

ADVANCED MOLECULAR TECHNIQUES FOR DETECTION OF GENETIC DISORDERS

Abstract

Unlike other diseases, genetic diseases are difficult to detect until the affected individual leaves infancy and reaches an age where he/she begins an active life. Detection of a genetic disease when the individual is still in the embryonal or fetal stage is important for the parents to make a decision that would be best for their child's quality of life in future. Hence, molecular detection tools like GAP-PCR (polymerase chain reaction), triple primed PCR, chromosomal microarray and others have become important to detect genetic anomalies in the fetus/ embryo using amniocentesis. GAP-PCR is important in detecting gene deletions which is important in the detection of diseases like thalassemia prenatally. Amplification Refractory Mutation System PCR (ARMS-PCR) is useful in prenatal detection of sickle cell anemia and also SNP (single nucleotide polymorphism) for differentiating mutations in different diseases. Other important detection techniques used in this chapter are multiplex PCR, Loop-mediated isothermal amplification (LAMP)-PCR, triple primed PCR, chromosomal microarray, Comparative Genomic Hybridization (CGH)

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I. INTRODUCTION

There are many techniques to detect genetic disorders like Polymerase chain reaction (PCR), Microarray techniques, Western blot, Dot blot analysis. However, they can only detect the expression or absence of expression of the genes. However, they have many disadvantages in that they cannot detect the deletions within the gene. They also cannot detect duplications in a gene. Further, they are highly incapable of finding aneuploidies and sub chromosomal disorders which are the major reasons for many of the advanced congenital disorders which are debilitating. Using the conventional molecular techniques, it is impossible to detect these anomalies of the chromosomes and this would lead to the carrying over of the disease to the next generation and creating more and more debilitated and suffering individuals.

There are many techniques which can overcome the disadvantages of the above-mentioned conventional techniques and the purpose of the present chapter is to throw light on the many important techniques which can detect genetic defects like deletions, duplications, aneuploidies

II. MLPA TECHNIQUE

Multiplex Ligation-dependent Probe Amplification (MLPA) is a type of multiplex PCR in which about 40 probes are used and each of them are specific for different DNA sequences (here different probes specific for each exon of a particular gene). This is done to quantify the relative amount of each of the exons that are expressed. What are these probes made of? These probes are made up of half probes for 5' and 3' end of each sequence specific for a target. In addition to this there are primers which are applicable to the gene as a whole. The inclusion of all these primers would allow for the simultaneous mode of amplification of these probes using multiplex PCR. These half probes also contain a stuffer sequence which are DNA sequences of different lengths (for each exon). These stuffer sequences facilitate the identification of the probes (and also the amplified PCR product) after the electrophoresis procedure.

The MLPA reaction consists of five steps:

- 1 Denaturation of DNA and hybridization of probes
- 2 Ligation of probes to gene sequences
- 3 Amplification of gene sequences using PCR amplification
- 4 Using electrophoresis to separate amplified PCR products
- 5 Analysis of data.

Firstly, denatured of DNA and incubation of the denatured DNA was done with all kinds of probes in MLPA. The pair of half probes used in this process are used to identify contiguous sequences of target. The two probes can only bind when there is a perfect match between probes and sequences. Here even a single mismatch between probes and gene sequences can prevent ligation and amplification of the half probes. Amplification of the gene sequences is then done using PCR. For this, one of the pairs of primers is fluorescently labelled. Here PCR amplifies the probes that are ligated to the gene. Hence the target sequences within a gene can be quantified by the number of products of ligated probes. These

PCR products are separated according to their sizes (due to stuffer sequence length) using Capillary Electrophoresis. This is done in denaturing conditions. The quantification of PCR products is done by measuring the height or area fluorescence peaks derived from PCR. This is done after normalizing the data and then comparing the normalized data with control DNA samples' data. Assessment of the quality of PCR reaction quality is done by means of control peaks. An important fact about MLPA is that here PCR amplifies the ligated probes only and there is no amplification of target DNA sequences.

MLPA can be used to detect deletions and duplications in gene sequences as a tool for genetic diagnosis of various hereditary diseases. When specific peaks for target gene sequences are absent when control probes are normally amplified, it can be interpreted as Homozygous deletions or hemizygous deletions. Heterozygous duplications, deletions, and Copy number variations create a pattern of variable heights and/or areas of the peaks relative to each other. For proper interpretation of MLPA raw data, Coffalyser software, a program based on Excel. Duplications or deletions of certain genes are responsible for many inherited neuromuscular disorders like Spinal Muscular Atrophy, Dystrophinopathies (Duchenne Muscular Dystrophy and Becker Muscular Dystrophy), Hereditary Neuropathy, Charcot Marie Thoot disease.

Recently, aneuploidies of 13, 18, 21, X and Y chromosomes have been reported to have been screened using MLPA (Stuppia et al. 2012).

III. LAMP-PCR

Loop-mediated isothermal amplification (LAMP)-PCR is a kind of PCR which uses Forward Inner Primer (FIP) (with an F2 region at the 3'- end, F1c region at the 5'- end, Forward Outer Primer (F3 Primer) with F3 region which is complementary to the F3c region of the sequence in template, Backward Inner Primer (BIP) consisting of B2 region present at the 3'- end and B1c region present at the 5'- end, Backward Outer Primer (B3 Primer) having a B3 region complementary to the B3c region of the sequence of the template. In LAMP-PCR first amplification begins with hybridization of F2 region of FIP with F2c region of the gene to be assessed. This starts synthesis of complementary strand. After this, F3 primer hybridizes with F3c region present on target DNA. Following this, there is extension and displacement of the FIP hybridized to complementary strand. This is followed by loop formation by the displaced strand at the 5'- end. This loop at the 5'- end which is made of single- stranded DNA acts as a template for BIP. After this, there is hybridization of B2 with B2c region present on template DNA. This is followed by initiation of DNA synthesis resulting in complementary strand formation, after which there is opening of the loop at 5'- end. B3 then undergoes hybridization to B3c region present in target DNA. This is followed by extension and displacement of the BIP linked to the complementary strand. This causes creation of a DNA which is dumbbell- shaped. 3'- end of F1 undergoes extension after which there is opening up of the loop at the 5'- end. This DNA which looks like a dumbbell then becomes transformed to a stem- loop structure. This structure which has the appearance of a stem-loop initiates LAMP cycle. This is the second phase of the LAMP process. Primers complementary to the loop are to facilitate exponential amplification of the process of LAMP. The end products of LAMP consist of a mixture of DNA looking like stem- loop with different lengths of stem and different cauliflower shaped structures containing multiple loops (Wong et al. 2018).

LAMP is different from conventional PCR, in that, it requires isothermal conditions and less infrastructure like a simple water bath or dry bath. LAMP is also quick, specific, sensitive, and allows easy visualization of end-products. LAMP has been used extensively to detect various bacteria, parasites and viruses in addition to being used for the detection of genetically modified plant products in pathology studies of plant and in diagnosis of diseases. LAMP also uses strand displacement causing polymerases lacking 5'-3' exonuclease activity which can cause amplification of nucleic acids in an auto-cycling manner at a uniform temperature of 60–75 °C. The different sizes of end product DNAs looking like stem-loop containing different inverted repeats of the target sequence, look like ladder in an agarose gel. (Srividya et al. 2019).

LAMP assay can be used for prenatal diagnosis and screening of α -thalassemia. This technique requires just 2.5 ng DNA. This assay does not require a PCR machine and the results can be deduced by naked eyes even without gel electrophoresis of the amplified products. (Jomoui et al. 2022).

IV. SINGLE TUBE MULTIPLEX PCR

The multiplex PCR is an efficient variant of the usual PCR. In this, different DNA templates or different sets of primers or different regions of a gene can be amplified, in one single reaction. Multiplex PCR is a combination of different PCR reactions. This type of PCR also requires the usual PCR reagents like dNTPs, nuclease-free water, reaction buffer, and Taq DNA polymerase. In case of real time PCR multiplex reaction, additional reagents like oligo (dT) primers, reaction buffer, TaqMan probe or SYBR green dye would be required. Multiplex PCR has the advantage of saving time although more reagents are needed to amplify each template. In this PCR, multiple PCR reactions can happen in one tube. Multiplex PCR follows 2 techniques:

- 1 Multi-template multiplex PCR
- 2 Uni-template multiplex PCR

In multi-template multiplex PCR, there is amplification of many different templates in one reaction. Here, it causes amplification of different templates present in one sample in the presence of many different primer sets. In this, each of the different set of primers is made for each template and hence cause amplification of their respective target sequence only. Multi-template multiplex PCR, employing more than one template, finds its application in microbial genetics, identification of pathogen and microbial detection. This technique is used to quantify, amplify and identify microbes and thus the amount of pathogens in a given sample. Multi-template multiplex PCR cannot be used to detect disorders where single genes are involved. However, uni-template multiplex PCR involves amplification of different loci or different regions of one template DNA with many different primer sets. For instance, if we want to detect 5 separate mutations of the beta-thalassemia, we need to use a single beta-globin gene for a single reaction. In uni-template multiplex PCR 5 primer sets can amplify 5 separate loci of mutation present on one beta-globin gene.

Multiplex PCR can be used for screening of single-gene inherited disorders like muscle dystrophy. Interestingly, the first multiplex PCR was conducted in different loci of one gene of dystrophin.

1. Multiplex PCR is Affected by Following Factors:

- **Two Crucial Factors that Affect a Multiplex PCR are:**
 - Designing of Primers
 - Type of Reagents used
- 2. **Primer Designing:** this is a very important step in success of multiplex PCR because primers which are wrong affect the reaction negatively. A wrong set primer either cannot amplify a target gene. In a multiplex PCR reaction, every set of primers needs to be unique and should not cross-react with each other.
- 3. **Type of Reagents Used:** Each reagent has a particular quantity and quality that can significantly impact multiplex PCR. A high concentration of each reagent causes non-specific binding and a low concentration of the reagent cannot amplify the template properly.

Multiplex PCR requires the following reagents:

- Taq DNA polymerase
- PCR reaction buffer
- dNTP cocktail
- Template DNA
- Sets of primers
- A TaqMan probe or SYBR green dye
- Nuclease-free water
- Multiplexing buffer

Reagent	Purpose
Taq DNA polymerase	An enzyme for amplification of DNA
PCR reaction buffer	To enhance the efficiency of the reaction and to reduce non-specific binding
dNTPs cocktail	To provide dNTP during the DNA synthesis.
Template DNA	A DNA target for amplification
A SYBR green dye or TaqMan probe	To quantify the PCR product
Primer sets	For amplification reaction by means of annealing with the template DNA. These

	create a3' end free for the polymerase to start the synthesis.
Nuclease-free water	To provide volume to the reaction and to dilute the reagents.
Multiplexing buffer	To increase efficiency of reaction, and also to increase specificity and to reduce instances of non-specific binding.

- 4. Procedure of Multiplex PCR:** Initiation of Multiplex PCR starts with DNA extraction, purification and dilution. After this, all the reagents are thawed to prepare the reaction mixture. First, master mix or dNTPs cocktail are added to the PCR tube. Next multiplex reaction buffer containing DMSO, MgCl₂, or KCl is added. After this, primer sets are added to the reaction mixture. Next, the Probe or dye is added, in case of a quantitative assay. template DNA is then added to the reaction mixture. Finally, nuclease-free water is added to make up the final volume of reaction mixture. After setting up the reaction mixture, set up the thermocycler to the standardized PCR conditions and then put all the tubes in the machine to start the process.

This process can be used to detect deletions in alpha-thalassemia. This can be done using deletion-sensitive primers for amplification of the DNA sequences. Deletions responsible for alpha-thalassemia fail to get amplified when these primers are used. These primers are taken along with primers which are made in such a way that they can bind to an unaffected control sequence from the hemoglobin alpha or beta chain. Here they get amplified simultaneously. In this way, heterozygotes for alpha-thalassemia-2 (in which one alpha locus is deleted) can be detected (Bowie et al. 1994).

- 5. Chromosomal Microarray:** In this technique, a microarray gene chip containing hundreds of tiny dots containing DNA from every one of 46 chromosomes, is used to detect Copy Number Variations (CNVs) (deleted or extra regions). Computer algorithms are used to identify and measure the amount of DNA sample that is bound to the microarray chips and then comparing them against a reference DNA sample. This will facilitate the study of the exact amount of missing or extra DNA. Chromosomal microarrays (CMAs) are now used as first stage tests and is preferred over other cytogenetic tests as prescribed by the International Standards for Cytogenomic Array Consortium.

Chromosomal microarrays (CMAs) can detect missing or extra Regions in DNA. CMAs can also detect chromosome number abnormalities like Trisomies – involving extra chromosome instead of a pair. It can also detect unbalanced translocations in which there is an extra chromosome piece or when a small missing piece of chromosome occurs resulting in an inadequate amount of genetic material in a child. CMAs are not capable of detecting balanced chromosomal translocations rearrangements between chromosomes. Even single gene disorders cannot be detected by CMAs. CMAs are unable to detect disorders brought about by a single letter change in a DNA). (<https://mapmygenome.in/blog/basics-of-chromosomal-microarray-analysis>)

Chromosomal microarray can measure DNA gains and losses in human genome. It can also detect aneuploidy in chromosomes and large changes in chromosomal structure. These are usually detected by karyotype analysis. However, CMAs are better than karyotype analysis as they can detect sub-microscopic genetic abnormalities which are too small to be detected by traditional techniques. CMAs do not require actively dividing cells. Hence CMA of fetal tissue (ie, amniotic fluid, placenta, etc) are used to evaluate intrauterine fetal death when more cytogenetic studies are required to detect abnormalities. CMAs can also detect Single nucleotide polymorphism much better than karyotyping. It can also detect maternal cell contamination, which is an important contributor to false-negative results (Reports 2016).

CMAs are popularly used for genetic testing of individuals with developmental delay/intellectual disability (DD/ID), multiple congenital anomalies (MCA) or autism spectrum disorders (ASD) (Miller et al. 2010).

CMAs can detect changes even in 5-10Kb size DNA. It has a resolution ability which is 1000 times higher than that of conventional karyotyping. CMA is also used to detect copy number variants (CNVs) responsible for congenital anomalies and neurodevelopmental disorders. It can be used as both prenatal or postnatal detection method (Batzir et al. 2015).

- 6. Microarray Based Comparative Genomic Hybridization (Array- CGH):** Comparative Genomic Hybridization (CGH) method was developed to detect chromosomal duplications and deletions on whole genomes. In CGH, metaphase chromosomes are first spread on glass slides. This is followed by co-hybridization of one test and one reference labeled probe DNA onto the chromosomes, in presence of Cot-1 DNA. This is to suppress high repeat sequences. CGH can detect numerical aberrations in chromosome. This is associated with solid tumor progression (Redon et al. 2009).

In the first step of array-CGH choice of the type of microarray has to be selected to screen test genomes. One of the types is selection of microarray covering the entire genome for screening all deletion or duplication in a test genome as compared to a reference DNA; another type is the microarray targeted to a single part of the genome (Redon et al. 2009). CGH is an alternative method to screen genome-wide copy number variations. It was developed initially to identify copy number changes in solid tumors. It uses test and control genomes. These are labelled differentially and hybridized competitively to metaphase chromosomes. The signal intensity of the fluorescently labelled test DNA in relation to that of the reference DNA is plotted linearly across each chromosome to identify copy number changes. CGH can also be used to scan an entire genome thoroughly for imbalances. Further, CGH does not require dividing cells. In array-CGH, metaphase chromosomes need not be used. Here, microarrays are made by the depositing and immobilizing small amounts of DNA on a solid support, like a glass slide, in an ordered fashion. In array CGH, a test sample like blood, skin, fetal cells are taken and DNA is isolated from the sample. This DNA from the test samples is then labeled with a particular-coloured fluorescent dye. Then DNA from a normal sample is taken as control which is also called reference sample. This is labeled with a different colored dye. The test and reference DNA samples are mixed and subjected to microarray. denaturation of these DNA samples makes them single stranded. Hence when they are

subjected to microarray, they hybridize with the single-strand probes present on the array. Quantification of relative fluorescence intensities of the labeled DNA is done using digital imaging systems on the probes hybridized to each target. The ratio of the test and reference hybridization fluorescence signals is identified at different locations along the genome. From this, we can get data on the existence of relative copy number of sequences in the test sample genome as compared to the normal sample genome.

Array-CGH technology can be used to detect submicroscopic abnormalities seen in developmental defects and congenital disorders caused by aneuploidies, duplications, deletions, amplifications. It can also be used to detect pericentromeric and subtelomeric rearrangements seen in idiopathic mental retardation. It can be used to detect common Rett syndrome wherein there is duplications of the MECP2, which causes developmental delay in males. It can detect microdeletion syndrome of 3q29 like Williams-Beuren syndrome (Theisen et al. 2008).

V. ARMS-PCR

One of the most accurate detection tools emerged recently in diagnosis of genetic disease is Amplification Refractory Mutation System PCR (ARMS-PCR). It is the most preferred method to detect sickle cell anaemia and thalassemia, detection of mutation in HIV and JAK2. This technique can also be used to detect SNP (single nucleotide polymorphism) for differentiating mutations in different diseases. This can be done by use of refractory primers. selective amplification of the mutant (with SNP) and normal (without SNP) alleles can be done by designing primers suited for this purpose. Electrophoresis can be used to analyze the results. One of these primers has a modified single base at the 3' end of in such a way that it matches the normal allele. The other primer is designed in such a way that it matches the allele which is mutant. Both of these primer types are mixed in one PCR reaction mixture. This is to facilitate the performance of PCR simultaneously. The detection of a variant allele happens when the primers attach and also amplify either the mutant allele or the normal allele. Here the primer matching the mutant allele cannot undergo normal PCR. The reverse is true for the normal allele. Because they are resistant to a PCR amplification, the process is called amplification refractory mutation system.

Primer modification of is one of the biggest highlights in the process of ARMS-PCR. Selective amplification of the genes happens because of primer sets' mismatch. When a mismatch is introduced at the primer 3' end the annealing temperature is altered in the variant of allele. Because of the inability of Taq DNA polymerase to conduct exonuclease activity, there is no repair of mismatch. The procedure of the ARMS-PCR happens with four important steps:

- 1 Primer Design
- 2 Amplification
- 3 Electrophoresis
- 4 Results

1. Primer Design: if a DNA sequence of our choice shows the presence of a A in place of G in the mutant allele and a G in the normal allele, then a forward primer designed for the mutant allele should contain T in place of C at the 3' end and the forward primer for

normal allele should contain C. To ensure that the ARMS-PCR is successful, a mismatched base is added at the 3' end near the SNP. C: T, G: A, and A: G were found to be the strong mismatch base pairs which reduce the amplification process up to about 100-fold. The entire PCR involves the reverse primer which does not undergo alteration. Just like conventional PCR, during primer design, the primer must have satisfied all the conditions for the ideal primer.

2. **Amplification:** compared to conventional PCR, ARMS-PCR has lower cycles (22 to 25 cycles) than the normal reaction (35 cycles). When the PCR cycles are increased, the chances of false-positive results increase. Therefore, internal control is very important in ARMS PCR. This provides more accuracy by reducing false-positive results' chances.
3. **Electrophoresis:** After amplification gets over, the loading of amplified fragments is done on 2% agarose gel for observation of the results. This obliterates the need for hybridization. After the sequential loading of samples, the electrophoresis is run for 45 minutes.
4. **Results:** Upon result interpretation, bands of internal control in all the wells shows the absence of false-positive results present. This is important because there are no clear differences in the banding patterns in the mutant and normal alleles.

ARMS-PCR cannot detect deletion, duplication and abnormalities in chromosomes. However, it has a very good capability of detecting SNPs. In fact, most of the SNPs already known were detected using this technique. However, thousands of SNPs cannot be detected by ARMS-PCR in a single run (Ehnert et al. 2019; Alyethodi et al. 2018; Little 1995)

VI. GAP-PCR

GAP-PCR is the PCR of choice to detect deletion in a gene. It is one of the most popular techniques in DNA diagnostics for screening the common defects associated with gene deletion responsible for alpha-thalassemia or beta-thalassemia. GAP-PCR operates on the following principle: here there are two primers complementary to the sense and antisense strand in the DNA segment surrounding the deletion and amplification using PCR. If there is a bigger distance between the two surrounding primers, the normal does not get amplified. However, the allele having deletion is amplified and a PCR product is obtained from only the deletion allele. The type deletion is indicated by the length of the product. Different primer sets specific for different deletions can be used in multiplex mode of the amplification reaction, to detect more than one deletion in a single experiment. This makes this PCR a quick, cost-effective and inexpensive method of screening. In this method, a positive control is used for each deletion, in addition to a blank and a wild type (normal) DNA. GAP-PCR is easily available in regular diagnostic laboratories and is fast and inexpensive. This PCR is mostly used to detect alpha thalassemia mutations that is alpha thalassemia type 2, in which there is a single functional alpha globin gene present on the chromosome. There are two common types of alpha thalassemia type 2 defects. In the first type, there is a 3.7 kb deletion called the right-ward deletion. The other type is a 4.2 kb deletion which is called the left-ward deletion. In addition to the right ward and left ward deletion, the third type of defect is called α^0 -thalassemia defect, in which both alpha-genes of one chromosome are erased. In this type

there is more than 20 kb deletions and is common among Mediterranean basin populations or SE Asia populations. All these deletions can be pre-screened by GAP-PCR. (Harteveld 2014).

VII. TRIPLE PRIMED PCR

Expanded alleles in myotonic dystrophy can be detected by a technique called triplet repeat primed PCR (TP PCR) method. This type of PCR involves a primer which is locus-specific surrounding the repeat and also paired primers which amplify different priming sites inside the repeat. To provide specificity to the detection reaction, locus-specific fluorescently labeled primers are added to the reaction mixture. Triple primed PCR showed a ladder-like characteristic pattern with a fluorescence trace. This facilitates the quick identification of large repeats of pathogenic nucleotide combinations which cannot be amplified using flanking primers as in other PCRs. Initially this technique was used to detect myotonic dystrophy. Later, this technique was used to detect frataxin GAA repeat expansion to screen Friedreich ataxia patients with GAA expansion interruption (Ciotti et al. 2004).

This technique can be used to detect the number of CAG repeats on huntingtin gene on chromosome number 4 in Huntington's chorea. It is known that if CAG repeats are more than 40, full blown Huntington's disease ensues. If CAG repeats are between 10-35, the person is normal. People with CAG repeats between 36-39 come under reduced penetrance types. These people have chances of developing Huntington's disease later in life or transfer the disease to the next generation and hence come under the category of carriers. Hence detection of the number of CAG repeats is very important to screen the reduced penetrance range of people to take appropriate steps to prevent the carry over of the disease to next generation. Hence triple primed PCR is a valuable tool to detect Huntington's disease (Ciotti et al. 2004).

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