleotide incorporated during DNA synthesis releases a pyrophosphate. ATP sulfurylase converts that pyrophosphate to ATP in the presence of adenosine 5′ phosphosulfate. That ATP then drives a luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light that can be measured. Unincorporated dNTPs are degraded by apyrase. The nucleotides are added in a specific order such that there is an expected pattern for the wild-type or mutant allele. When compared to other sequencing techniques such as Sanger sequencing, pyrosequencing offers the advantage of short read length when analyzing genetic variants for applications such as SNP genotyping or detection of known mutations. PCR fragments can be small and the assay is relatively fast in which 96 samples can be processed in approximately 20 min. Pyrosequencing is used in clinical laboratory settings for a variety of tests including pharmacogenetic testing in the analysis of polymorphisms within genes such those involved in drug metabolism [23, 24]. A quick analysis of these polymorphisms provides information on whether a patient may be a poor or rapid metabolizer of a particular drug, allowing clinicians to make more informed choices as to patient dosage.

2.6 Real-Time PCR

All of the previously discussed assays require post-PCR manipulation. In the mid-1990s, a technique involving the analysis and quantification of DNA or RNA in real time was developed [25, 26]. This sensitive assay allows for accurate quantification of a PCR product during the exponential phase of PCR. The first reports of real-time PCR were performed using hydrolysis or TaqMan probes [25, 26]. These probes specifically hybridize to the region around a target allele internal to the primer binding sites. The TaqMan probe is generally labeled on each end with a fluorescent molecule, a reporter dye and a quencher. As long as the two are in close proximity, the quencher prevents the reporter from fluorescing. As the PCR cycle progresses,

the exonuclease activity of Taq polymerase degrades the probe and the fluorophore become separated from the quencher allowing fluorescence emission which can then be measured. The increase in fluorescence is measured at every cycle and directly correlates to the amount of PCR product formed [26, 27].

Another method utilized in real-time PCR is the use of fluorescent DNA intercalating dyes. The first use of this method measured the increase in ethidium bromide fluorescence in double-stranded DNA molecules and was referred to as kinetic PCR [25]. In later years, SYBR Green I was used since it incorporates into double-stranded DNA and is less toxic than ethidium bromide. As the amount of double-stranded DNA increases exponentially during the PCR reaction, the amount of dye incorporation and emission also increases and is measured.

There are multiple pros and cons in the use of either TaqMan probes or SYBR Green dye in real-time PCR. In a TaqMan assay, specific hybridization between probe and target is required for fluorescent signal, which greatly decreases background and false positives. In addition, probes can be labeled with different reporters so two distinct assays can be performed in one tube. However, individual probes must be constructed for every allele of interest, so it can be costly. Off-the-shelf kits containing probes for a variety of disorders are commercially available. An advantage to using SYBR Green is that since no special probes are required, the cost is much cheaper. However, SYBR Green will bind to any double-stranded DNA species including nonspecifically amplified products leading to an increase in background and false positives. The reproducibility and accuracy of real-time PCR assay is also highly dependent on factors such as normalization of samples and controls. Regardless, the ability to quickly measure factors such as DNA copy number in real time with little DNA manipulation makes this assay common in molecular diagnostic laboratories.

3 Detection of Unknown Mutations

All of the techniques described in the previous section require a prior knowledge of the mutation in question and the nucleotide sequences around it. Here we describe techniques that were developed to screen unknown changes in targeted genomic regions.

3.1 Gradient Gel Electrophoresis

Gradient gel electrophoresis (GGE), including temperature (TGGE) and denaturing (DGGE), is based on the principle that the electrophoretic mobility of double-stranded DNA fragments is altered by their partial denaturation. The technique was first used in characterizing human mutations in the mid-1980s when it was applied to detect β-thalassemia mutations [28]. At that time both RFLP-Southern blotting

and ASO were being used to analyze single-base-pair mutations leading to a disease state or polymorphisms linked to mutant alleles. Still, many base pair substitutions did not lead to altered restriction sites and using ASO probes required knowledge of the DNA sequence around the allele of interest. In addition, as more mutations were identified in disorders such as β-thalassemia, the number of probes required for ASO continued to increase. GGE allowed for the detection of allelic changes without the requirement of knowing the exact DNA sequence of the region in question and multiple nucleotide changes in a single region could be simultaneously screened. The initial GGE assays were performed using digested genomic DNA mixed with a synthesized oligonucleotide probe of the region of interest. In later years, amplified PCR fragments of the region of interest were used. These DNA fragments are denatured then re-annealed, followed by the analysis on denaturing gradient gels. Fragments move through the gel based on their melting temperatures (T_m). Since the T_m is dependent on the overall DNA sequence, even a single-nucleotide substitution can alter the dissociation and mobility. Heteroduplexes of wild-type and mutant DNA fragments generally migrate slower than homoduplexes in polyacrylamide gels under denaturing condition due to mismatching of alleles and can therefore be separated by gradients of linearly increasing denaturant such as urea (DGGE) or temperature (TGGE). However, some base substitutions will not lead to a shift in position for the heteroduplex. As duplex DNA moves through the gradient, dissociation occurs in discrete regions known as "melting domains" that are 50–300 base pairs in size. All of the nucleotides in a particular region dissociate in an all-or-nothing fashion in a given temperature interval. If the mutation is located in the highest temperature region, or if the entire fragment dissociates as a single domain, no shift is observed.

Both DGGE and TGGE require a gradient of either denaturant or temperature. Temporal temperature gradient gel electrophoresis (TTGE) was first introduced by Yoshino et al. as a modification of TGGE [29]. In TTGE, the temperature of a gel plate increases gradually and uniformly with time which allows for easier temperature modulation. This increases the sensitivity as the separation range expands. One of the first reports showing successful application of this method to clinical diagnosis was in the detection of mutations in mitochondrial DNA [30]. Subsequently, TTGE has been used as a method of detection for germline mutations in a variety of disorders including cystic fibrosis [31] and somatic mutations in cancer tissues [32].

3.2 Single-Strand Conformation Polymorphism and Heteroduplex Analyses

Single-strand conformation polymorphism (SSCP) and heteroduplex analyses (HDA) were developed shortly after the introduction of PCR amplification [33, 34]. SSCP is based on the theory that single-stranded short DNA fragments migrate in a non-denaturing gel as a function of their sequence as well as size. In SSCP, during electrophoresis the single-stranded fragments adopt a unique conformation

depending on their nucleotide sequence. Even a single-base-pair change can alter the conformation leading to a change in migration on a gel. Fluorescent SSCP (F-SSCP) using fluorescently labeled PCR products and an automated DNA sequencer was developed in the early 1990s [35]. Its advantages included nonradioactive labeling of PCR products, greater reproducibility, and lower overall cost. HDA is also based on the migration of PCR products through a non-denaturing gel in a similar fashion to GGE in which heteroduplexes are analyzed in relation to homoduplexes. These heteroduplexes are formed by mixing denatured, single-stranded wild-type and mutant DNA PCR products, followed by slowly reannealing them to room temperature to form duplexes. These duplexes migrate differently depending on whether they are heteroduplexes of wild-type and mutant PCR fragments or homoduplexes of wild-type or mutant PCR fragments. Therefore, mutations can quickly be detected through simple gel migration analysis. One of the first reported uses of HDA in molecular diagnostics was in the detection of the p.F508del three base pair deletion in cystic fibrosis [36]. These techniques have been used for years in the detection of mutations in a number of disorders including a variety of cancers, phenylketonuria, and retinoblastoma [37–39].

3.3 Denaturing High-Performance Liquid Chromatography

Denaturing high-performance liquid chromatography (DHPLC) was first reported in 1997 [40] and was designed to combine some of the best features of methods currently available at that time. Sensitive methods such as DGGE were very labor intensive and required much optimization and analysis by gel electrophoresis whereas less complex methods such as SSCP and HDA lacked sensitivity. DHPLC was introduced as a highly sensitive method that facilitates the analysis of a large number of samples in a high-throughput capacity. Briefly, in a manner similar to HDA, DNA fragments are denatured and allowed to reanneal and homo- or heteroduplexes are formed. However, instead of gel electrophoresis, the DNA duplexes are applied to a positively charged chromatography column. The PCR fragments then bind to the column at different strengths depending on whether they are homo- or heteroduplexes and will elute from the column at different times generating distinct chromatograph patterns.

While relatively fast with easy automation and high specificity since no labeling or purification of the PCR products is required, DHPLC does have drawbacks. Each allelic change in any given PCR fragment will have a characteristic heteroduplex elution pattern. Although this technique has been used routinely to analyze a large number of samples for known mutations, it does not distinguish among different mutations in the same fragment. Therefore, its utility in clinical diagnostic laboratories, similar to other mutation detection methods, is limited to the detection of mutations, not the final identification, which will require Sanger sequencing. In addition, the elution conditions must be optimized for each assay in order to get the correct degree of denaturation and separation.

3.4 The Protein Truncation Test

The protein truncation test (PTT), also known as the in vitro synthesized protein assay (IVSP), does not rely on analysis of changes at the genomic level. Instead, this method is based on the change in size of proteins resulting from in vitro transcription and translation of a gene target [41]. Briefly, an RNA template is reverse transcribed to generate a cDNA copy. That cDNA is then amplified with primers specifically designed to facilitate in vitro transcription and translation. The resulting proteins are then analyzed by SDS-PAGE electrophoresis. Proteins of lower mass than the expected full-length protein represent translation products derived from truncating frameshift or nonsense mutations. The PTT was initially developed in the early 1990s to detect early termination mutations in the dystrophin gene responsible for Duchenne and Becker muscular dystrophy [42]. At that time, analysis of genes with as many exons as the dystrophin gene (79 exons over 2.4 Mb) was extremely time consuming and laborious, and a substantial number of cases were a result of truncating mutations. The most frequent application of the PTT is in detection of premature truncation mutations in cancer-causing genes in which many truncating mutations have been identified such as APC and BRCA1 [43, 44]. However, the PTT has a number of limitations and is not commonly used in most clinical diagnostic laboratories today. It only detects mutations that lead to truncated proteins, and missense mutations are not detected. Also, the requirement for electrophoresis of translation products does not translate easily to high-throughput analyses. In addition, the dependence on RNA as an amplification source precludes its easy use in most clinical diagnostic laboratories which generally work with genomic DNA. Although it is possible to use genomic DNA as a source, exons must be then analyzed individually. Therefore, while effective in the determination of truncation mutations in specific target genes, the PTT is not a commonly used test to screen for mutations in most genes.

3.5 Sanger Sequencing

Although all the mutation scanning methods described in the above sections are relatively easy to perform and fairly sensitive, they often require an extensive amount of design and optimization. In addition, any mutations detected by these scanning methods need to be ultimately confirmed by Sanger sequencing. Therefore, today, the capillary electrophoresis-based Sanger sequencing has become the most widely used approach for DNA analysis in molecular diagnostic laboratories. For a more in-depth review of the history and development of sequencing technologies, see Chap. 1. First described by Sanger et al. in 1977 [45], it has become the “gold standard” for mutation analysis particularly for very rare disorders and genes that do not harbor common mutations. In general, Sanger sequencing-based clinical assays use amplified PCR products of a particular region of interest. This may be a single-PCR product if a specific target allele is of interest or all coding exons plus flanking intronic sequences

of a gene. Each amplicon must then be sequenced independently. For clinical diagnostic laboratories, 2X coverage of a sequence is generally required. This is most often accomplished by sequencing once each in the forward and reverse directions. However, sometimes that is not possible due to repetitive sequence around the region in question, and two separate forward or reverse sequences are used. Therefore, while faster, safer, and often cheaper than some of the other techniques, it is still somewhat laborious with a high operating cost. In recent years, "next-generation" or massively parallel sequencing technologies have been developed that will provide clinical diagnostic laboratories the ability to offer analysis of multiple genes or even the whole exome at a cost that is competitive with single-gene Sanger testing.

4 Detection of Copy Number Variations

Chromosome analysis is important in the diagnosis of conditions such as intellectual disability, developmental delay, and congenital anomalies. Routine chromosomal analysis has the capacity to detect both balanced and unbalanced structural rearrangements as well as deletions and duplications larger than ~5 Mb in size, in addition to whole chromosome aneuploidy. However, conventional karyotype analysis is unable to detect submicroscopic deletions and duplications that are a common cause of intellectual disability. While Southern blotting is able to detect copy number changes in a number of genes, as previously discussed, it is very labor intensive, requires large amounts of DNA, and is only able to analyze a single region at a time. In addition, copy number changes in small regions such as single exon deletions may not be detected by Southern blot. Real-time PCR can be used to detect copy number changes in small regions; however, its use in a multiplex assay is also limited by the number of fluorescent dyes available and quantification of the data can be problematic if multiple primer pairs are desired. The requirement for the ability to analyze copy number variations across multiple exons/genes simultaneously is essential for disorders like DMD, BRCA1-related breast cancer, and mental retardation. Therefore, a variety of techniques have been developed to increase the resolution of detection for chromosomal alterations, such as multiplex ligation-dependent probe amplification (MLPA), array comparative genomic hybridization (aCGH), and single-nucleotide polymorphism (SNP) arrays. The clinical implementation of these assays has revolutionized the ability of diagnostic laboratories to detect copy number variations down to the exonic level in a multitude of genes simultaneously, with SNP arrays providing the additional ability to detect large regions of homozygosity in the genome.

4.1 Southern Blotting

As previously noted in Sect. 2.1, Southern blotting and RFLP were commonly used to track mutations in particular disorders. However, after the discovery of PCR,

mutations and deletions that previously required Southern blot mapping were routinely analyzed using PCR-based techniques. Even though RFLP analysis became PCR based, Southern blotting techniques still provide additional information for some diseases such as fragile X, although prescreening or tandem PCR analyses are often performed. The fragile X mental retardation syndrome was one of the earliest disorders in which Southern blotting and RFLP were used in clinical diagnostics. Mapped in the late 1980s and early 1990s using linkage and RFLP [46–50], analysis of the number of CGG repeat expansions and their methylation status in the 5′ untranslated region of the FMR1 gene has become one of the most common assays performed in clinical diagnostic laboratories today. PCR-based methods can be used to amplify the region containing the repeats and the size of the PCR product is therefore indicative of the number of repeats. However, the efficiency of the reaction is somewhat inversely related to the number of repeats and the larger the size, the more difficult it is to PCR. In addition, no methylation information is provided by PCR. Southern blotting allows both the size of the repeat region and its methylation status to be assayed at the same time. During restriction enzyme digestion, methylation-sensitive restriction enzymes can be used to distinguish between methylated and unmethylated species. Even though it is laborious and requires a large amount of DNA, the Southern blot is still used today in many clinical molecular diagnostic laboratories in the analysis of many diseases in particular trinucleotide repeat expansion disorders.

4.2 Multiplex Ligation-Dependent Probe Amplification (MLPA)

For many clinical diagnostic laboratories, MLPA is an attractive assay to use for the detection of copy number variations. MLPA has the advantage of analyzing multiple regions of interest simultaneously with a low operating cost requiring only a PCR thermocycler and capillary electrophoresis equipment. Briefly, MLPA is essentially a combination of two techniques: amplified fragment length polymorphism (AFLP) in which up to 50 different multiple DNA fragments are amplified in a single reaction with a lone primer pair and multiplex amplifiable probe hybridization (MAPH) in which multiple target oligonucleotide probes are hybridized to specific nucleotide sequences [51]. These probes are then also amplified with a single primer pair. However, similar to Southern, MAPH requires the immobilization of samples to a membrane and multiple washing steps to remove unbound probes. MLPA allows for the amplification of multiple oligonucleotide probes in a single reaction without the immobilization of sample to a membrane and removal of excess probe is not necessary. Each MLPA probe set consists of two oligonucleotides that hybridize to adjacent sides of the target sequence. Only when both oligonucleotides are hybridized to the correct nucleotide sequence can they be ligated into a single

probe. Therefore, only ligated probes are amplified using M13 primers at the 5′ends of the oligonucleotides. Each probe set gives rise to a unique amplification product of a particular size that can then be separated using capillary electrophoresis.

As previously discussed, using MLPA in a clinical laboratory setting is cost-effective and fast, and the universal tags allow multiple amplicons to be produced in a single reaction. However, MLPA does have its disadvantages. Due to the limits of multiplexing, each gene in a kit generally only has a limited number of probes per exon, with a maximum probe number of about 50 per reaction. SNPs in probe regions can cause a decrease in the binding efficiency of oligonucleotides resulting in false positives. Also, if a deletion is detected and the break points of that deletion are desired, further studies requiring additional PCR reactions are necessary. Finally, extensive design "rules" sometimes make the development of an MLPA assay difficult. Therefore, the inclusion of MLPA in a clinical diagnostic laboratory can increase the detection rate of mutations in a number of genes; however, the caveats listed above must be taken into consideration.

4.3 Array Comparative Genomic Hybridization (aCGH)

First described in the early 1990s [52], aCGH is now widely used for the identification and characterization of chromosomal abnormalities in many different cell types. The principle of aCGH analysis is the detection of chromosomal deletions and duplications by comparing equal amounts of genomic DNA from a patient and a normal control. Briefly, patient and control DNA are each labeled with a different fluorescent dye, typically Cy5 (green) for the patient and Cy3 (red) for the control. Equal amounts of labeled patient and control DNA are then mixed together and co-hybridized to the array, which is a microscope slide onto which small DNA fragments (targets) of known chromosomal location have been affixed. Current oligonucleotide arrays use targets made from short oligomers of approximately 60 base pairs in length. If the oligo density in a particular region is high enough, even small single exon deletions and duplications may be detected [53, 54]. Some of the first clinical diagnostic arrays were constructed using bacterial artificial chromosome (BAC) clones as targets [55]. At that time, constructing microarrays for use in a clinical laboratory was complicated due to the fact that mapping information for some BAC clones was inaccurate, and cross hybridization to multiple regions of the genome often occurred. While these first studies highlighted multiple challenges in using aCGH technology such as equipment costs, proper mapping and FISH confirmation of BAC clones, and interpretation of data, most of those challenges have now been overcome. Today, some clinical molecular diagnostic laboratories continue to use arrays based on BAC clones; however, cDNA clones, PCR products, or synthesized oligonucleotides immobilized on glass slides are increasingly more common [56].

4.4 Single-Nucleotide Polymorphism Arrays

Single-nucleotide polymorphism (SNP) arrays were originally designed to genotype human DNA by simultaneously analyzing thousands of SNPs across the genome [57]. Since their inception, SNP arrays have been used for a variety of other applications including detection of copy number changes and absence of heterozygosity. Like aCGH, SNP arrays are also based on oligonucleotide probes immobilized to glass slides. However, unlike CGH arrays that use both patient and control samples for comparison, SNP arrays use only a single patient DNA. The patient DNA binds to the oligonucleotide probes differently depending on the target SNP allele. Therefore, the resolution of the array is limited by SNP distribution. One major advantage of SNP arrays is their ability to detect copy number neutral differences in cases of absence of heterozygosity (AOH) that may occur as a result of uniparental isodisomy (UPD) or consanguinity (two copies), or deletion (one copy) such as loss of heterozygosity (LOH) associated with tumors. They can also detect copy number variants, but do not have the exon-by-exon coverage that most CGH arrays have nowadays.

5 Pitfalls of Conventional PCR-Based Methods

In most clinical molecular diagnostic laboratories, a single set of primers is used for PCR amplification of regions of interest. As a result, an SNP present within a primer site may disrupt the binding of that primer, and allele dropout could unknowingly occur. If a mutation is located within that region of interest, it may be missed. If a heterozygous deletion encompasses the region of amplification, only one chromosome will be amplified which would also result in incorrect analysis. Failure of allele amplification for one chromosome will also cause heterozygous mutations to appear homozygous. These problems can be minimized by continuous reassessment of the presence of SNPs in primer sites using the constantly updated dbSNP database. In addition, the identification of an apparently homozygous point mutation in an affected proband with an autosomal recessive disease should be followed up by parental testing whenever possible. If testing of the parents does not confirm their carrier status, additional molecular analyses can be performed to identify the underlying molecular etiology [58]. In general, allele dropout due to SNPs at primer sites should always be ruled out first for any PCR-based analyses. Capture-based next-generation sequencing will not have the problem of allele dropout since they do not rely on PCR primers. However, some regions of the genome may have poor coverage due to high GC content or the interference of pseudogenes, which will still require Sanger sequence analysis, and all positive results obtained by next-generation sequencing should be confirmed by a secondary method, which is usually Sanger sequencing.

6 Conclusions

With the advent of next-generation sequence analysis, we are entering a new era for molecular diagnostics. However, PCR-based testing methodologies still currently predominate most clinical molecular diagnostic laboratories. The choice of detection method used in the analysis of gene mutations depends on a variety of factors and can range from laboratory to laboratory. Sample volume, the spectrum of mutations in a given gene of interest, and equipment investment required can all play a role in what type of assays a molecular diagnostic laboratory chooses to perform.

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