MUTATION ANALYSIS OF DYSTROPHIN GENE USING MULTIPLEX LIGATION DEPENDENT PROBE AMPLIFICATION (MLPA)

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Acronyms

µUtr	Micro-utrophin
2-OMePS	2′-*O*-methyl-phosphorothioate
ADHD	Attention deficit hyperactivity disorder
AICAR	5-aminoimidazole-4-carboxamide ribotide
AON	Antisense oligonucleotides
ASD	Autism spectrum disorder
BGE	Background electrolyte
BISRS	Bread-induced serial replication slippage
BMD	Becker Muscular Dystrophy
CCD	Charge coupled device
CMDN-Nepal	Center for Molecular diagnostic Nepal
CMT	Charcot Marie Tooth
CNV	Copy number variation
CPK	Creatine protein kinase
DAG	Dystrophin associated glycoprotein
DAPC	Dystrophin associated protein complex
DHPLC	Denaturing high performance liquid chromatography
DMD	Duchenne Muscular Dystrophy
DOVAM-S	Detection of virtually all mutations-SSCP
EOF	Electro-osmotic flow
ESCE	Entangled solution capillary electrophoresis
FAM	Fluorescein amidite
FoSTeS	Fork stalling and template switching
GLB	Gel loading buffer
HEX	Hexacholorofluorescein
HNPP	Hereditary Neuropathy with liability to Pressure Palsies
LAM-111	Laminin-111

MAPH	Multiplex amplifiable probe hybridization
MDF-Nepal	Muscular Dystrophy Foundation Nepal
MDSCs	Muscle derived stem cells
MLPA	Multiplex ligation-dependent probe amplification
MMBIR	Micro-homology mediated break-induced replication
MMRDR	Micro-homology-mediated replication-dependent recombination
NAHR	Non-allelic homologous recombination
NHEJ	Non-homologous end joining
OCD	Obsessive compulsive disorder
PMOs	Phosphorodiamidate morpholino oligomers
POP	Performance optimized polymer
PPMD	Parent project muscular dystrophy
PRINS	Primed in situ labeling
PTC	Pre-termination codon
PTD	Protein transduction domain
PTT	Protein truncation test
qmfPCR reaction	Quantitative multiplex fluorescence polymerase chain
RDOs	RNA/DNA chimeroplasts
rhBGN	Recombinant human biglycan
SCAIP	Single condition amplification/internal primer sequencing
SH3	src-Homology-3
SMA	Spinal muscular atrophy
SNP	Single nucleotide polymorphism
SSCP	Single strand confirmation polymorphism
TAT	Trans-activator of transcription
VIQ	Verbal Intelligence Quotient
WW	Tryptophan-Tryptophan

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// Abstract

Duchenne muscular dystrophy (DMD), an allelic X-linked progressive muscle-wasting disease, and its allelic form Becker muscular dystrophy (BMD) are one of the most common single-gene disorders caused by mutations in the DMD gene (also dystrophin gene), the longest known human gene ranging 2.4 Mb, encoding a 427 kDa cytoskeletal protein called dystrophin. Due to the lack of reliable genetic diagnostic tool in Nepal the diagnosis of these genetic diseases are narrowed to phenotypic and clinical diagnoses. Besides, the lack of even the base line data of these genetic diseases has barred the progression of the further research of these genetic diseases in Nepal.This research has been carried out with the aim to introduce Multiplex ligation dependent probe amplification (MLPA) as one of the convenient molecular diagnostic tool in diagnosis of the genetic diseases as DMD/BMD.

In this research, DNA was extracted from the blood samples of DMD/BMD patients and from the normal male and female to be taken as the reference samples. DNA samples so extracted were then amplified in the thermocycler by using the MLPA assay. The PCR products of the test samples and the reference samples so obtained were run on the capillary electrophoresis (CE) and the data were analysed. Using an algorithm of MLPA, 26 total samples were assayed. The capillary electrophoresis run (ABI-310 genetic analyzer) demonstrated that it could pick up the deletions in 14 of the 21 test samples considered. Consequently, MLPA was efficient in accurately confirming mutations in about 67% of all cases. Most prevalent exonic deletion regions were found to be confined in the exon 7-14, the proximal zone and 45-53, the first half of C-terminal domain. The reading frame (in-frame or out-frame) were determined by using the "DMD exonic deletions/duplications reading frame checker 1.9" as recommended by MRC-Holland, which need to be confirmed by sequencing. No novel mutations were identified in this study. Overall, this approach confirmed mutations in 67% of the patients in our study which is compatible with the recent studies in Chinese and Indian population.
Among the 21 test samples used MLPA could not diagnose the mutation in some of the samples which were clinically diagnosed as DMD/BMD. This result aware us that in order to know the exact point of mutation and to know exactly which of the exon is deleted or duplicated further sequencing should be done. But still the efficiency of this MLPA assay makes it a rapid, robust, efficient and reliable genetic tool in the diagnosis of genetic disorders. The systematic approach/algorithm used in this study offers the best possible less invasive and effective mutation analysis in the context of Nepal.

Key words: Dystrophin gene, Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), Multiplex Ligation Dependent Probe Amplification (MLPA), capillary electrophoresis (CE)

CHAPTER I: INTRODUCTION

1.1 Background

A genetic disorder is the condition that is caused by the alteration and abnormalities in the genome. A disease-causing mutation generally leads to the abnormality in the function of the targeted gene and interfere with the body's production of a particular protein. A genetic disorder may be inherited from the parents or it may be due to the de novo mutation. There are many genetic diseases known till date that may be a single gene disorder or multifactorial and polygenic (complex) disorder.

Although the majority of human hereditary diseases are due to abnormalities in the DNA sequence of specific genes (point mutations), gene deletions or duplications represent a relevant portion (about 5%) of all disease-causing mutations, and in some cases are the most frequent cause of a genetic disease, such as in the cases of Duchenne Muscular Dystrophy (DMD) or Spinal Muscular Atrophy (SMA) (Armour JA et al., 2002). It has been recently demonstrated that the genetic basis of several human diseases is related to the Copy Number Variation (CNV), generally defined as a DNA segment, longer than 1 kb, showing a variable copy number compared with a reference genome (Lee C et al., 2007). At present, the real proportion of genetic diseases caused by CNVs is unknown, but it may be substantial, when considering that it has been suggested that germ line CNVs can also predispose an individual to syndromic malformations (Choy KW et al., 2010). Among the many genetic diseases under study, muscular dystrophies like dystrophinopathies are widely studied. Muscular dystrophies are genetic disorders characterized by progressive muscle wasting and weakness that begin with microscopic changes in the muscle. As muscles degenerate over time, the person's muscle strength declines (Lewis J, 2000).

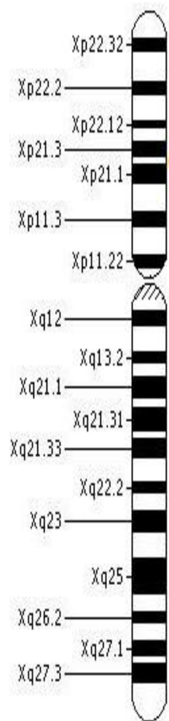


Fig.1.1: X-chromosome

Dystrophinopathies include the genetic diseases called Duchenne muscular dystrophy (DMD) and its less severe allelic form Becker muscular dystrophy (BMD). These genetic diseases are caused by the mutation in the dystrophin gene located in the X chromosome (Xp21.2). The dystrophin protein acts as the anchoring protein in the dystrophin associated glycoprotein complex (DAG).The dystrophin gene is the largest gene known of about 2.4 Mb producing 14-kb mRNA transcripts forming a protein product dystrophin. The large size of the dystrophin gene makes it susceptible to the

higher rate of mutations than in other genes. DMD is one of the most common inherited paediatric neuromuscular disorders affecting 1 in 3500 live male births (Emery AE, 1991).

There is a report on proportion of patients with deletion in dystrophin gene made early in the 19th and 20th century throughout world. But in the context of Nepal, there is no report even in the hot spot region (i.e. proximal 1-20 exon and central region from exon 42-53; where the frequency of mutation is more centered) and no baseline data has been generated. Though the factual report regarding the prevalence of DMD and BMD cases in Nepal is not available, depending upon the number of cases that are recorded in the MDF-Nepal, it is estimated that the number of patients of DMD and BMD show the same proportion as in other countries of the world. But the secondary survey, data collection and diagnostic researches are yet to be done. In our country where most of the patients are diagnosed to be suffering from various genetic diseases only through phenotypic characteristics and some clinical diagnostic procedures the use of precise diagnostic tool in genetic level as MLPA is an essence. It is important to find the true picture of the Duchenne muscular dystrophy (DMD) and less severe allelic form of DMD i.e. Becker muscular dystrophy (BMD) in the genetic level and further document the proportion of mutation (intragenic and intergenic deletion) in Nepalese population. So even distinguishing the types of deletion (that is frequent in DMD/BMD present in ~2/3 of cases) on the basis of failure to amplify exon using DMD multiplex PCR based study (as first described by Chamberlain et al., 1988) could be used in the context of Nepal to get fast and earlier detection of types of dystrophy in children to improve their condition as well as timely application of physiotherapy and further application of therapeutic and pre-implantation technique coming in the world arena using PCR based diagnostics (Yang J et al., 2013).

Chamberlain et. al had described a series of primers that can amplify 6 exons (exons 8, 17, 19, 44, 45, 48) that are deleted in many DMD/BMD patients. The addition of primers for three more exons (exons 4, 12 and 51) makes the Chamberlain multiplex test informative in about 80% of DMD/BMD patients having cDNA detectable deletions (Chamberlain JS et al., 1988). Alan H. Beggs et al described the primers for 9 additional exons (3, 6, 13, 43, 47, 50, 52, 60, 49), that when used in combination of Chamberlain et al. detect all 71 deletions known till date in BMD patients and over 97% of deletion in DMD patient(Beggs AH et al., 1990). Various other studies made on the past indicate that the central hot spot region is prone to most deletion and duplication (i.e. exon 1-20m, the proximal region and exon 42-53, the cysteine rich domain), the peak of recombination is on intron 44, and exon 50 is most deleted (Rao et al., 2003; Thong et al., 2005). The overall studies have shown that most of the cases of DMD and BMD are caused due to three forms of mutation in one or more exons of 79 exons of dystrophin

gene viz. deletion (~65% of cases), duplication (~5% of cases) and point mutation (~30% of cases).

Neither conventional cytogenetic analysis nor DNA sequencing is able to detect gene deletions/duplications and CNVs. As a consequence, these mutations must be investigated by using specific approaches. At the beginning, the detection of gene deletions/duplications was mainly based on the use of Southern Blot and FISH techniques. However, both approaches are time consuming, with low throughput analysis, and are not able to detect small intragenic rearrangements. On the other hand, CNV detection is mainly based on the use of array Comparative Genomic Hybridization (CGH), but results provided by this approach must in some cases be validated by other quantitative PCR methods, such as microsatellite genotyping, long-range PCR or different array CGH or genotyping platform (Lee C et al., 2007). Among the different approaches used in recent years for the detection of gene deletions/duplications or for the validation of array CGH results in the analysis of CNVs, particular interest has been devoted to the Multiplex Ligation-dependent Probe Amplification (MLPA) assay (Schouten JP et al., 2002). There are many other approaches that are on the list such as FISH, quantitative/Sq-PCR, Southern blot, CGH array and SNP array but with their own limitations such as FISH and SNP array cannot detect small rearrangements (e.g. Deletions <100 kb or duplications >500 kb), CGH array has costly equipment and reagents and low throughput and Southern blot cannot detect copy neutral loss of heterozygosity and is laborious and time consuming.But the use of multiplex PCR spotting the mutation on hot spot region only is being replaced by a new technology that can detect the copy number variation in all the exons of a single gene as in case of the dystrophin gene that contain 79 exons. It has also overcome some major drawbacks of the other approaches as FISH, SNP and Southern blotting. This assay called MLPA (Multiplex Ligation Dependent Probe Amplification) is capable of detecting at least 98% of the mutation along with the point mutation unlike the multiplex PCR that can detect at most 98% of the deletion or duplication mutation in the gene.

MLPA assay is a recently developed technique that is able to evidence the variation in copy number of several human genes. Thus it has been used in the molecular diagnosis of several genetic diseases whose pathogenesis is related to the presence of deletions or duplications of specific genes as dystrophin genes. This assay can also be used in the molecular diagnosis of genetic disease characterized by the presence of abnormal DNA methylation. MLPA technique is able to analyze in a single reaction up to 50 DNA sequences and to detect copy number variation of specific genes, including small intragenic rearrangements. MLPA assay has become in a few years a widely used technique in laboratories performing genetic testing for the molecular diagnosis of several diseases. A search in the Pubmed database using the word “MLPA” displays the presence of a total of 978 scientific articles, of which 45 in 2005, 74 in 2006, 124 in 2007,

170 in 2008, 163 in 2009, 229 in 2010, and 203 up to October 2011, thus demonstrating the growing interest devoted by the scientific community to this technique.

1.1.1 Principle of MLPA Assay

MLPA is a multiplex PCR assay that utilizes up to 50 probes, each specific for a different DNA sequence (mainly exons of a specific gene of interest), to evaluate the relative copy number of each DNA sequence. Each probe is composed of two half-probes (5' and 3' half-probes), consisting of a target-specific sequence and a universal primer sequence allowing the simultaneous multiplex PCR amplification of all probes (Schouten JP et al., 2002). In addition, one or both half-probes contain a stuffer sequence allowing differentiation during electrophoresis of the length of the probe itself, and, as a consequence, the size of the amplification product.

The MLPA reaction can be divided into five steps: (1) DNA denaturation and probes hybridization; (2) ligation reaction; (3) PCR amplification; (4) separation of amplification products by electrophoresis; (5) data analysis.
In the first step, the DNA is denatured and incubated with a mixture of MLPA probes. The two half probes are able to recognize contiguous target-specific sequences. The two half-probes can only be ligated and amplified in the presence of a perfect match without a single gap after the hybridization. PCR amplification is performed using only one PCR primers pair, one of which is fluorescently labelled. Because only ligated probes will be amplified during the subsequent PCR reaction, the number of probe ligation products is a measure of the number of target sequences in the sample. PCR products are then separated by size using Capillary Electrophoresis under denaturing conditions. The height or area of the PCR derived fluorescence peaks is measured, quantifying the amount of PCR product after normalization and comparing it with control DNA samples. It indicates the relative amount of target DNA sequence in the input DNA sample (Kozlowski P et al., 2008).

The quality of the reaction is assessed by the presence of control peaks providing information about the efficiency of the amplification and the correct amount of DNA used for the reaction. A key point in the MLPA reaction is that PCR does not amplify the target sequences, but the ligated probes. Thus, in MLPA a single pair of PCR primers is used for the amplification unlike the typical multiplex PCR which requires the use of specific PCR primers for each target sequence.

1.1.2 Applications of MLPA in Genetic Testing of dystrophinopathies

Several types of inherited neuromuscular disorder are due to deletions or duplications of specific genes. Among these, Dystrophinopathies (Duchenne Muscular Dystrophy, DMD, and Becker Muscular Dystrophy, BMD), Spinal Muscular Atrophy (SMA), Charcot Marie Tooth (CMT) disease and Hereditary Neuropathy with liability to Pressure Palsies (HNPP) represent a large portion of all mendelian neuromuscular disease for which genetic testing is routinely carried out for diagnostic purposes, for the identification of healthy carriers and for the evaluation of the recurrence risk. MLPA assay represents a powerful tool for the study of these different conditions.

DMD and BMD are X-linked diseases affecting 1:3500 and 1:18,000 birth males, respectively, both caused by mutations of the *DMD* gene on Xp21.2. In about 65% of DMD cases and up to 85% of BMD cases the pathogenic mutation is represented by large deletions of the *DMD* gene, while duplications of the same gene account for 5–10% of cases and point mutation are responsible for the remaining 25–30% of cases (Forrest SM et al., 1988).

In affected males, about 98% of deletions are easily detectable using a multiplex PCR approach that is able to analyze two hot spot regions (exons 2–20 and 44–53) (Chamberlain JS et al., 1988). However, this approach is not able to detect heterozygous deletions in female carriers, which represents a crucial point for the calculation of the recurrence risk of the disease within a family and the prevention of the birth of affected children. In fact, about one third of DMD cases are due to "*de novo*" mutations in children whose mothers are not healthy carriers and are thus at very low risk of recurrence of the disease. Moreover, DMD gene duplications cannot be detected by multiplex PCR approach either in affected males or in female carriers. As a consequence, a number of different approaches have been suggested for the identification of DMD duplications and heterozygous deletions, such as linkage analysis (Clemens PR et al., 1991), quantitative analysis of gene dosage (Prior TW et al., 2005) , FISH analysis (Vokova-Goldman A et al., 1997) , Entangled Solution Capillary Electrophoresis (ESCE) (Fortina P et al., 1997) ,Primed In Situ Labelling (PRINS) combined with FISH (Cinti C et al., 2002) , Multiplex Amplifiable Probe Hybridisation (MAPH) (White S et al., 2006), quantitative real time PCR (Jancourt F et al., 2004) and CGH array (Hegde MR et al., 2008).

MLPA analysis, based on the use of two SALSA kits able to investigate all the exons of the DMD gene and several control probes on sex chromosomes and autosomes, have been used by several groups in the study of DMD and BMD, both in affected patients and in female carriers (Schwartz M et al., 2004). All these studies reported MLPA as a simple,

rapid and reliable tool in the screening of deletions and duplications of the DMD gene, based on its ability to simultaneously hybridize and amplify all of the 79 DMD exons in only two reactions tubes, allowing a reduction in labour intensity compared with ESCE, PRINS, real-time PCR and MAPH. The usefulness of MLPA assay is evident in the study of suspected carrier females, where this approach represents a first choice method for the detection of heterozygous deletions/duplications and thus for the assessment of the carrier status in female relatives of affected males. In the study of affected patients, the MLPA ability to analyze all of the DMD exons provides high sensitivity and specificity and a sharp identification of the breakpoints of the rearrangements. This latter represents a crucial point in the management of DMD affected patients, since the determination of the full extent of the DMD gene deletions/duplications is critical knowledge for possible gene therapy strategies based on the skipping of specific exons involved in the deletion (Lalic T et al., 2005; Meow KT, 2005).

However, although some authors suggested that the identification of all exons involved in the deletion is critical for predicting the progression of the disease, it must be stressed that MLPA analysis is not able to provide information about the “in frame” or “out of frame” status of the deletions, which represents the crucial difference between DMD and BMD causing mutations. The frame-shift mutations in DMD patients result in the complete absence of dystrophin in their skeletal muscle because the translational reading frame of the mRNA is not maintained, whereas muscle tissue from BMD patients contains truncated dystrophin translated from the in-frame mRNA. The difference between “in frame” or “out of frame” deletions can be due to the involvement of even a single nucleotide, and is thus not detectable by MLPA, able to evidence the involved exons but not to identify the specific break points of the deletion. A crucial point in the interpretation of MLPA results is represented by the detection of deletions involving a single *DMD* exon. In these cases, in fact, the apparent deletion could actually consist of a change in the exon sequence hampering the correct hybridization of the specific probe. This sequence variation can be represented either by a *DMD* pathogenic point mutation or by a polymorphism not affecting gene function. Thus, apparent single exon deletions detected by MLPA should be checked by an independent method (Janssen B et al., 2005).

In order to further improve the throughput and speed of the MLPA approach in the diagnosis of *DMD* gene rearrangements, a modification of the original protocol has been described involving the use of a 96-well flow-through microarray system for the detection of the different probes, allowing the hybridization to be completed in 5 to 30 min (Zeng F et al., 2008)**.** In addition, a possible improvement in the detection rate of MLPA analysis is represented by the use of probe multiplexes, including specific probes

for common point mutations of the *DMD* gene, allowing both full dosage analysis and partial point mutation analysis in a single test (Bunyan DJ et al., 2007).

Due to the large number of genes that can be analyzed by a single technique, MLPA assay represents the gold standard for molecular analysis of all pathologies derived from the presence of gene copy number variation.

1.2 Hypothesis

a. MLPA has been used as a simple, rapid and reliable tool in the screening of mutations (deletions, duplications, point mutation) of the DMD gene, based on its ability to simultaneously hybridize and amplify all of the 79 DMD exons in only two reactions tubes providing high sensitivity and specificity and a sharp identification of the breakpoints of the rearrangements. The MLPA assay also compensates the limitations of those diagnostic procedures that are cost effective but have many limitations such as multiplex PCR and Southern blotting and those diagnostic assays that are quite effective but are very expensive as CGH array and sequencing. So, it can be used as an effective diagnostic tool at the genetic level for the diagnosis of genetic disease as DMD and BMD in the context of Nepal.
b. The major site of mutation of dystrophin gene in Nepalese population is likely to be in the hot spot regions as in the population of other countries of the world.

1.3 Objectives

1.3.1 Broad Objectives

- Screening of Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD) patients in central Nepal using MLPA (Multiplex Ligation Dependent Probe Amplification).
- Examination of the hot spot regions of mutation in DMD and BMD patients in Nepalese population.
- Introducing the MLPA assay as a rapid and reliable molecular method of genetic disease diagnosis as dystrophinopathies (DMD and BMD).
- Generation of the base line data of the Duchenne and Becker muscular dystrophy in Nepal.

1.3.2 Specific Objectives

- To collect the blood sample of the patients, clinically diagnosed as DMD or BMD from MDF-Nepal.

- To extract the DNA from the collected blood samples using the standard DNA extraction method or kit.
- To quantify the DNA samples extracted using the spectrophotometric technique.
- To amplify the DNA samples using the multiplex PCR based MLPA assay.
- To run the capillary zone electrophoresis (CE) of the PCR products using the standard genetic analyzer.
- To analyze the electropherograms obtained from the capillary electrophoresis and determine the site of mutations (deletion and duplication) in the individual patients.

1.4 Justification of study

The preponderance of genetic diseases in Nepal is not much different than that of the other countries, as suggested by some of the clinically diagnosed genetic diseases. But due to the lack of reliable method of diagnosis even the base line data of most of the genetic disease are not created yet. Thus the application of reliable molecular diagnostic tool has been very important. The introduction of the reliable, rapid and cost effective molecular method as MLPA for the diagnosis of the genetic diseases would be helpful in rapid diagnosis of the genetic diseases. Consequently, it would be of more help in genetic counselling which may contribute to more or less extent in reducing the number of cases of such genetic diseases. Among more than twenty different muscular genetic diseases known nine of the muscular dystrophies (Duchenne muscular dystrophy, Becker muscular dystrophy, Congenital muscular dystrophy, Distal muscular dystrophy, Emery-Dreifuss muscular dystrophy, Facioscapulohumeral muscular dystrophy, Limb girdle muscular dystrophy, Oculophryngeal muscular dystrophy and Myotonic muscular dystrophy) are well studied. And among these nine muscular diseases Duchenne muscular dystrophy is the commonest and best-known of the muscular dystrophies followed by Becker muscular dystrophy (Goh KJ et al., 2005; Laing NG et al., 2011)**.** But in the context of Nepal, the diagnosis of such widely known genetic disease is confined to physical examination, general personal medical history or family medical history and some laboratory tests that cannot entirely be trusted. Therefore this research would be a preliminary step towards the use of reliable molecular assay in the diagnosis of genetic diseases as muscular dystrophy (Murakami N et al., 2005).

In developing countries like Nepal the cost of diagnosis is another important criterion that should be considered. MLPA assay fulfil this need to some extent along with the reliable diagnosis. Thus the introduction of this highly sophisticated, cost effective and reliable molecular diagnostic method would turn out to be highly effective in genetic disease diagnosis in country like Nepal.

1.5 Scope of the study

MLPA assay has been applied efficiently in the genetic disease diagnosis and analysis of various mutation patterns in many forms of genetic diseases whose pathogenesis is primarily related to the presence of deletions or duplications of specific genes. Moreover, this assay can also be used in the molecular diagnosis of genetic diseases characterized by the presence of abnormal DNA methylation. This is the technique that can analyze a large number of genes containing the copy number variation.

In context of Nepal, a reliable diagnostic procedure for the disease diagnosis is yet to be introduced. This study is the pioneer work in the use of molecular tool as the rapid and reliable diagnostic tool against the genetic diseases. It will also help to create the base line data of the genetic diseases as DMD and BMD in Nepal. This study is likely to pave the pathway for the genetic counseling of parents. Thus this research is likely to stand in favor of sufferers and their parents to know about the genetic status of the diseases (as BMD or DMD), further documenting the gene mutation proportion and making them aware of possible familial pattern (X-linked pattern) of transmission of disease through carrier mother to the affected child.

It'll also give an rapid, accurate and reliable result in genetic disease diagnosis as PCR is sensitive, and multiplexing meant to study at gene level show the corresponding defects due to mutation in multiple exons of DMD gene (for instance, deletion can be determined by failure to amplify the targeted exon by PCR) at a single run of an isolated DNA. It is likely to make the people aware of the PCR based molecular diagnostics and makes them ready for receiving recent gene therapy based treatment and ensures people right of health service on the basis of whether patient are DMD or BMD suffer. Once the MLPA assay is established it can be used as the faster, reliable as well as easier diagnostics method of genetic disease diagnosis in the context of Nepal, where genetic tests aren't affordable and are not easily available in the present context.

CHAPTER II: LITERATURE REVIEW

2.1 Genetic and chromosomal disorder

Genetic disorders can be described as discrete events that affect the gene expression in a group of cells related to each other by gene linkage and may be manifested by the phenotypic deformities. Most genetic disorders are caused by an alteration in DNA sequences that alter the synthesis of a single-gene product. But there are many other genetic disorders that are caused by chromosome rearrangements that result in deletion or duplication of a group of closely linked genes or by mistakes during mitosis or meiosis that result in an abnormal number of chromosomes (Barsch, 2002).

There are number of genetic disorders known till date, but for the convenience of study they can be included in one of the following sub-heads, Single gene disorder and multifactorial inheritance disorder, depending upon the site of defective gene (as nucleus or mitochondria), type of defect (single or multifactorial), mode of inheritance and causative agents of the disease or disorder.

2.1.1 Single gene disorder

Single-gene disorders are caused by a single defective or mutant gene which may be present on an autosome or the X-chromosome. Thus they may be autosomal or X-linked disorders. But the single gene disorder also includes the disorders due to codominance and mitochondrial gene disorder in which the rate of mutation is higher than the nuclear DNA and has no repair mechanism. The single gene disorders follow the Mendelian patterns of inheritance and are often referred to as Mendelian disorders.

The **autosomal disorders** affect only one member of an autosomal gene pair (matched with a normal gene) or both members of the pair. Most of the single gene disorders are autosomal dominant and autosomal recessive are less common accounting approximately one third of the single gene disorders.

The **X-linked or Sex-linked disorders** are almost always associated with the X-chromosome, or female chromosome, and the inheritance pattern is predominantly recessive. Because of a normal paired gene, female heterozygotes rarely experience the effects of a defective gene. Currently, all sex-linked genetic disorders are thought to be X linked, and most are recessive. The only mutations affecting the Y-linked genes are involved in spermatogenesis and male fertility and hence are not transmitted (Nussbaum, 2001).

2.1.1.1 Pedigree Characteristics of X-Linked Recessive Inheritance

A male child of a carrier mother has a 50% risk of inheriting the disorder. A female child of a carrier mother has a 50% risk of inheriting the gene mutation and thus being a carrier herself. An affected male will pass on the mutation to all daughters, who are therefore obligate carriers but never on to a son. A woman who has both a brother and a son affected with an X-linked disease is also an obligate carrier (Evans et al, 2005).
There are certain pitfalls in recognizing X-Linked Recessive Inheritance with accuracy. It may be due to small family size with few male children that may make the pattern of an X-linked recessive disorder difficult to discern. It may also be due to the de novo mutation as the affected male may be the first person in the family with the condition. A new mutation may arise in testis or ovary, resulting in a parent who can pass on the condition or the carrier state to children, without being either affected (in the case of a male parent) or a carrier (in the case of a female parent). The germ line mosaicism can be another probable cause. X-linked recessive disorders include the fragile X syndrome, glucose-6-phosphate dehydrogenase deficiency, hemophilia A, and X-linked agammaglobulinemia, Bruton-type hypogammaglobulinemia, Duchenne and Becker muscular dystrophy etc.

2.1.2 Multifactorial inheritance disorders

Multifactorial inheritance disorders are caused by multiple chromosomal genes and, in many cases, environmental factors. Chromosomal defects usually develop because of defective movement during meiosis or because of breakage of a chromosome with loss or translocation of genetic material. There are many congenital disorders that are thought to arise through multifactorial inheritance as cleft lip or palate, clubfoot, congenital heart disease etc. (Muchir A et al., 2007).
The environmental factors that the embryo shares with the mother and the physiologic status of the mother—her hormone balance, her general state of health, her nutritional status, and the drugs she takes—undoubtedly influences the development of the unborn child. For example, diabetes mellitus is associated with increased risk for congenital anomalies. Other agents, such as radiation, drugs and chemicals (as alcohol, anticoagulant as warfarin, anticonvulsants, cancer drugs as aminopterin, methotrexate and 6-mercaptopurineetc), infectious agents (such as viruses as cytomegalovirus, herpes simplex virus, measles (rubella), mumps, varicella–zoster virus (chickenpox)) and non-viral factors as syphilis and toxoplasmosis can also lead to mutagenic effects (Maitra and Kumar, 2003). Environmental factors play a greater role in disorders of multifactorial inheritance that develop in adult life, such as coronary artery disease, diabetes mellitus, hypertension, cancer, and common psychiatric disorders such as manic depressive psychoses and schizophrenia.

2.2 Muscular Dystrophy

The muscular dystrophies (MDs) are inherited muscle disorders that are characterized by progressive skeletal muscle weakness, defects in muscle proteins and the death and wasting of muscle cells and tissues (Harrison, 2005). The most prominent account of muscular dystrophy began in the decade of 1860s. In the following decade, the French neurologist Guillaume Duchenne gave a comprehensive account of thirteen boys with the most common and severe form of the disease, which now carries his name- Duchenne muscular dystrophy (Emery AE, 2002).

The general signs and symptoms of patients suffering from muscular dystrophy include progressive muscle wasting, poor balance, drooping eyelids, atrophy, scoliosis, inability to walk, frequent falls, waddling gait, calf deformation, limited range of movement, respiratory difficulty, joint contractures, cardiomyopathy, arrhythmias and muscle spasms (http://www.sciencedirect.com/science/article).

2.3 DYSTROPHIN: GENE AND PROTEIN

2.3.1 Evolution of the DMD gene family

To gain an insight into the evolution of the large and complex DMD gene and its function in lower organisms, Nudel et al. identified and cloned the sea urchin homologue of the gene and began to complete the cloning of the drosophila gene. The characterization of the sea urchin and drosophila genes and their products helped them to construct an evolutionary tree connecting the dystrophin gene family in the vertebrates with related genes in invertebrates. Uri Nudel et al. had studied the structure and evolution of the huge and complex DMD gene, the regulation of expression and the function of the various products emphasizing on Dp71, and the possible involvement of Dp71 in brain function and in embryonic development. The X-gal staining of Dp71 null embryos revealed a very specific and interesting pattern of Dp71 promoter activity which includes parts of the nervous system, eyes, limb buds, lungs, blood vessels, vibrissa and hair follicles (Nudel U et al., 2001).

2.3.2 Dystrophin gene and gene sequence

The identification of the DMD gene on the X-chromosome was the first triumph of positional cloning. It opened up a new era in DMD research. The gene was localized to Xp21 by studies of rare female DMD patients with balanced X-autosome translocations with the translocation breakpoint in Xp21. The DMD gene was found to be the largest gene described, spanning ~2.5 Mb of genomic sequence (Monaco AP, 1992) or roughly

0.1% of the genome and composed of 79 exons (Roberts RG et al., 1992) that together account for only 0.6% of its sequence. The full-length 14-kb mRNA transcribed from the DMD locus was found to be predominantly expressed in skeletal and cardiac muscle with smaller amounts in brain and covered a large genomic region (MRC Holland, 2013).

2.3.3 Dystrophin protein family and dystrophin protein

Dystrophin protein is a cytoskeletal protein that is a member of the β-spectrin/α-actinin protein family (Koening M et al., 1990). This family is characterized by an NH_2-terminal actin-binding domain followed by a variable number of repeating units known as spectrin-like repeats.

Dystrophin protein is a rod shaped cytoskeletal protein of 427 kDa translated from a 14 kb mRNA localized to the cytoplasmic face of skeletal and cardiac sarcolemma. It can be organized into four separate regions based on sequence homologies and protein-binding capabilities: the amino-terminal domain (N) which has a high homology with the actin-binding region of α-actinin and α-spectrin, a series of 24 repeats of 109 amino acid motif that are similar to the triple helical repeats of spectrin in the form of a triple helix (rod) a cysteine-rich domain, homologous to the calcium binding region of α-actinin and the C-terminus domain (C) (Angelini C et al., 1990, 1994, 1996). The repeating unit accounts for the majority of the dystrophin protein and is thought to give the molecule a flexible rod like structure similar to β-spectrin. These α-helical coiled-coil repeats are interrupted by four proline-rich hinge regions (Hayashi YK et al., 1998).

At the end of the 24th repeat is the fourth hinge region that is immediately followed by the WW domain. The WW domain binds to proline-rich substrates in an analogous manner to the Src homology-3 (SH3) domain (Macias MJ et al., 1996). This region mediates the interaction between β-dystroglycan and dystrophin. The WW domain separates the rod domain from the cysteine-rich and COOH-terminal domains.

The ZZ domain is also part of the cysteine-rich domain and contains a number of conserved cysteine residues that are predicted to form the coordination sites for divalent metal cations such as Zn^{2+} (Ponting CP et al., 1996). The ZZ domain of dystrophin binds to calmodulin in a Ca^{2+}-dependent manner and may have implications for calmodulin binding to other dystrophin-related proteins. The ZZ domain does not appear to be required for the interaction between dystrophin and β-dystroglycan (Rentschler S et al., 1999).

The COOH terminus of dystrophin contains two polypeptide stretches that are predicted to form α-helical coiled coils similar to those in the rod domain (Blake DJ et al., 1995). Each coiled coil has a conserved repeating heptad $(a, b, c, d, e, f, g)_n$ similar to those

found in leucine zippers where leucine predominates at the "d" position (Lupas A, 1996). This domain has been named the CC (coiled coil) domain. The CC region of dystrophin forms the binding site for dystrobrevin and may modulate the interaction between syntrophin and other dystrophin-associated proteins (Sadoule-Ouccio HM et al., 1997)

2.4 Mutation in Dystrophin gene

The extremely large size of the dystrophin gene leads to a complex mutational spectrum. Deletions account for approximately 65% of DMD mutations (Monaco AP et al., 1985) and 85% of BMD mutations. Duplications occur in approximately 6 to 10% of males with either DMD or BMD. The remaining 30% to 35% of mutations consist of small deletions, insertions, point mutations, or splicing mutations, most of which introduce a premature stop codon (Madhuri R et al., 2008). The vast majority of large deletions detected in dystrophin gene causing BMD and DMD cluster around two mutation "hot spots" (Koenig M et al., 1990), although the reasons for this are unclear. Deletion cluster region I spans exons 45–53 (Beggs AH et al., 1990) and removes part of the rod domain, while deletion cluster region II spans exons 2–20 and removes some or all of the actin-binding sites together with part of the rod domain (Liechti-Gallati S et al., 1989).

Despite various setbacks due to mutation a small number of useful mutations have also been identified that generate a mutated or truncated protein and convey information regarding the functional importance of the different dystrophin domains (Hoffman EP et al., 1987; Chaudhary AG et al., 2009).

2.4.1 Cause of mutation in dystrophin gene

The genomic rearrangements that range from simple deletions or duplications to complex rearrangements are mediated by mutational mechanisms that are not completely understood. However, several mechanisms, including non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ), fork stalling and template switching (FoSTeS), and micro-homology-mediated replication-dependent recombination (MMRDR), have been hypothesized to explain the cause of different genomic rearrangements (Stankiewicz P et al., 2006).

Micro-homology-mediated mechanisms (BISRS, MMBIR, and FoSTeS) explain genomic deletions and duplications, as well as complex rearrangements as happening by several template slippages (Chen JM et al., 2005) or switch events due to micro-homology. According to these mechanisms, deletions and duplications are a result of occasional dislodging of the replicating polymerase or primer and re-engagement at a different template position based on micro-homology, to continue replication (Chen JM et al., 2010). The common hypothesis is that upon replication fork stalling, the leading/lagging

strand primer/polymerase disengages from its original template, translocates, and then re-associates. These models further postulate that during replication, downstream fork switching (forward invasion) results in a deletion, whereas switching to an upstream fork (backward invasion) results in a duplication, and repeated switches back and forth result in complex rearrangements that can include triplications and inversions (Hastings PJ et al., 2009). Aberrant origin firing is a plausible explanation for duplication copy number variations (CNVs). Firing of dormant origins may cause re-replication of the DNA template, leading to DNA duplications. Plasticity of origin selection during replication stress has been shown to potentiate dormant origin firing and subsequent duplications (Rosenfeld JA et al., 2010).

Furthermore, since DMD is a late replicating gene, it has less time to recover from any stress and complete replication prior to the end of S phase and subsequent chromosome condensation, thus contributing to the high frequency of unrecovered deletion events seen (McAvoy S et al., 2007).

2.5 Duchenne and Becker muscular dystrophies (DMD and BMD)

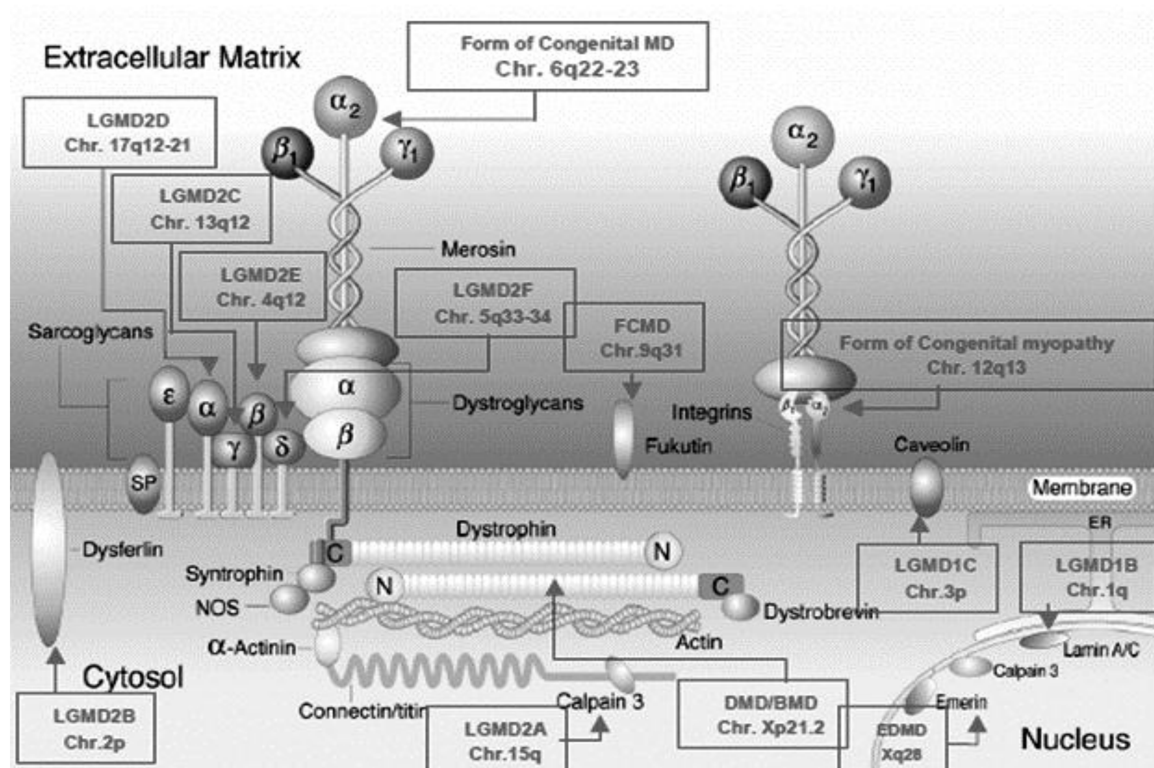


Fig.2.5: Proteins and muscular dystrophies identified by molecular analyses

Photo Source: Corraado Angelini (2002)

The dystrophin associated glycoprotein complex (DAG) spans through the sarcolemma and provides a linkage between actin and laminin α-2 (merosin) in the extracellular matrix. The DAG is composed of dystrophin (427 KDa), α-dystroglycan and β-dystroglycan (which bind to merosin by the sarcoglycan (SG) complex). The SG complex is composed of five transmembrane glycosylated proteins i.e. alpha (α), beta (β), gamma (γ), delta (δ) and epsilon (ε)-sarcoglycan. Partial or complete absence of one of these proteins might lead to muscle cell necrosis and initiate a cycle of degeneration-regeneration in muscle, this will result in various types of muscular dystrophies, with a predominant proximal muscle involvement (Matsumura K et al., 1994; Brown C et al., 2008).

DMD and BMD (DMD; MIM# 301200 and BMD; MIM#300376) are X-linked recessive neuromuscular disorders caused by mutations in the DMD, affecting approximately 1 in 3,500 and 1 in 30,000 live male births, respectively. The vast majority of DMD mutations result in the complete absence of dystrophin, whereas the presence of low levels of a truncated protein is seen in BMD patients (Mehler MF et al., 2000; Skuk D et al., 2007).

2.5.1 Dystrophinopathy in female

Women with translocation involving X-chromosome with the break point in Xp21.1 gene and an autosome show the preferential inactivation of normal X gene with normal DMD allele. It may give rise to the phenotype of females with full blown dystrophinopathy. A mutation in the DMD gene in the only X-chromosome of girls with Turner syndrome (a chromosomal disorder in female that have only one X chromosome) results in a phenotype similar to that of affected males. Uniparental disomy for the X-chromosome with a mutation in the DMD gene has been described once. Women with a 46,XY karyotype and DMD caused by the co-occurrence of mutations in both the dystrophin and the androgen-receptor genes have been described. And two women have been described with a normal karyotype and mutations in both *DMD* genes. One, a 14 year old girl with consanguineous parents, is homozygous for the mutation (Lindenbaum et al., 1979). The other is a 15 year old girl with compound heterozygous mutations. The female patients are usually heterozygous carriers, who carry a random, or non-random, inactivation of the X chromosome gene (Mendell JR et al., 2002).

2.5.2 Duchenne muscular dystrophy (DMD)

DMD or Duchenne muscular dystrophy, named after Dr. Duchenne de Boulogne, is one of more than 20 types of muscular dystrophy caused by the defect in dystrophin gene located in the short arm of the X-chromosome (Xp21.2) that results in the defect in the single important protein in muscle fibers called dystrophin. DMD is an X-linked recessive

disorder that affects only boys (with extremely rare exceptions). In DMD the "out of frame rule", demonstrable in most cases, predicts that frame-shift mutations will produce no dystrophin or an unstable dystrophin molecule that is rapidly degraded leading to DMD disorder.

2.5.2.1 Signs and symptoms: DMD

Typically, DMD patients are clinically normal at birth, although serum levels of the muscle isoform of creatine kinase (CK) are elevated. Duchenne dystrophy boys show disease onset in early childhood between the ages of 2 and 5 years, and there is often a delay in the achievement of motor milestones including a delay in walking, unsteadiness, with difficulties in running and later, climbing stairs. Their walking ability is lost around 5 years of age and the eventually decreased lower-limb muscle strength and joint contractures result in wheelchair dependence usually by the age of 12 (Emery AEH, 1993). Some boys also have learning and or behavioural difficulties, which may begin to manifest at this stage. Weakness of the arms occurs later along with progressive kyphoscoliosis. Subsequently, the onset of pseudohypertrophy of the calf muscles, proximal limb muscle weakness, and Gowers' sign suggest DMD (Gowers W, 1897). Patients normally die in their late teens or early twenties usually of respiratory failure due to intercostal muscle weakness and respiratory infection. Death can also be the result of cardiac dysfunction with cardiomyopathy and/or cardiac conduction abnormalities (Emery AEH, 1993).

Thus depending on the stage of the disease the patient may show delayed motor development, frequent fall, awkward standing posture, Gower's sign-positive and they may become wheel chair bound, be unable to hold body even the head, get bed ridden and show cardio respiratory dysfunction.

2.5.3 Becker muscular dystrophy (BMD)

Becker Muscular Dystrophy, named after the German doctor Peter Emil Becker (Becker PE, 1957), is the allelic form of DMD. "In frame" deletions in the rod domain give rise to intermediate or milder BMD. According to the clinical severity the groups of BMD patients are usually classified as asymptomatic or sub-clinical, benign, moderate and severe. The dystrophin molecule in BMD is often a truncated protein as predicted from the genetic defect, the size of deletion being inversely proportional to the size of protein produced. However in some cases duplications create an enormous dystrophin molecule (Angelini C et al., 1990; Bushby KMD et al., 1993) leading to the disorder. Being the X-linked genetic disease, women rarely develop symptoms of BMD. However, they may do so due to mosaicism.

2.5.3.1 Signs and Symptoms: BMD

In individuals affected by BMD, the clinical course is similar to that of DMD, although the onset of symptoms and the rate of progression are delayed. Symptoms usually appear in men at about ages 8–25, but some men are not diagnosed with BMD until they are in their thirties. More than 90% of patients are still alive in their twenties, with some patients remaining mobile until old age (Emery AEH, 1993).

The CPK (creatine phosphokinase) levels in blood is elevated at younger ages and decreases later in life, perhaps because muscle degeneration occurs more rapidly at younger ages, when there is also more muscle mass to deteriorate. The patient show toe-walking (equinus) and there is the use of Gower's Manoeuvre or a modified form of Gower's Manoeuvre to get up from floor. Patients can lose the ability to walk as early as age 15 in the very rare severe form. Skeletal deformities of chest and back (scoliosis) and the muscle deformities as contractions of heels, legs and pseudohypertrophy of calf muscles are seen. But the cardiomyopathy does not occur in BMD as commonly as in Duchenne's muscular dystrophy. The other complications that BMD can give rise to include deformities (particularly kyphosis), mental impairment (less common as compared to DMD), cardiomyopathy (noncompaction myopathy), pneumonia or other respiratory infections and respiratory failure (Dezawa M et al., 2005).

2.6 Position of DMD gene in National Centre for Biotechnology Information (NCBI)

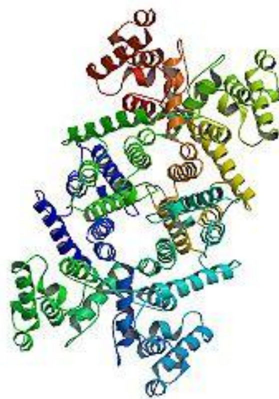


Fig 2.6 Dystrophin protein

In NCBI, the dystrophin gene is officially full named as 'dystrophin' and symbolized as 'DMD' and is categorized as a 'protein coding' gene. As cited in NCBI, Dystrophin gene code for the protein called dystrophin. Dystrophin (as encoded by the Dp427 transcripts) is a large, rod-like cytoskeletal protein which is found at the inner surface of muscle fibers. The gene was identified through a positional cloning approach, targeted at the isolation of the gene responsible for Duchenne (DMD) and Becker (BMD) muscular dystrophies. The dystrophin gene located in Xp21.2 chromosome is highly complex, containing at least eight independent, tissue-specific promoters and two poly A-addition sites.

The dystrophin RNA is differentially spliced, producing a range of different transcripts, encoding a large set of protein isoforms. NCBI has listed out some details on various

isoforms of the dystrophin protein viz. Dp427c, Dp427m, Dp427l, Dp427p1 , Dp427p2 , Dp260-1, Dp260-2, Dp140, Dp116, Dp71, Dp71b,Dp71a, Dp71ab, Dp40, Dp140c, Dp140b, Dp140ab andDp140bc.

2.7 Muscular dystrophy in Nepal

Nepal is one of the least developed countries in terms of health and medical scenario having the average doctor to patient ratio of 1: 10,000 (WHO, 2010) for health care within more than 26-million populations in total. The available data on the number of DMD patients shows the same proportion of 1:3500 in Nepal, as in the world population. But there are few recorded cases of patient suffering from BMD. However, the secondary survey, data collection and diagnostic research are yet to be done to get the factual report of the disease and the patients.

2.7.1 Parent project muscular dystrophy (PPMD)

A project called PPMD (Parent project muscular dystrophy) was established in 2002 A.D by the common effort of some of the parents of the patients suffering from DMD/BMD and relatives. This project deals with the various fields of muscular dystrophy as planning and finalizing on different programs/activities for Research, Treatment and Hope in relation to DMD, help in the search for a care/viable treatment for Duchenne Muscular Dystrophy globally, activate the role of Parent Project for treatment and further action and conduct public awareness programs regularly in association with the supporting agencies and volunteers.

2.7.2 Muscular Dystrophy Foundation-Nepal (MDF-Nepal)

The MDF-NEPAL was registered in HMG District Administrative office, Lalitpur as a non-profit making voluntary organization on 2003 A.D. It is a voluntary organization, formulated in initiation of Parents of Muscular Dystrophies (especially Duchenne Parents), Medical professionals and some Social activists (Physiotherapists, Occupational therapists, Counselors, Medical professionals, Artists, Fund raisers and Event managers etc.). MDF-Nepal is dedicated to disseminate the information about the disease and the way to comfort both the patients and their family to prolong the life span of the patients as far as possible. This organization was established to maintain a links with the global research organization, parent organization and associations, so that the latest information on research and rehabilitation management for muscular dystrophy can be obtained.

This foundation aims to create nation-wide awareness of the dystrophinopathies and other muscular disease and find the feasible treatment and cure for MD. It is directed towards the diagnosis and research of Muscular Dystrophy cases, facilitating the national level rehabilitation approach and participatory management. It has the novel objective of counseling the patients, parents and their families in order to create awareness about the disease on general public, parents and professionals. It also intends to provide the medical cure, treatment and equipment support to the patients.

2.7.3 Patients profile of patients suffering from muscular dystrophy in Nepal (2065/66)

The MDF (Muscular Dystrophy Foundation)-Nepal has recorded the patients profile with their name, type of MD, age, sex and the corresponding address. The profile was created on the year 2065/66 and there is no updated record till date. But still it gives us a lot of information about the type of MD that are present in Nepal and the prevalence rate of DMD and BMD in Nepal as per the record.

According to the patient profile of MDF-Nepal there were 463 MD patients recorded till the fiscal year 2065/66, among which 279 of them were DMD patients, 57 were BMD patients, 5 of which were not confirmed to be DMD or BMD and 122 were suffering from other muscular dystrophies as Limb-girdle muscular dystrophy (LGMD) and Fascioscapulohumeral muscular dystrophy (FSHMD). Thus the total patients suffering from the dystrophinopathies (DMD and BMD) were 336. Among the 463 recorded patients 443 of them are alive whereas the remaining 20 patients are already expired. Among the live DMD patients 5 of them are recorded to be female whose genotypic mutational confirmations are yet to be done. In case of BMD 13 out of 56 patients are recorded to be female. The age distribution of DMD/BMD patients shows that most of the DMD patients are within 10 to 20 years of age, whereas BMD patients above 20 years of age are recorded to be higher in number. Most of the DMD and BMD patients are found to be wheel chair bound as recorded. The exact distribution of DMD/BMD patients all over the country is not exactly known, but most of the patients are recorded from the capital which is likely to be due to the facilities available in the capital. Bheri and Karnali have least number of DMD/BMD patients recorded in the foundation as per the records.

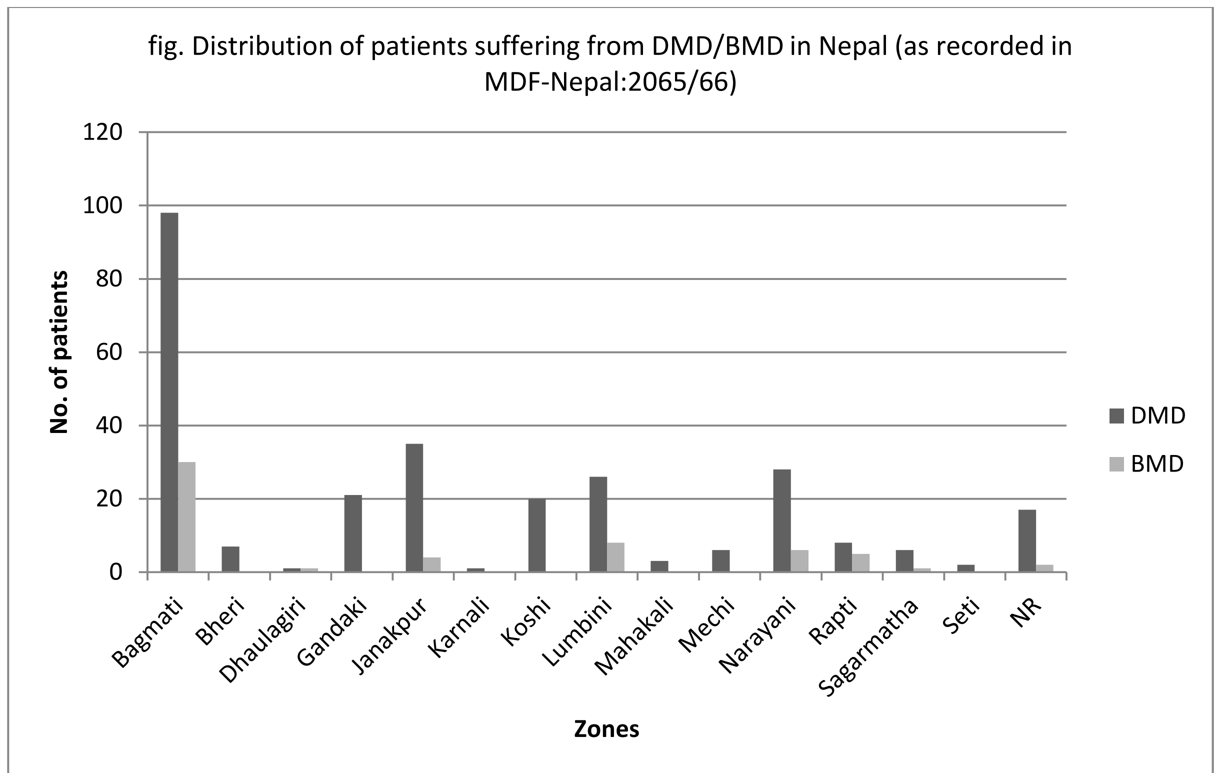


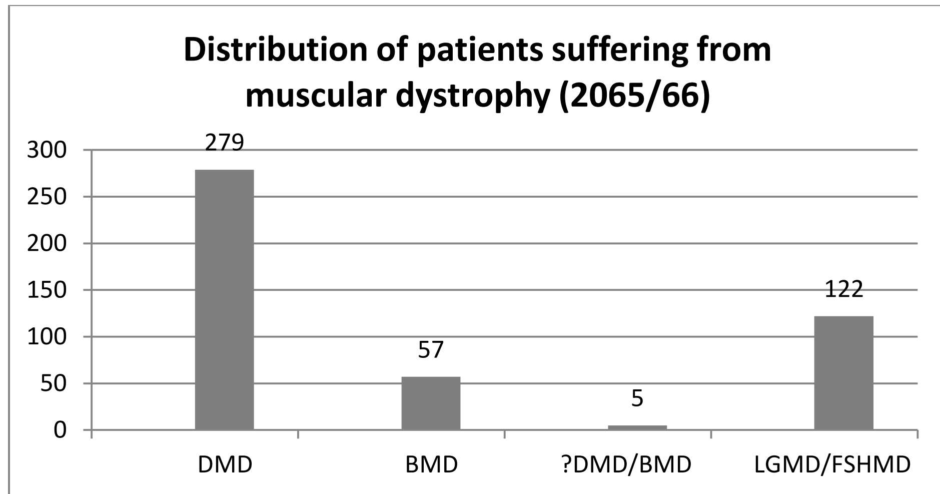
Fig. 2.7.3 (a): Distribution of patient suffering from DMD/BMD (MDF-Nepal (2065/66))

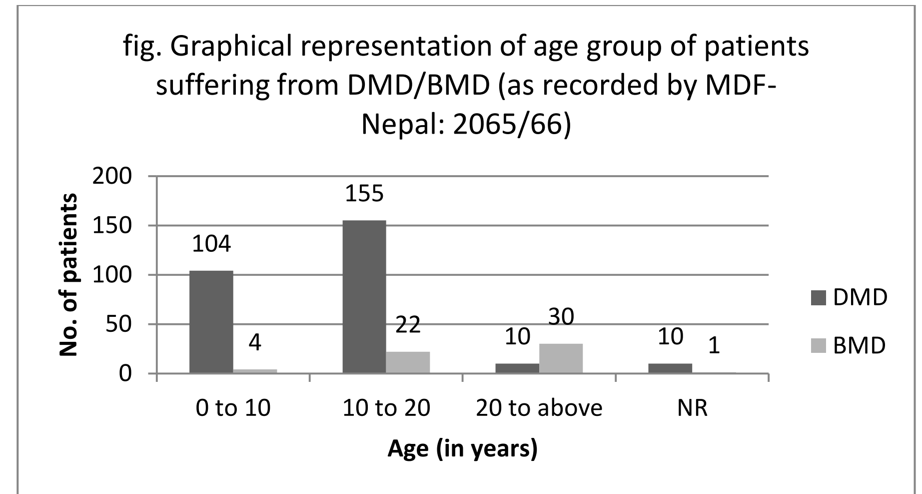
2.8 Diagnosis of DMD and BMD

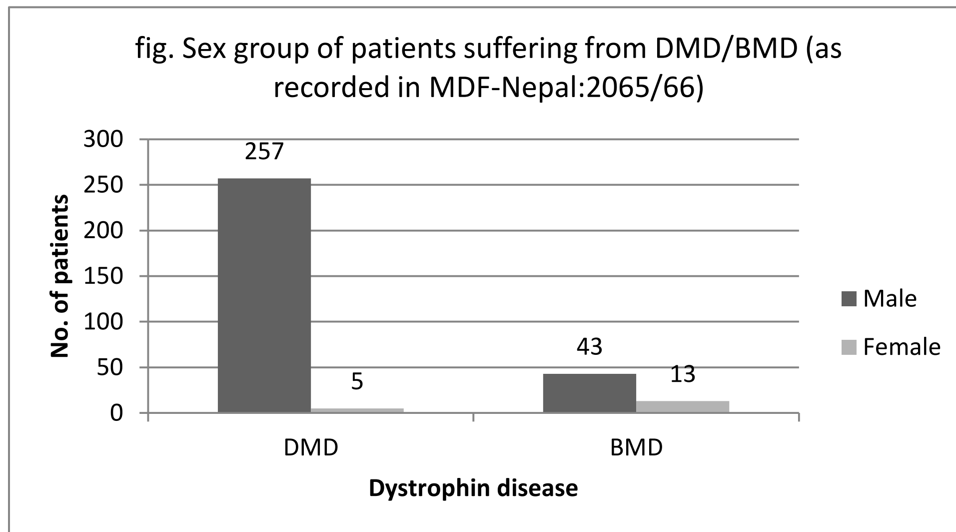
The suspicion of the diagnosis of DMD/BMD should be considered irrespective of family history and is usually triggered by the observation of abnormal muscle function in a male child, detection of increased transaminases (aspartate aminotransferase and alanine aminotransferase), detection of an increase in serum creatine kinase and various signs and symptoms associated with the disorders. The diagnosis of the disorder is confirmed by using various diagnostic measures that can be studied under two headings viz. Classical diagnostic measures and Novel diagnostic measures.

2.8.1 Classical diagnostic measures

The classical yet important diagnostic methods include Muscle biopsy, Southern and or Western blotting, Elevated creatine kinase level in blood and the Family history. Though these classical diagnostic methods can be a part of novel diagnostic methods, it is essential to apply the molecular methods for the confirmation and detection of disorder.







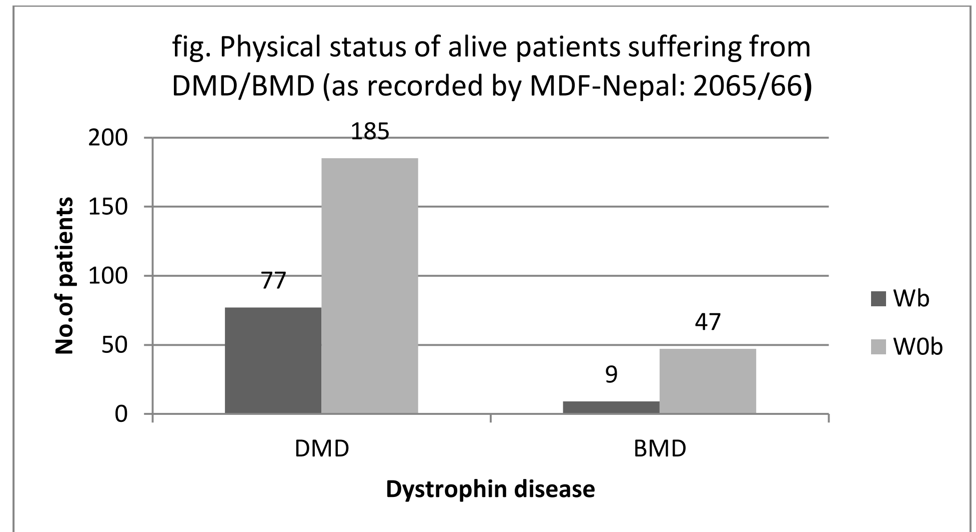


Fig 2.7.3 (b): Graphical representation of data recorded in MDF-Nepal (2065/66)

2.8.2 Novel diagnostic methods

The Novel diagnostic measures applied for the diagnosis of muscular dystrophies like DMD and BMD can be divided into two main heads depending upon the genetic testing of range of mutation: Gene testing for large deletion and duplication and Mutation scanning for point mutation.

2.8.2.1 Gene testing for large deletion and duplication

There are various techniques applied to detect the large mutations in dystrophin gene. If analysis by one or more of these techniques leads to the identification and full characterization of a dystrophin mutation, then no further testing is required. If deletion/ duplication testing is negative, then dystrophin gene sequencing should be done to look for point mutations or small deletions/insertions (Dent KM et al., 2005). The diagnostic measures applied in the diagnosis of muscular dystrophies worth mentioning include multiplex PCR, multiplex ligation dependent probe amplification (MLPA), Linkage by Quantitative multiplex fluorescence PCR (qmf PCR), STR (CA) segregation analysis, SSCP and Detection of virtually all mutations– Single strand conformation polymorphism (DOVAM- SSCP), Denaturing High Performance Liquid Chromatography (DHPLC) and Microarray based Mutation detection (Stockley TL et al., 2006).

Multiplex PCR is widely available and the least expensive method of detecting the mutation in dystrophin gene. But it only detects deletions and does not cover the whole gene so that a deletion might not always be fully characterized (Bushby K et al., 2012). MLPA is much more accurate and is suggested as the ideal method used to confirm the deletions and, more importantly regarding clinical outcome, to reliably determine its boundaries (Schwartz M et al., 2004). But the single-exon deletions must be confirmed by a second method (such as sequencing), because single nucleotide polymorphisms (SNPs) in some regions tested will appear falsely as deletions (Schouten JP et al., 2002). Linkage by Quantitative multiplex fluorescence PCR (qmfPCR) and STR (CA) segregation analysis methods are the methods that are helpful in validating the mutation patterns as detected by MLPA technique. The strategy, termed “DOVAM-S” (detection of virtually all mutations–SSCP) relies on multiplexing as many as 23 amplicons per lane, with SSCP in as many as five conditions. DHPLC has become a standard pre-screening tool for mutation detection, offering very high efficiency and sensitivity of detection. Microarray based Mutation detection is a novel CGH -based testing strategy that cover the entire 2.4-Mb genomic region of the dystrophin gene enabling the mutation detection in both male and female patients.

2.8.2.2 Mutation scanning for point mutation

Testing of dystrophin point mutation requires sequencing of all 79 exons and 8 promoters. There are no particularly common point mutations or point mutation hotspots, and each affected family may carry a unique mutation (private mutations) in this enormous gene. The two major methods that are generally applied for the detection of point mutation include **SCAIP (Single condition amplification/ internal primer) and Sanger sequencing**.

SCAIP is a direct sequence analysis that relies on amplification of a large number of exons at a single set of PCR temperatures. In case of the dystrophin gene, the method applied results in complete double-stranded sequencing coverage of all known coding regions and in seven of the eight tissue-specific promoters (Dp427m, Dp427p, Dp427c, Dp427l, Dp260, Dp140, and Dp116). This method also allows detection of ~2% of the patients with exonic deletions not detected by the widely available multiplex PCR technique. The chain termination or dideoxy method of nucleic acid sequencing is a very sensitive analytical technique. Though there are many diagnostic procedures that can determine the various forms of mutations in the dystrophin gene, the reliable test of detecting the point mutation is the sequencing. But this technique is not affordable in developing countries due to the economic reason.

2.9 MLPA: General introduction

Multiplex ligation dependent probe amplification (MLPA) was first described in 2002 A.D by Jan. P Schouten and his colleague as a new method of relative quantification of 40 different sequences as an easy to perform reaction requiring only 20 ng of human DNA (Schouten JP et al., 2002). MLPA is a method for the detection of unusual copy number changes of genomic sequences. It is a sensitive technique for the relative quantification of up to 50 different nucleic acid sequence in a single, easy to perform, reaction. It is primarily used for the detection of small copy number changes of DNA/RNA sequences like, all exons deletions or duplications of a gene. MLPA is much more accurate and is suggested as the ideal method used to confirm the deletions and to reliably determine its boundaries (Schwartz M and Duno M., 2004).

2.9.1 Principle of MLPA

MLPA is a special form of multiplex PCR in which the MLPA probe is amplified instead of the sample DNA. One MLPA probe consists of two probe oligonucleotides, one synthetic

and one M13 derived. If the probe target sequence is present in the sample, the two probe oligos hybridize next to each other. The hybridized probe oligos are connected by ligation. All ligated probes have identical end sequences, permitting simultaneous PCR amplification using only one primer pair. Each probe gives rise to an amplification product of unique size between 130 and 480 bp. The probe target sequences are smaller (50-70 nt). The prerequisite of a ligation reaction provides the opportunity to discriminate single nucleotide differences. The ligated probes are amplified in a multiplex PCR. Amplification of probes by PCR depends on the presence of probe target sequences in the sample. PCR reaction is very reproducible, as only one pair of PCR primers is used for amplification of all fragments.
The amplification products are analyzed by capillary electrophoresis. The peak patterns are generated on a patient sample and a reference DNA sample and the relative peaks are compared. Differences in relative amounts of probe target sequences in the sample result in different relative peak heights between patient sample and reference sample. The difference reflects the copy number changes of sequences detected by the MLPA probe.

2.9.2 An outline of the MLPA reaction

DNA (20-500 ng) is denatured and fragmented by a 5 minute heat treatment at 98^0C. The MLPA probes are added and allowed to hybridize for 16 hours at 60^0C in a thermocycler with a heated lid. The dilution buffer including the ligase enzyme is added and ligation is allowed to proceed for 15 min at 54^0C. After the heat inactivation of ligase and addition of PCR primers, dNTPs and polymerase, PCR amplification of the ligation products is started. One PCR primer is fluorescently or isotopically labeled. Amplification products are detected and quantified by capillary electrophoresis.

2.9.3 Reaction Conditions

All M13-derived MLPA probe oligonucleotides contain the complement sequence of one of the two PCR primers at their 3' end, and will thus be linearly amplified during the PCR. This PCR primer is unlabeled in order to prevent background signals. To prevent most of this PCR primer being consumed by linear amplification, low amounts of probe oligonucleotides are used. Despite the use of low amounts of probes, hybridization of probe oligonucleotides to their target sequence has to be complete in order to obtain reproducible results. So, a hybridization period of about 16 hours is used in an 8 µl reaction volume containing 1-4 fmol of most probe oligonucleotides.

The NAD requiring Ligase-65 enzyme is chosen for the ligation reaction which is active at 50-65^0C, but can easily be heat inactivated (95^0C) before the start of the amplification reaction. Ligase-65 is very sensitive to probe-target mismatches next to the ligation site. Apart from the copy number of the probe target sequence, the major factors influencing

the relative signal strength of MLPA probes is the amount of polymerase used in the PCR and the nature of the first nucleotide following the labeled PCR primer. The average signal strength increased in the order A<T<G<C. Primers that are not, or not sufficiently elongated will be melted during the transition from annealing temperature to the elongation temperature, but might also be more easily displaced by the complementary strand during the last PCR cycles.

2.9.4 Preparation and design of MLPA probes

Each MLPA probe consists of two oligonucleotides, one short synthetic oligonucleotide and one phage M13 derived, long probe oligonucleotide that can be ligated to each other when hybridized to a target sequence. All ligated probes have identical sequences at their 5’ and 3’ ends, permitting simultaneous amplification in a PCR containing only one primer pair. Each probe gives rise to an amplification product of unique size between 130 and 480 bp. The short synthetic oligonucleotide of each probe contains a target-specific sequence (21-30 nt) at the 3’ end and a common 19 nt sequence, identical to the labeled PCR primer, at the 5’ end. Each second long probe oligonucleotide contains a sequence of 25-43 nt at the 5’ end that is able to hybridize to the target sequence immediately adjacent to the first probe oligonucleotide, a common sequence used for PCR amplification at the 3’ end and a stuffer sequence of 19-370 nt in between.

For the preparation of each long MLPA probe, a target sequence-specific oligonucleotide of 25-43 nt is cloned in one of the M13-derived SALSA vectors. Each clone obtained is used to infect 1-1 culture of *E. coli* strain TG1. Since the chemically synthesized nucleotides of this size (80-440 nt) are not commercially available in the quality needed for MLPA, ssDNA from M13 clones containing small target-specific sequences are used for the preparation of these oligonucleotides. ssDNA is purified by polyethylene glycol (PEG) precipitation of phage particles, heat disruption of the virus and cetyl-trimethyl-ammonium bromide (CTAB) precipitation of the DNA. The single stranded DNA is made partially double stranded at the EcoRV and BsmI sites by the annealing of two short complementary oligonucleotides. Digestion by two restriction endonucleases by EcoRV and BsmI results in the formation of a large 7200 nt M13 fragment and the MLPA probe oligonucleotides (80-420 nt).

Table 2.9.4: Enzyme digesting to form M13 fragment and MLPA probe oligonucleotide

Enzyme	Source	Recognition site	Cut
EcoRV	*Escherichia coli*	5'GATATC 3'CTATAG	5'---GAT ATC---3' 3'---CTA TAG---5'
BsmI	*Escherichia coli*	GAATGCN, NGCATTC	5’---GAATGCN ---3’ 3’---CTTACGN---5’ (Isoschizomer)

One of these enzymes cuts the DNA outside its recognition sequence, resulting in a 5' phosphorylated end that is perfectly complementary to the target sequence. Most probe mixes made contain 35-42 probes with length differences between consecutive amplification products of 6-9 bp. All probes used in a specific MLPA probe mix are made in a different M13-derived vector and have different stuffer and hybridizing sequences. This probe oligonucleotide contains the 25-43 nt target-specific sequence at the 5' phosphorylated end, a 36 nt sequence that contains the complement of the unlabeled PCR primer and is common to all probes at the 3' end, and a stuffer sequence of variable length in between.

Amplification products of different probes have common sequence only at their ends in order to prevent heteroduplex formation during later stages of the amplification reaction when competition between duplex formation and PCR primer annealing takes place. A set of 118 different M13-derived MLPA vectors were prepared initially containing a stuffer sequence of different length and sequence in each. Target sequence-specific synthetic oligonucleotides can easily be inserted in these vectors, allowing flexibility to create all required fragment lengths. The sequence of the labeled primer is 5'-GGGTTCCCTAAGGGTTGGA-3' and that of the unlabeled primer is 5'-GTGCCAGCAAGATCCAATCTAGA-3'.

2.9.5 SALSA MLPA probemix PO34-A3 / PO-35 DMD / Becker

This SALSA MLPA probemix is designed to detect deletions/duplications of one or more sequences in the DMD gene in a DNA sample. A mutation or polymorphism in the sequence detected by a probe can also cause a reduction in relative peak area, even when not located exactly on the ligation site. In addition, some probe signals are more sensitive to sample purity and small changes in experimental conditions. Therefore, deletions and duplications detected by MLPA should always be confirmed by other methods.

2.9.5.1 Probe description

The SALSA MLPA probemix for DMD gene mutation analysis consists of two probe mixes named as "SALSA MLPA probemix PO34-A3 and PO35-A3". This probemix was developed by J. Coffa & J.P. Schouten at MRC-Holland.

The PO34/35 probemix contains probes for each of the exons of the DMD gene (79 exons) on Xp21.1 chromosome. In addition, one probe is present for the alternative exon 1 DP427c of DMD. These 80 probes have been divided into two probe mixes: PO34 and PO35 in an alternative fashion. Performing two probe mixes is thus sufficient to investigate the copy number of all exons. The probemix is designed to detect deletions/duplications of one or more sequences in the DMD gene in a DNA sample. Deletions of a probe's recognition sequence on an X-chromosome will lead to a

complete absence of the corresponding probe amplification product in males, whereas female heterozygotes are recognizable by a 35-50% reduction in relative peak value.

The P034-A3 DMD probemix contains 45 different probes with amplification products between 129 and 490 nt, as well as 10 control fragments generating an amplification product smaller than 120 nt. The control fragments include two (105-118 nt) chromosome Y-specific probes. The P035-A3 DMD probe mix contains 45 different probes with amplification products between 129 and 490 nt, as well as 9 control fragments generating an amplification product smaller than 120 nt. The control fragments includes one (105 nt) chromosome Y-specific probe.

During the data analysis, data generated by this probemix are normalised intra-sample by dividing the peak area of each amplification product by the total area of only the reference probes in the probe mix (block normalisation). Secondly, inter-sample normalisation can be achieved by dividing the intra-normalised probe ratio in a sample by the average intra-normalised probe ratio of all reference samples. The operation is automatically performed after the capillary electrophoresis in most advanced genetic analysers (MRC-Holland, 2013).

2.9.6 Probe Specificity

The major cause of the specificity of the MLPA probes is the need for two probe oligonucleotides to anneal at immediately adjacent sites of a target nucleic acid. In order to increase specificity, MLPA hybridization and ligation reactions are performed at 54-60^{0}C. Thermophilic NAD requiring ligases are not able to ligate oligonucleotides that leave a one base gap or have an overlap when bound to their target sequence. Furthermore, the ligase-65 enzyme used is very sensitive to mismatches between probes and target sequences next to the ligation site. NAD requiring ligases are, in general, most sensitive to mismatches at the last oligonucleotide (3' OH site) of the short probe oligonucleotide (Tong J et al., 1999). Omitting one of the probe oligonucleotides, or replacing it by an identical oligonucleotide having a mismatch at the 3' end prevents the appearance of probe amplification. The probes specific for sequence on human chromosome Y doesn't give any signal in female DNA.

2.9.7 MLPA vs. other diagnostic techniques

At present there are several techniques that are used for the detection of copy number changes of chromosomal sequences including standard chromosomal analysis, comparative genomic hybridization (CGH) (Kallioneimi A et al., 1990), fluorescence in

situ hybridization (FISH) (Klinger K et al., 1992), BAC arrays (Snijder AM et al., 2001), Southern blots and loss of heterozygosity (LOH) (Devilee P et al., 2001) assays. But most of these techniques have their own limitations that make them less suitable than MLPA technique.

Most of these techniques applied are not able to detect deletions or duplications of single exons. Besides, they are time consuming, difficult to implement as multiplex assays (FISH, LOH) and cannot detect small mutations (FISH). They may require large amount of sample DNA and may be labour intensive (Southern blots). The oligonucleotide and cDNA microarrays are still insufficiently sensitive and reproducible to detect a deletion or duplication of one copy of a small chromosomal sequence like a single exon. The real-time PCR provides the possibility to detect several fold amplification of chromosomal sequences, however, it is less sensitive to small copy number changes and its use in a multiplex assay is severely limited by spectral overlap of the fluorescent dye used. The presence of multiple primer pairs in a multiplex reaction also reduces the robustness of PCRs and the reliability of the quantification.

As demonstrated by the nucleic acid fingerprinting techniques such as amplified fragment length polymorphism (AFLP) and differential mRNA display, PCR can be used for simultaneous reproducible amplification of many DNA fragments in one reaction if a single primer set is used for amplification of all the fragments. Multiplex amplifiable probe hybridization (MAPH) is a similar method in which not random fragments but 40 different specific target sequences are detected and quantified (Armour JA et al., 2000). MAPH uses oligonucleotide probes that hybridise to specific nucleic acid sequences. Each hybridised probe can be simultaneously amplified with the use of a single primer pair that yields amplification products of unique size. The copy number of target sequences is reflected in the relative intensities of the MAPH probe amplification products (Armour JA et al., 2000). However, MAPH requires immobilization of sample nucleic acids and tedious washing of unbound (amplifiable) probes making it difficult to implement in a routine diagnostic setting. Sequencing, DHPLC (Denaturing high performance liquid chromatography), Size-sieving capillary electrophoresis (SSCE) and other techniques for the detection of point mutations, will not detect copy number changes of complete exons. MLPA is capable of overcoming most of these difficulties.

2.9.8 Features of MLPA

MLPA can detect the copy number of 40-50 genomic DNA sequences in a simple to perform, single PCR based reaction. It requires only 20 ng human DNA (3000 cells / 0.5 ml amniotic fluid) and can discriminate sequences that differ in only a single nucleotide. MLPA can also be used on partially degraded DNA, such as DNA extracted from paraffin imbedded, formalin treated tissues. It is an easy to use experimental work that only

requires a thermocycler and a sequence type electrophoresis system to perform. The high throughput and the results are obtained within 24 hours and up to 96 samples can be processed in one experiment.

MLPA can be used for various genetic implications as the copy number changes, determining the methylation status of imprinted and promotor regions, relative quantification of mRNAs, detection of the known point mutations and SNP's (single nucleotide polymorphisms) and much more. Besides, the amplification reaction of MLPA is very robust because MLPA makes use of a single primer pair. The large amount of probes and primers in the reaction makes the reaction stable for minor changes in reaction conditions.

2.9.9 Applications of MLPA

The major application of this technique includes the detection of the mutation as exon deletions and duplications in the human genes as DMD, BRCA1, MSH2 and MLH1 genes, micro-deletion syndromes as in Di-George syndrome, sub-telomeric and centromeric screening (P181/P82), imprinted regions and methylation detection (as in Hypermethylation of the MGMT promoter in brain tumors and detection of aberrant CpG island methylation), detection of trisomies such as Down's syndrome, detection of gain and loss of chromosomal regions, characterization of chromosomal aberration in cell lines and tumor samples and SNP (single nucleotide polymorphism) or SNP like mutation detection, DNA fingerprinting, homozygote/heterozygote discrimination in agriculture etc. MLPA probe mixes are also available for many other genes as MSH6, APC, NF1, NF2, VHL, FBN1, PARK, CFTR, SPAST, RB1, NSD1, MECP2 and many more. The MLPA assay can also be applied in relative quantification of mRNAs, detection of known mutations and in research.

2.9.10 Limitations of MLPA

MLPA is used to detect both deletions and duplications of coding regions of the dystrophin gene. However, finding duplications in males and some deletions in females is difficult. Single-exon deletions must be confirmed by a second method (such as gene sequencing), because single nucleotide polymorphisms (SNPs) in some regions tested will appear falsely as deletions. Besides, MLPA is more sensitive to impurities in the sample than ordinary PCR. The decreased signal of a probe can also be due to a rare polymorphism/SNP other than the deletion which gives the false positive result (Madhuri R et al., 2008). Design of the best possible probe sets requires sufficient sequence information. MLPA detects changes in relative amounts of the target sequences. But the female diploid and triploid cells cannot be distinguished by this method and MLPA assays for mRNA quantification have to be optimized for specific tissues. MLPA cannot be used on single cells and require at least 3000 cells. It cannot yet

be used on samples amplified by WGA (Whole Genome Amplification) methods. MLPA cannot detect balanced translocations and the dynamic range of MLPA is limited.

2.10 CAPILLARY ELECTROPHORESIS

2.10.1 Introduction

Capillary electrophoresis or capillary zone electrophoresis is generally used to separate ionic species by their charge and frictional forces and hydrodynamic radius. Capillary electrophoresis (CE) was developed from combining several features of different methods including the principle of gel electrophoresis, the fused silica capillary of gas chromatography (GC), and the highly sensitive detectors of high-performance liquid chromatography (HPLC). Separation in CE is based on different mobility of analytes under an electric field, which occurs in a capillary filled with buffer. Unlike other media (e.g., paper, agarose, and polyacrylamide gel), electrophoresis in a capillary can use a high voltage (up to 800 V/cm) due to the physical properties of fused silica capillaries. A large surface area to volume ratio of a capillary provides effective heat dissipation. The small dimension of a capillary requires small amounts of samples and buffer and the automation of CE requires less time and labour. Additionally, the high separation efficiency [number of theoretical plate (N) > 10,000], the on-column detection, and the various modes of CE make the technique attractive for solving challenging problems.

2.10.2 Principle of CE

The principle of CE is based on the different migration of solutes in an electric field as the electrophoresis is performed in narrow-bore capillaries filled with electrolyte. The mobility of analytes depends upon their sizes, charges, and degree of ionization, viscosity, temperature, and dielectric constant of the background electrolyte (BGE). Upon application of voltage, analytes are driven by two forces, the electrophoretic migration and the electro-osmotic flow (EOF). Analytes are usually introduced at the anode and are detected at the cathode. Cations migrate first with the highest velocities toward the cathode by the electrophoretic force and the EOF. Neutral compounds migrate with the same velocity of the EOF and are detected as one peak. Lastly, anions migrate by the force difference of the EOF toward the cathode and the electrophoretic flow from the anode in the opposite direction. By using sensitive detectors (e.g. laser-induced fluorescence), sensitivity in the atto-mole range can be achieved (Suntornsuk L, 2007).

2.10.3 Instrumentation

The main components of a system consists of a sample vial, source and destination vials, a capillary, electrodes, a high-voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample and then returned to the source vial. The migration of the analytes is then initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. The analytes separate as they migrate due to their electrophoretic mobility and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different retention times in an electropherogram.

Separation by capillary electrophoresis can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. In general the capillaries used in capillary electrophoresis are coated with a polymer for increased stability. The portion of the capillary used for UV detection, however, must be optically transparent. For the samples that naturally fluoresce or are chemically modified to contain the fluorescent tags fluorescence detection system is used in capillary electrophoresis. This mode of detection offers high sensitivity and improved selectivity for these samples. The laser induced fluorescence has been used in CE systems with detection limits as low as 10^{-18} to 10-^{21} mol.

2.10.5 ABI-310 Genetic Analyzer

The ABI PRISM 310 Genetic analyzer is a system composed of instrument hardware, a Macintosh computer, several types of software and consumables. It is a proven multicolor fluorescent labeling technology to achieve a completely automated, yet flexible system to sequence, size and quantitate nucleic acids. The ABI 310 Genetic Analyzer can perform two main operations, DNA sequencing and DNA fragment analysis using the GeneScan Software.

2.10.5.1 Instrument Hardware

The instrument hardware of ABI PRISM 310 Genetic Analyzer consists of Led Status Indicators (red, amber and green lights), doors, Autosampler region, Gel block region and Detection region. The Hardware located inside the ABI PRISM 310 is responsible for automated sample handling, Electrophoresis and Fluorescence detection.

The autosampler regions consist of four main parts: Autosampler, Cathode electrode, 48 or 96 well tray and electrode thumbscrew. The gel block region consists of various parts:

Gel pump button, Tray button, Syringe dive, Syringe dive toggle, Syringe guide, Luer valve, Anode electrode, Anode buffer valve, Anode buffer reservoir, Glass syringe, Waste valve, Waste vial, Capillary ferrule, Anode buffer valve actuator and Drip tray. The detection region consists of various important parts: Charge-Coupled Device (CCD), Laser Detector Window and Door, Heat Plate, Capillary, Heater Door and Thermal Tape.

Consumables: The consumables used during the operation include capillary tube having an opaque polyimide coating (except in the window area), Septa strips for the 96 well tray and GeneScan Polymer(Performance Optimized Polymer, 4% (POP-4) (P/N 402838)). The running buffer used for the electrophoresis is 10X Genetic Analyzer Buffer with EDTA (for use with POP-4) (P/N 402824).

2.10.5.2 Principal of operation

During the operation samples are generally labelled using a set-four dye that fluoresces at different wavelengths. In the fragment analysis as MLPA, a single dye is labelled in all the probes but they differ in the lengths due to the presence of different stuffer sequences of different length. A dye-labelled internal size standard is also placed in the tube. Three samples and a size standard can be electrophoresed simultaneously, as dyes come in sets of four colours. The sample tubes are placed in a tray in the instrument's autosampler. The cathode electrode and one end of a polymer-filled capillary are located near the autosampler. The autosampler successively brings samples in the sample tray into contact with the cathode electrode and one end of a glass capillary filled with polymer. An anode electrode at the other end of the capillary is immersed in buffer. A portion of the sample enters the capillary as current flows from the cathode to the anode. This is called electrokinetic injection that injects the sample into the capillary that forms a tight band in the capillary. The autosampler then moves the cathode buffer reservoir to the capillary and electrode to continue electrophoresis. Samples are electrophoretically separated as they travel through polymer in the capillary. As the DNA fragments pass through the window of the capillary, an argon-ion laser located behind the detector door excites the attached dye labels and they fluoresce. Emitted fluorescence from the dyes is collected by a CCD camera.
The Data Collection software creates a real-time display of the light intensities collected on the virtual filters. Virtual filters C, D and F are used only with the GeneScan Analysis Software and thus are the virtual filters that are compatible for the fragment analysis as MLPA. The software interprets the result, calculating the size or quantity of the fragments from the fluorescence intensity at each data point (ABI 310 Genetic analyser, User manual, 2010, Applied Biosystems).

In the experiment that we performed the dye labels are incorporated using 6-FAM labeled primers. The entire MLPA PCR uses a single pair of primer, one of which is FAM labelled. The sequence of the labelled primer is 5'-GGGTTCCCTAAGGGTTGGA-3' and that of the unlabelled primer is 5'-GTGCCAGCAAGATCCATCTAGA-3'. Besides, the MLPA probes of the MRC-Holland for the analysis of the DMD gene mutation analysis also contain 92 nucleotide control fragment which is a ligation dependent probe that behaves similarly to the other MLPA probes and forms a benchmark to compare other control fragments to. The other internal quality control fragments include Q-fragments, D-fragments and XY-fragments. The GeneScan™-500-LIZ® Size Standard, a product of the Applied Biosystem (Product P/N 4322682) is used as the size standard to ensure the efficiency of the capillary electrophoresis. The size standard fragments all migrate consistently resulting in better sizing precision, data migration, and reproducibility. The software used in this experiment is the GeneScan Analysis software, the Gene Mapper version 4.

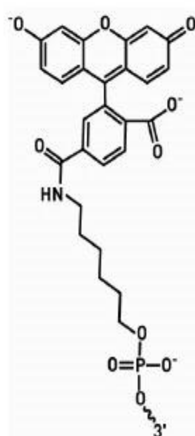


Fig 2.10.5.2: 6-FAM TM Fluorescein

Table 2.10.5.2: Colour guide for GeneScan Application

Corresponding dye				
Colour	**Virtual filter A (Fluorescent dNTPs)**	**Virtual filter C**	**Virtual filter D**	**Virtual filter F**
Blue	R110	6-FAM	6-FAM	5-FAM
Green	R6G	TET	HEX	JOE
Yellow	TAMRA	HEX	NED	NED
Red	ROX	TAMRA	ROX	ROX

2.10.5.3 Software files and their purposes

The ABI PRISM 310 Genetic Analyzer consists of various softwares with their definite purposes such as the Sample Sheet that associates sample information, Injection List that specifies the order for running samples, Modules that control the run parameters, Matrix File that contain information that corrects for "spectral overlap", Size Standard Files that holds the results of a run performed with fragments of known length, Analysis Parameters File that holds the default size-calling method for data analysis and Preference Files that record the default for a number of different selections in the software.

2.10.5.4 Software for data collection and analysis

The softwares that are used with genetic analyzer for fragment analysis include ABI PRISM® 310 Data Collection Software and GeneScan® Analysis Software.
The ABI PRISM® 310 Data Collection Software controls, monitors, and collects raw data from GeneScan® Analysis Software runs. It allows real time run monitoring in the software windows that show Sample raw data, Run status, Electrophoresis record and Instrument log file. It also contains information about the dyes' emission (fluorescence) spectra and processes the raw data as fragment size, or relative concentration for use by one of the analysis software applications.
The GeneScan® Analysis Software for fragment analysis sizes and quantifies DNA fragments. It also provides flexibility to interactively confirm and fine-tune the data analysis, and allows us to display the results of an experiment in several different ways.

2.10.5.5 Summary of Procedures

To perform a GeneScan® Analysis Software experiment with pooled DNA using the ABI PRISM® 310 Genetic Analyzer, it is necessary to Prepare the 310 Genetic Analyzer by Preparing the reagents, Cleaning the pump block, Installing the capillary, Cleaning and installing the syringe and Cleaning the electrode. We need to recalibrate the autosampler if the electrode is removed, replaced or cleaned. The capillary is replaced, priming the pump block, loading buffers and preheating the instrument. The matrix standards is prepared (if necessary), the samples are amplified and denatured and the samples are loaded. Then the run is started by selecting or creating a Sample Sheet or filling out an Injection List.

2.11 ANALYSIS OF MLPA DATA USING NOVEL SOFTWARE COFFALYSE.NET BY MRC HOLLAND

A crucial point in the use of MLPA assay as a genetic test for the molecular diagnosis of gene deletions/duplications is the interpretation of the MLPA results. Homozygous or hemizygous deletions are clearly evidenced by the absence of the specific peaks for the target gene in the presence of a normal amplification of control probes. On the other hand, heterozygous deletions, duplications and CNVs produce a different height and/or area of the relative peaks, and the interpretation of these results can be challenged by the presence of different efficiencies of the PCR reaction among the different probes and sample-to-sample variations. As a consequence, different MLPA data analysis strategies have been developed to allow a correct interpretation of the reaction raw data. Among these, the most widely used is the Coffalyser software developed at MRC Holland, an Excel-based program that is able to perform all data normalization steps and

corrections for signal sloping. There are many other types of software that have been recently released. But few numbers of data are generally analysed manually.

Coffalyser.net is software that was developed by MRC Holland for the sophisticated analysis of the data obtained after the capillary electrophoresis. In some circumstances reliable results can be obtained by comparing unknown samples and the reference samples by visual assessment, simply by overlaying two fragment profiles and comparing relative intensities of fragments. It may however not be feasible to obtain reliable results out of such visual comparison in circumstances such as if the DNA quality of the samples and references is incomparable, if the MLPA kit contains probes targeted to a number of different genes or different chromosomal regions resulting in complex fragment profiles, if the data set is very large making visual assessment very laborious and if the DNA was isolated from the tumor tissue which often shows DNA profiles with altered reference probes.

Thus MRC-Holland has developed reliable and novel Coffalyser.net software for the analysis of the MLPA data. All applied algorithm in the software are specifically designed to suit MLPA applications. The algorithms are designed for peak detection and quantification specifically for MLPA peak patterns (Coffa. J and Berg JVD, MRC-Holland; 2008).

2.12 RECENT ADVANCES IN MD TREATMENT

There is currently no effective therapy for dystrophinopathies and the classical treatment is just aimed at lessening the symptoms. But the new insights into the pathophysiology of dystrophic muscle, the identification of compensating proteins, and the discovery of new binding partners are paving the way for novel therapeutic strategies to treat this fatal muscle disease. Recently, unprecedented advances in strategies devised to correct the primary defect through gene and cell based therapeutics hold particular promise for treating dystrophic muscle.
Several promising approaches have emerged due to advances in experimental design, delivery, and efficacy for all three subgroups: cell therapy, gene therapy and pharmacological therapy.

2.12.1 Cell-based therapeutic approaches

Cell-based therapies involve transplantation or transduction of allogenic or autologous cells to engraft with existing myofibers or repopulate the cellular niche to promote functional muscle regeneration (Meregalli M et al., 2012).

Myoblast transplantation was the first cell-based strategy to be assessed in immunologically tolerant mice, providing evidence of host–donor fusion and stimulating

myofiber development. But there are several unfavorable characteristics of using allogenic myoblasts, including poor intramuscular migration, low efficiency of dystrophin production, limited cell survival, and immune complications. Then the direct satellite cells (SC) engraftment studies began that faces two major hurdles: the rapid decline of their autologous isolation potential, and their individual isolation (Collins CA et al., 2005; Maguire K et al., 2009). A number of these parameters can be alleviated through the use of muscle-derived stem cells (MDSCs), which are commonly thought to represent a predecessor of the SC. Despite these encouraging results, the typically heterogeneous nature of MDSCs may affect their efficacy, depending on their isolation and culturing conditions (Gussoni E et al., 2011).
In recent years, a number of pluripotent and non-muscle derived progenitor cells, as MSCs, which can differentiate to form myogenic cells in-situ and the use of vessel-associated meso-angioblasts (MABs) have demonstrated myogenic potential (Ichim TE et al., 2010).

2.12.2 Gene-based therapeutic approaches

The gene-based therapeutic approaches include gene replacement therapy and gene editing. Delivery of exogenous functional dystrophin is an attractive prospect to benefit all DMD patients. The major challenge involves developing suitable delivery vectors and gene cassettes while avoiding a destructive immune response and the large size of the dystrophin gene (Sakamoto M et al., 2002). An increasingly promising alternative strategy to deliver functional dystrophin involves the ex-vivo combination of cell and gene therapies using the genetically modified cells as autologous delivery vehicles to circumvent immune challenges and reduce the risk of implant rejection (Bachrach E et al., 2004; Wilton SD et al., 2006).

The present prime focus on gene editing includes gene repair and exon skipping. The initial approaches to gene editing were aimed at repairing the gene by correcting point mutations in the dystrophin gene using synthetic RNA/DNA “chimeraplasts” (RDOs), (Rando TA et al., 2000; Bertoni C et al., 2005).

2.12.2.1 Framedness and exon skipping

The RDO-mediated editing has now been replaced by antisense oligodeoxynucleotide (AON) approaches called exon skipping. Exon skipping is a type of molecular plaster therapy that generates an in-frame mRNA encoding a shorter, but moderately functional dystrophin protein product, resembling the protein in BMD patients (Denti MA et al., 2008). Exon skipping has immense clinical potential, as 60% of DMD patients harbor deletions in exons 45–55 and sole targeting of exon 51 can address the majority of patients (Matsuo M et al., 2003; Yokota T et al., 2011). Two different AON chemistries

have been under extensive study for clinical application: 2′-*O*-methyl-phosphorothioate (2-OMePS) and phosphorodiamidate morpholino oligomers (PMOs) (Arechavala-Gomeza V et al., 2012).

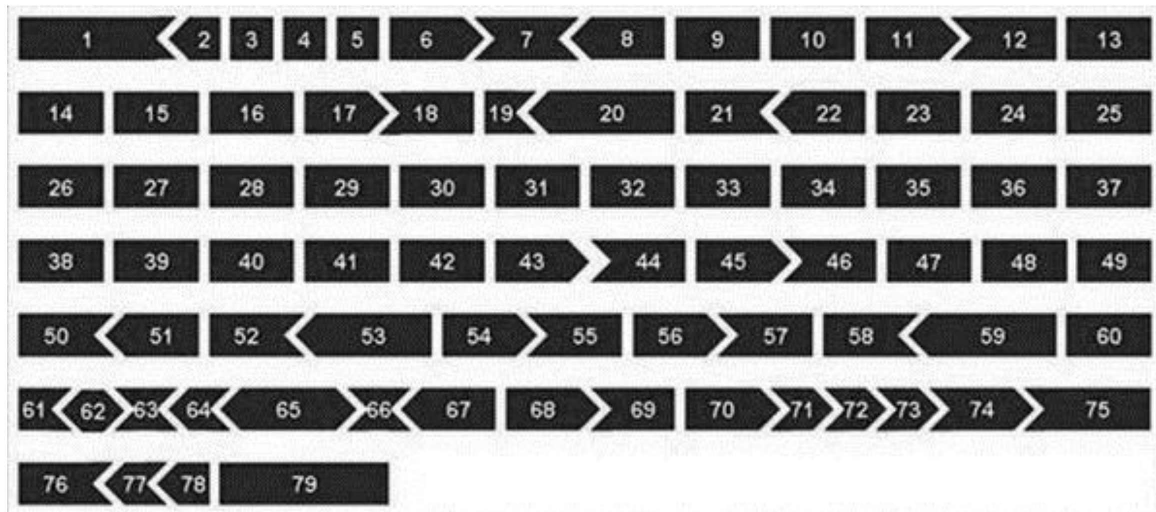


Fig 2.11.2: Exonic arrangement of 79 exons of dystrophin gene

The reading frame theory of Monaco et al. (Monaco AP et al., 1988) helps to explain many genotypic and phenotypic correlation of the mutation in dystrophin gene. It argues that if a deletion leads to the expression of an internally truncated transcript without shifting the normal open reading frame, then a smaller, but functional version of dystrophin could be produced. This scenario would be consistent with a BMD phenotype. If, on the other hand, the deletion creates a translational frame-shift, then premature termination of translation will result in the synthesis of a truncated protein (Yokota T et al., 2009).

The above figure (fig. 2.11.2) depicts the alignment of seventy nine exons of dystrophin gene on the basis of the reading frame rule. The variously shaped gaps between the two exons indicate how the exons can be aligned to produce useful proteins even after the deletion mutation. This framedness of the exons also helps us to determine the in-frame or out-of-frame mutation. For example: If there is the deletion of the exons 49 in the dystrophin gene, exon 48 and 50 can exactly align and thus all the triplet codons in the remaining exons code for the useful protein product. This mutation that does not destroy the reading frame is the in-frame mutation. This in-frame mutation leads to the less severe form of muscular dystrophy i.e. Becker muscular dystrophy. But if there is the deletion of the exon 50 in the dystrophin gene, exon 49 and 51 do not align perfectly such that all the remaining triplet codons in the exons do not code for any meaningful protein product as the reading frame for subsequent codons is shifted according to the changes made. It leads to the out-of-frame mutation and hence the severe form of dystrophinopathy i.e. Duchenne muscular dystrophy.

As the reading frame rule suggests, the Duchenne form of muscular dystrophy occurred due to the deletion of exons 50, 52, 45-50, 48-50 or 59-60 can be converted into less severe form by targeting the exon 51. Exon skipping is the method aimed to convert the DMD into BMD based on this strategy. Drisapersen (GSK2402968) is one of such experimental Prosensa-GSK drug that targets exon 51 of the dystrophin gene. It has encouraging results with respect to walking ability and dystrophin protein production in a phase 2 trial outside the US. Drisapersen is intended to treat about 13% of the DMD patients-those with deletions of dystrophin exons 50, 52, 45-50, 48-50 or 59-60. PRO045 is another drug that targets exon 45 of the dystrophin gene. This drug is intended to treat approximately 8% of DMD patients-those with deletions of dystrophin exons 44, 46, 46-47, 46-48, 46-49 or 46-51. It is being tested in a phase 2b trial outside the U.S. PRO044 is another drug that targets exon 44 of the dystrophin gene. It is aimed at approximately 6% of the DMD patients-those with deletions of exons 43, 45, 38-43, 40-43, 42-43 or 45-54 (van Deutekom JC et al., 2007).

2.12.3 Pharmacological approaches

The pharmacological approach include specific restoration of the DAPC by suppressing nonsense mutations in the dystrophin gene, up regulation of its autosomal paralogue, utrophin, to provide a scaffold on which components of the DAPC can be restored to the sarcolemma and compensatory formation of integrin–laminin complexes, which have mechano-signalling similarities to the DAPC (Mitrpant C et al., 2009).

The suppression of non-sense mutations is mediated by aminoglycosides as gentamicin and less toxic negamycin (Yukihara M et al., 2011). PTC124 or ataluren was recently described as a drug that induces read through of a premature stop codon, without affecting the normal termination codon (Welch EM et al., 2007; A new approach to drug discovery, 2012).

Upregulation of utrophin that is 74% similar to dystrophin at the amino acid level is performed by the utrophin promoters like nabumetone (Moorwood K et al., 2011). A promising approach is the administration of the adenosine monophosphate analog 5-aminoimidazole-4-carboxamide ribotide (AICAR) that elevates sarcolemmal utrophin and β-DG protein levels (Ljubicic V et al., 2011). An alternate promoter-based utrophin strategy involves artificial zinc-finger proteins fused with effector domains (ZF-ATF), which is currently being evaluated in mdx (Passananti C et al., 2010). Immunosuppressant steroids (as prednisone) have been known to help slow the progression of dystrophinopathies (Biggar WD et al., 2002). The drug contributes to an increased production of the protein Utrophin. A hepatitis C drug, "Debio-025", which is a known inhibitor of the protein cyclophilin D shows much promise for halting the muscle

necrosis seen in the disease, which regulates the swelling of mitochondria in response to cellular injury.

2.11.4 Protein-based therapy

Direct protein replacement of utrophin in dystrophin-deficient muscle uses deliverable chimeras constructed by fusing the trans-activator of transcription (TAT) protein transduction domain (PTD) of human immunodeficiency virus (HIV-1) with micro-utrophin protein (TAT-μUtr) (Sonnemann KJ et al., 2009). A related protein-based pharmacological candidate is biglycan, a small leucine-rich proteoglycan found at elevated levels within the extracellular matrix (ECM) of DMD patient skeletal muscle. To mitigate off-target effects, active rhBGN is currently being manufactured without biglycan-associated complex carbohydrate side chains for preclinical evaluation (Tivorsan, compound TVN-102) (Tivorsan Pharmaceuticals, 2012). Besides, α7-integrin and laminin-111 (LAM-111) showing the structural and signaling role of the DAPC, (Rooney JE, 2009) are also used.

The development of a definitive DMD therapy is increasingly likely to involve synergism between adjunctive pharmaceuticals with gene-based approaches (such as exon skipping) to target multiple aspects of dystrophic pathology (Pichavant C et al., 2010).

2.12 PSYCHOLOGY IN DMD

Expression of the full-length dystrophin transcript is controlled by three independently regulated promoters: the brain (B: dp427c), muscle (M: dp427m), and Purkinje (P: d427p) promoters (Boyce FM et al., 1991). The B promoter drives expression primarily in cortical neurons and the hippocampus of the brain (Gorecki et al., 1992), while the P promoter is expressed in the cerebellar Purkinje cells and also skeletal muscle (Holder E et al., 1996). The M promoter results in high levels of expression in skeletal muscles and cardiomyocytes and also at low levels in some glial cells in the brain. Due to the lack of full expression of dystrophin in brain (hippocampus) and cortical neurons BMD and DMD patients also present with mild cognitive impairment, indicating that brain function is also abnormal in these disorders (Blake DJ and Kroger S, 2000). So, they are likely to act different than their pairs.

2.12.1 Intelligence And Behavioral disorder in DMD/BMD

In case of the patients suffering from the DMD, the mean IQ is found to be 80 which is considered as the "Low Average IQ". About 30% of the DMD patients have IQ below 70 which is the result of "mental retardation". But the IQ of the DMD patient even range from 14 to 134, referring that even the DMD patients can be very bright and have a high

IQ. The patients suffering from Becker muscular dystrophy do not show such extreme cases as DMD. But it is always suggested not to overestimate them when looking at their strengths alone or to underestimate them when looking at their weakness (Robert JS et al., 2001).
The behavioral disorders relatively common in DMD/BMD include ASD (Autism spectrum disorder), ADHD (Attention deficit hyperactivity disorder) and OCD (Obsessive compulsive disorder). The parents of DMD/BMD patients (especially DMD) are also psychologically affected along with their children. The researches dated from 1983 already showed that the parent's biggest worry is 'when to tell my son and what'. Social organizations like parent projects (PPMD) help the parent a lot to cope with the situations (Bushby K et al., 2012).

2.13 INTERDISCIPLINARY MANAGEMENT OF DMD

Dystrophinopathies (DMD/BMD) is a single gene disorder. But the consequence of the disorder comes in the form of multiple complications. Thus the diseases need to be managed in an interdisciplinary mode.
Bushby K et al., provides a multidisciplinary care setting in which the individual and family can access expertise for the required multisystem management of DMD in collaboration. The aspects of management of DMD is generally divided into eight major areas viz. Diagnostics, Rehabilitation management, Orthopaedic management, Psychological management, Cardiac management, Pulmonary management, GI, speech/swallowing, nutrition management and Corticosteroid management.
Among these various aspects considered the Psychological management, cardiac management, pulmonary management and GI, speech/swallowing and nutrition management are under the management of other complication that may arise in patients suffering from the disease besides the neuromuscular and skeletal management (Turner C and Hilton JD, 2010).

CHAPTER III: MATERIALS AND METHOD

3.1 DNA EXTRACTION AND QUANTITATION

3.1.1 Patients

All patients clinically diagnosed with either DMD or BMD who were referred to MDF-Nepal for physiotherapies from Teaching Hospital were included in this study.

3.1.2 Collection Of Blood Sample

The blood samples of the patients who were clinically recorded as the DMD or BMD clients were collected from the Muscular dystrophy foundation-Nepal (MDF-Nepal) located at Kupondol (Gunsigal), Lalitpur, Nepal. The blood was extracted by the trained personnel in an ethylene diamine tetra-acetic acid (EDTA) coated vacunator tube (vacuum tube) under the written consent as provided by the MDF-Nepal. The blood so collected was delivered to the Central Department of Biotechnology lab in the ice box and preserved at -20°C until further use. The use of heparinized blood is strictly avoided as MLPA is more sensitive to contaminants than simple monoplex PCR assays and the traces of heparin are very difficult to remove from DNA preparations that can distort the MLPA PCR reaction (MLPA DNA protocol, 2012).

3.1.3 Extraction Of DNA Using QIAamp DNA Mini Spin Column Method

As recommended by the MRC-Holland, the extraction method of the QIAamp DNA mini kit (QIAamp® DNA Mini and Blood Mini Handbook Cat no 51104, Third Edition, 2010, Qiagen, Sample and Assay technologies) was applied to extract the DNA from the blood which is considered to be suitable for reliable polymerase chain reactions (PCR) based molecular tool as MLPA (MLPA DNA protocol, 2012).

3.1.3.1 Spin protocol

Before starting, the blood samples were equilibrated to the room temperature and the water bath was heated at 56°C for use in the fourth step of the extraction. The elution buffer (AE) was equilibrated to room temperature for the elution. The washing buffer 1 (AW1) and washing buffer 2 (AW2) were prepared according to the instruction. QIAamp DNA Mini Kits contained ready-to-use proteinase K solution dissolved in a specially formulated storage buffer.

3.1.3.2 Preparation of the Buffer AW1

Buffer AW1 was supplied in concentrated form (95 ml). Before using for the first time, 125 ml of ethanol (96–100%) was added to make the final volume of 220 ml as instructed.

3.1.3.3 Preparation of the Buffer AW2

Buffer AW2 was supplied in concentrated form (66 ml). Before using for the first time, 160 ml of ethanol (96–100%) was added to make a final volume of 226 ml as instructed.

3.1.3.4 DNA Isolation Procedure

20 µl of the QIAGEN proteinase K was pipetted into the bottom of a 1.5 ml sterilized micro-centrifuge tube. 200 µl of the blood sample was added to the micro-centrifuge tube containing 20 µl of the proteinase K. To the mixture 200 µl of the Buffer AL (Lysis buffer containing chaotropic salt) was added and the mixture was further mixed by pulse-vortexing for 15 secounds. The micro-centrifuge tube containing the mixture of the proteinase K, the blood sample and the Buffer AL was then incubated at 56°C for about 10 minutes for the maximum yield of the DNA. The 1.5 ml micro-centrifuge tube was then briefly centrifuged (Eppendorf AG, Centrifuge 5418, Germany) to remove the droplets from the inside of the lid. 200 µl of the ethanol (96–100%; analytical grade) was added to the mixture, and mixed again by pulse-vortexing for 15 secounds. After mixing, the 1.5 ml micro-centrifuge tube was briefly centrifuged to remove the droplets from the inside of the lid. Now the mixture was carefully applied from the micro-centrifuge tube to the QIAamp Mini spin column with the help of a micro-pipette without wetting the rim. The cap of the mini spin column was closed tightly ensuring the balance to avoid the aerosol formation during the centrifugation step and the content was centrifuged at 6000Xg (8000 rpm) for 1 minute. The QIAamp Mini spin column was carefully opened and 500 µl of the Buffer AW1 was added without wetting the rim. The cap was closed tightly and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube provided in the kit, and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and then 500 µl Buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The filtrate alone was discarded and the QIAamp Mini spin column was placed in the same 2 ml collection tube again and centrifuged at full speed for 1 minute to eliminate the chance of possible Buffer AW2 carry over. The QIAamp Mini spin column was then placed in a clean and sterilized 1.5 ml micro-centrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and 150 µl of Buffer AE was added. It was then incubated at room temperature (15–25°C) for about 5 min.

Finally, the content was centrifuged at 6000 x g (8000 rpm) for 1 min. The filtrate so obtained was the DNA sample. The tubes were properly labeled with the number and date of extraction. The DNA so extracted was stored at –20°C for the long term storage.

3.1.4 Quantification Of DNA

The determination of the average concentration and purity of DNA is the major step in molecular biology as the reactions that use DNA often require particular amounts and purity for the optimum performance.

The spectrophotometric quantification was performed to determine the concentration and the purity of the DNA extracted from the blood. The absorbance was measured at 230 nm, 260 nm and 280 nm using the UV spectrophotometer (Eppendorf-AG22331, Germany). The ratio of the absorbance at 260 nm and 280 nm (A260/280) was calculated and the purity of the DNA was assessed as for pure DNA, the ratio of A260/280 is approximately 1.8.

The ratio 230/260 is also used as a secondary measure of nucleic acid purity. The significant absorbance at the wavelength of 230 nm can be caused by the contamination by phenolate ion, thiocyanates and other organic compounds. For a pure DNA sample the A230/260 should be around 0.556. If the ratio is appreciably higher than expected, it may indicate the presence of contaminants which absorb at 230 nm (e.g. the phenolate ions and thiocynates) (Nanodrop Technologies, Inc. 2007). Hence, the quantitation and quality check of investigated DNA in present study was observed adequate for the MLPA reaction.

The DNA concentration and purity were also determined by carrying out the electrophoresis of the samples in 1.0-1.5% agarose gel and comparing the intensities and discreteness of bands with the Genes Ruler^{TM} 1000/100 bp plus DNA ladder (Fermentas LIFE SCIENCES, #SM0323).

3.1.5 Gel Electrophoresis Of Extracted DNA

3.1.5.1 Preparation of general use gel electrophoresis reagents

50X TAE stock buffer preparation (Tris-acetate-EDTA)

Tris-acetate-EDTA (TAE) buffer is the most common buffer used for agarose gel electrophoresis in the analysis of DNA products resulting from PCR amplification, DNA purification protocols or DNA cloning experiments.

To prepare (50 X) TAE stock buffer, Tris base (242gm; Qualigens Fine Chemicals, Mumbai) was dissolved in approximately 750 ml deionized water. To this solution, glacial

acetic acid (57.1mL; Qualigens Fine Chemicals, Mumbai) was added followed by 0.5 M EDTA (pH 8.0, 100mL; Promega Corporation, USA). The final volume was made up to 1L. This stock solution can be stored at room temperature.

Preparation of the working solution

The working solution of 1X TAE buffer was made by diluting the stock solution of 50X in deionized water. Final solute concentrations were 40 mM Tris-acetate and 1mM EDTA. The buffer is now ready for use in running an agarose gel.

3.1.5.2 Gel electrophoresis on agarose Gel (1%)

0.5 gm of the agarose (Promega, Product of Spain) was dissolved in 50 ml of 1XTAE buffer in a conical flask. The mixture was then slightly dissolved and heated in the microwave oven for few minutes. It was then cooled to approximately 55^{0}C (luke warm). The gel was prepared such that it contained 0.5 µg/ml of Ethidium Bromide (EtBr) in it. The gel was then poured on to the gel casting tray with an appropriate comb (12 to 20 toothed) fixed in place for the well formation.

The DNA so extracted were loaded in the well such that the total volume loaded in well was 12µl (10µl DNA +2µl GLB (6X)). The electrophoresis was performed on the 1% Agarose gel in TAE buffer (1X) at 50 V (8.47 V/cm) for half an hour and 25V (4.2V/cm) for one and half hour respectively using the gel tank (Biorad). Then, the gels were visualized on a UV illuminator (MS, Major Science, UV tray, MUV21-254/365-220, Taiwan).

3.2 Multiplex Ligation-dependent probe amplification (MLPA) Assay

The MLPA reaction consists of five major steps: Denaturation, Hybridization, Ligation, PCR and Capillary Electrophoresis.

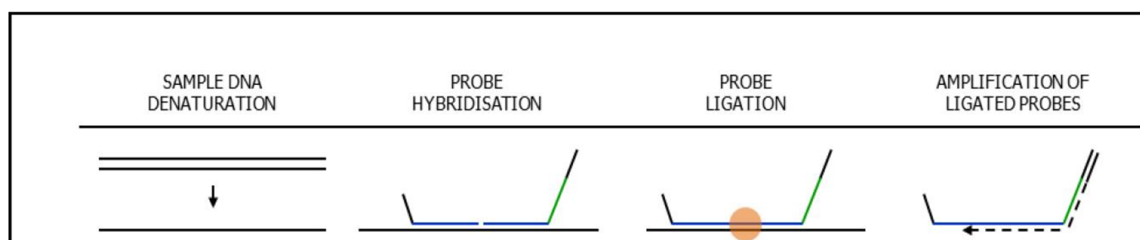


Fig. 3.2: MLPA reaction

DNA denaturation: DNA extracted from the blood sample is heated at 98°C for five minutes to denature.

Hybridization: The SALSA probemix and the necessary MLPA buffer are added to the content which is then incubated for one minute at 95°C followed by the 16 hours of hybridization at 60°C.

Ligation: The ligase mix is added and incubated at 54°C for 15 minutes for the ligation. The ligase is then inactivated by heating at 98°C for 5 minutes.

Polymerase chain reaction (PCR): The primers, dNTPs and polymerase are then added for the normal PCR.

Capillary Electrophoresis: The sequence type capillary electrophoresis is done to determine the relative size of the fluorescent peaks and the results are compared to the reference samples.

3.2.1 Instruments

MLPA is an easy to perform reaction that requires only a thermocycler and a sequence type electrophoresis system (Capillary Electrophoresis). For the experiment the MRC thermocycler was used which was compatible with the MLPA reaction to be performed which was kindly provided by the Muscular Dystrophy Foundation-Nepal (MDF-Nepal).

3.2.2 Sample Treatment

Total quantity of 50-250 ng of genomic DNA was used in 5 ul volume for each MLPA reaction.

The sample was in dilution in elution buffer as provided in the QIAamp DNA mini kit. The pH of the DNA preparation was maintained in between 8.0 – 8.5 in order to prevent the depurination during the initial heat treatment at 98 ^{0}C. It is recommended that the extraction method should not leave a high concentration of contaminants such as salt. So, the preferred extraction method as QIAamp DNA mini kit was applied to extract the DNA. The high concentration of the samples should not be higher than 2.5 mM that will negatively affect the ligation and PCR reaction. So, the vacunator tube containing less than 2.5 mM of EDTA was used.

3.2.3 Selection Of Reference And Control Samples

Reference samples: The reference samples were included in each MLPA experiment. The samples were then compared that were run within the same experiment and tested with the same probemix lot.

Selection of reference samples: The DNA samples in which the target and reference probe sequence are assumed to have a 'normal' copy number were taken as the reference samples. They were obtained from the healthy individuals.

No DNA control: A no DNA control reaction was included in which DNA was replaced by the elution buffer. This may reveal the possible contamination of the reagents used in the test.

3.2.4 Preparation before starting the actual reaction

All parts of the MLPA reaction (DNA denaturation, probe hybridisation, ligation of probes and PCR amplification) were performed in a thermocycler with heated lead. So, the thermocycler program was set in the thermocycler as instructed. The thawed buffer and probemix was vortexed briefly before use to evenly distribute the salts and other constituents at the bottom of the tubes after a freeze-thaw cycle. The MLPA reagent tubes were centrifuged for a few seconds before use, as drops might have adhered to the lid during transport or vortexing. The solution containing enzymes were not vortexed. And during the preparation of the master mixes, enzymes were always added at the last. The ligase-65 master mix and polymerase master mix was made just before use and stored in ice. A 5-10% volume surplus was prepared to compensate the pipetting errors.

3.2.5 METHODS: (MLPA)

3.2.5.1 DNA DENATURATION (DAY 1)

Two 0.2 ml tubes were labeled for each of the test sample, reference samples (controls) and no DNA controls corresponding to the two probe sets. 5 µl of DNA sample (50-250 ng) was added to each tube (or elution buffer for the 'no DNA control' reaction). The tubes were placed in a thermocycler and the MLPA thermocycler program was initiated. The sample DNA was denatured for 5 minutes at 98°C and the samples were cooled to 25°C.

3.2.5.2 HYBRIDISATION REACTION (DAY 1)

The viscous MLPA buffer and the MLPA probemix were vortexed before use. A hybridisation master mix was prepared containing 1.5 µl MLPA buffer (yellow cap) + 1.5 µl probemix (PO34-A3 and PO35-A3) (black cap) for each reaction. The hybridisation master mixes (containing probe set PO34-A3 and probe set PO35-A3) were mixed well by pipetting or vortexing. After DNA denaturation, 3 µl of the hybridisation master mixes

were added to each sample tube according to the probe sets PO34-A3 and PO35-A3. The mixtures were mixed well by pipetting up and down. The thermocycler program was continued. Thus the tubes were incubated for 1 minute at 95°C, then for about 16 hours (overnight) at 60°C. During this stage a tube containing 8 µl of distilled water was also added to test the evaporation problem.

3.2.5.3 LIGATION REACTION (DAY 2)

The next day after the completion of the hybridization reaction, two Ligase buffers were vortexed before use. A Ligase-65 master mix was prepared. For each reaction: 25 µl dH_2O + 3 µl Ligase buffer A (transparent cap) + 3 µl Ligase buffer B (white cap) was mixed. Then 1 µl of the Ligase-65 enzyme (green cap) was added. The mixture was mixed well by pipetting gently up and down. The enzyme solution was not vortexed. The thermocycler program was continued: Thus the reaction was paused at 54°C. When the samples were at 54°C, 32 µl of the ligase master mix was added to each reaction tube and gently mixed by pipetting up and down. The thermocycler program was continued. The tubes were allowed for 15 minutes incubation at 54°C (for ligation), followed by 5 minutes at 98°C for heat inactivation of the Ligase-65 enzyme and then paused at 20°C. The ligation reaction products were stored at room temperature for several hours or at 4°C for up to one week.

3.2.5.4 PCR REACTION (DAY 2)

The complete ligation reaction was then used for the PCR. The PCR was started at room temperature. The SALSA PCR primer mix was vortexed before use. The polymerase was warmed for 10 seconds in hand in order to reduce viscosity. A polymerase master mix was prepared by adding 7.5 µl dH_2O + 2 µl SALSA PCR primer mix (brown cap) + 0.5 µl SALSA Polymerase (orange cap) for each reaction. The reaction mix was mixed well by pipetting up and down. Vortexing was avoided. The mixture was stored on ice until use. At room temperature, 10 µl of the polymerase mix was added to each tube. The mixture was mixed by pipetting gently up and down and the thermocycler program was continued: 35 cycles: 30 seconds 95°C; 30 seconds 60°C; 60 seconds 72°C. The program was ended with 20 minutes incubation at 72°C and then paused at 15°C. The PCR reaction products were stored at 4°C for up to one week. For longer periods, the temperature was maintained between -25°C and -15°C. As the fluorescent dyes used are light sensitive, the PCR products were wrapped in Aluminium foil.

3.2.6 GEL ELECTROPHORESIS OF THE MLPA PRODUCTS

3.2.6.1 Preparation of general use gel electrophoresis reagents

50X TAE stock buffer preparation (Tris-acetate-EDTA)

To prepare (50 X) TAE stock buffer, Tris base (242gm; Qualigens Fine Chemicals, Mumbai) was dissolved in approximately 750 ml deionized water. To this solution, glacial acetic acid (57.1mL; Qualigens Fine Chemicals, Mumbai) was added followed by 0.5 M EDTA (pH 8.0, 100mL; Promega Corporation, USA). The final volume was made up to 1L. This stock solution could be stored at room temperature.

Preparation of the working solution

The working solution of 1X TAE buffer was made by diluting the stock solution of 50X in deionized water. Final solute concentrations were 40 mM Tris-acetate and 1mM EDTA. The buffer is now ready for use in running an agarose gel.

3.2.6.2 Electrophoresis on agarose Gel (4%)

2 gm of the agarose (Promega) was dissolved in 50 ml of 1XTAE buffer in a conical flask. The mixture was then slightly dissolved and heated in the microwave oven for few minutes. It was then cooled to approximately 55^{0}C (Luke warm) and immediately poured on to the gel casting tray with an appropriate comb (12 to 20 toothed) fixed in place for the well formation.

The extracted MLPA products were analysed on a 4% Agarose gel in TAE buffer (1X) at 50 V (8.47 V/cm) for half an hour and 25V (4.2V/cm) for one and half hour respectively using the gel tank (Biorad). The total volume loaded in well was 12µl (10µl DNA +2µl GLB (6X)) for DNA analysis. The gel was prepared such that it contains 0.5 µg/ml of Ethidium Bromide (EtBr) in it. Then, the gels were visualized on a UV illuminator (MS, Major Science, UV tray, MUV21-254/365-220, Taiwan).

3.3 CAPILLARY ELECTROPHRESIS: ABI-310 GENETIC ANALYZER

The ABI-310 Genetic Analyzer is generally used to sequence the DNA and quantify the DNA fragments. The GeneScan Analysis Software in the analyzer analyses the raw data to quantify the DNA fragments and determine the size of the fragments by comparing them to fragments in a size standard. Each sample is labeled with the FAM fluorescent dye. When the DNA fragments reach a detector window in the capillary coating, a laser

excites the fluorescent dye labels. Emitted fluorescence from the dyes is collected by a CCD camera. The software interprets the result, calculating the size or quantity of the fragments from the fluorescence intensity at each data point.
For this experiment, the ABI-310 genetic analyzer present in Centre for Molecular Dynamics Nepal (CMDN) was used, which fulfilled all the necessary criteria required to perform the capillary electrophoresis.

Centre for Molecular Dynamics Nepal (CMDN), located in Prashuti Griha Marg, Thapathali-11, Kathmandu, is a research driven non-governmental organization dedicated in promoting research in the country and is recognized as the leading public health and wildlife research organization of the country. It works closely with Intrepid Nepal Pvt. Ltd. (Nepal), a biotechnology company to provide consultancy services in various country programs, particularly in diseases diagnosis, surveillance and training activities. Beside the introduction of effective innovative approaches to address many of the challenges in the areas of epidemiological studies, disease surveillance, health research, environmental and biodiversity research, CMDN also rendered it's potentiality in the field of human genetic disease diagnosis by permitting the application of the ABI-310 Genetic Analyzer for capillary electrophoresis in the molecular analysis of the dystrophin gene in this study.

3.3.1 MATERIALS REQUIRED

- Deionized formamide
- ABI PRISM® 310 10X Genetic Analyzer Buffer with EDTA
- Filter sterilized, deionized water
- ROX labelled Liz size standard
- One 1.0-mL glass syringe (or one 2.5-mL glass syringe)
- Performance Optimized Polymer, 4% (POP-4™)
- Two 4.0-mL Genetic Analyzer Buffer Vials with cap adapters
- Two septa for the 4.0-mL buffer vials
- ABI PRISM® 310 Genetic Analyzer Capillary: 47-cm, 50-μ i.d., labeled with a green mark. Gene scan capillaries labelled with yellow mark is not recommended to use with POP-4 polymer.
- A thermal cycler to denature the sample

3.3.2 Setting up the reagents: Preparation of the polymer, formamide and buffer

Preparation of the polymer: The performance optimized polymer POP-4 (4%) was equilibrated to room temperature and then mixed thoroughly by inversion. It was allowed to stay for at least 5 minutes after mixing.

Preparation of formamide: 50-mL of the high quality formamide (Hi-Di formamide) was mixed with 5 g of ion-exchange resin. The mixture was stirred at room temperature for about 30 minutes. The pH was maintained at 7.0–9.0. The mix was then filtered through a 2-micron filter. 500-μL of the aliquots was prepared and stored at minus 20°C.

Preparation of buffer: 5-mL of 10X Genetic Analyzer Buffer with EDTA was diluted to a 1X concentration (50-mL) with filter-sterilized, deionized water and brought to room temperature.
The total volume of the buffer required was about 15 ml to fill the anode buffer reservoir and cathode buffer reservoir. The buffer was available in the form of a mixture with the EDTA, in 10X concentration. So, to make the 1X buffer, 1.5 ml of the 10X buffer and 13.5 ml of the distilled water was added. The buffer so prepared was then filled in the anode buffer reservoir and cathode buffer reservoir considering the lower meniscus.

3.3.3. Preparation of the 310 Genetic Analyzer for a GeneScan Run With POP-4

All the parts of the analyzer were thoroughly examined for all possible hazards and maintained before the sample run.

3.3.3.1. Cleaning the Pump Block

The pump block was removed from the instrument. All the valves and ports were opened and the pump block was thoroughly rinsed under warm water. Air was forced through the channel using the plastic syringe until the channels are dry. The pump block and the buffer reservoir were then replaced.

3.3.3.2 Cleaning and Preparing the Syringe for Use

1.0-mL syringe was used with Performance Optimized Polymers (POP). The syringe was rinsed with distilled water. Excess water was removed but it was not dried completely. Care was taken that plunger is not completely dried as excessive wear occurs to the Teflon fitting of the plunger if it is completely dry.

3.3.3.3 Loading the polymer in the syringe

The polymer was allowed to equilibrate to room temperature before loading it into the syringe. A small amount of polymer solution was used into the syringe and the plunger was pulled up to the 0.60-mL marker after the polymer solution was added. The syringe

was gently rinsed five or six times to coat the walls with polymer and the polymer solution was discarded to ensure that the running polymer was not diluted when added to the syringe. Then the syringe was manually filled with a maximum of 0.5-mL of polymer. All air bubbles were removed by inverting syringe and pushing air bubbles out. The outside of the syringe was rinsed with distilled water to remove any polymer on the outside of the syringe and was dried with a lint-free paper. The syringe drive toggle was moved to the left to attach the syringe to the pump block. The syringe was placed through the right-hand port of the plastic syringe guide plate and the syringe was screwed into the pump block finger-tight. The waste valve below the syringe and the luer valve to the left of the syringe were manually closed and tightened.

3.3.3.4 Filling the pump block channels with polymer

The Manual Control window was opened. Buffer Valve Close in the Function pop-up menu was selected to close the pin valve at the anode buffer reservoir on the pump block. The waste valve below the syringe was manually opened. The syringe plunger was pressed until a drop of polymer formed on the bottom of the waste valve to ensure that all the air bubbles at the valve site were removed. The waste valve was manually closed. The Manual Control menu was opened and the Buffer Valve Open was selected in the Function pop-up menu to open the pin valve at the anode buffer reservoir on the pump block. The syringe plunger was pressed until polymer filled the polymer channel in the block. This removed all of the air bubbles from the polymer channels, using about 0.1-mL of polymer. Buffer Valve Close was selected in the Function pop-up menu. The syringe drive toggle was moved to the right to position it over the syringe plunger. Syringe Down was selected in the Function pop-up menu. 50 step intervals were selected and Execute was clicked until the toggle made contact with the syringe plunger.

3.3.3.5 Cleaning the Electrode

The 310 Data Collection software was opened. The Tray button on the 310 Genetic Analyzer was pressed to lower the autosampler and present the tray. The electrode was wiped with lint-free paper that has been dampened with distilled, deionized water. The electrode was dried with fresh lint-free paper. The Tray button was pressed to return the autosampler to its original position and the capillary was immersed in buffer. The autosampler was recalibrated after cleaning, trimming or replacing the electrode.

3.3.3.6 Installing the Capillary

Connecting the Capillary to the Pump Block

The capillary window was cleaned and the door covering the heat plate was opened. The capillary fitting on the right side of the pump block and the capillary fitting back into the pump block were checked to ensure that the capillary are properly inserted through the fitting. The end of the capillary was checked either it was positioned directly below the opening to the glass syringe. The end of the capillary must protrude well beyond the opening at the tip of the capillary fitting.

Positioning the Capillary in the Detector

The laser detector door was opened and the laser port was well cleaned using 100% methanol which is usually preferred than other alcohols being highly and readily volatile. The slight white portion of the capillary that fits in front of the laser detector was gently wiped to prevent any bits of particles attached on it.

The capillary was positioned in the vertical track of the detector. The colored labeling mark was labeled on the capillary with the top edge of the detector plate and laser detector door. So, during the fitting of the capillary the green dots in laser device was overlapped with the green dots in capillary, the red dots in laser device was over lapped with the red dots in capillary and the laser detector and the faded part of the capillary was exactly overlapped. The capillary was taped to the heat plate with thermal tape to secure the position of the capillary labeling mark relative to the detector plate. The laser detector door was closed to secure the position of the capillary window.

Positioning the Capillary near the Electrode

The capillary was attached to the capillary ferrule in the gel block region along the heat plate and then threaded through the capillary hole in the electrode thumbscrew until it protruded past the tip of the electrode by about 0.5 mm. The capillary was taped to the heat plate with thermal tape to secure the position of the capillary tip relative to the electrode. The capillary was taped just above the electrode thumbscrew and just above the detector door. The door was closed over the heat plate. With the heat plate door closed, it was checked that the capillary has not moved relative to the electrode.
In the middle of the front and back border of the sample tray was the presence of a small silver tip like dots. They were used to calibrate the capillary tip and the electrode that imports the mixture of the master mix and the sample into the capillary. During the calibration the tip of the capillary was perpendicularly fixed above the silvery tip present in the middle of the front and back border of the autosampler tray. The tray could be moved up and down using PgUp and PgDn keys, and left and right using the corresponding keys in the computer keyboard. The TRAY button in the analyzer was pressed to check the movement of the sample tray.

3.3.3.7 Calibrating the Autosampler

Autosampler Calibration was chosen from the Instrument menu of the Collection software. The Autosampler Calibration window appeared. The Start was clicked and the directions were followed that appeared on the screen. The autosampler was moved using the arrow keys on the Macintosh® computer keyboard. The arrow keys were held down to move the autosampler with larger steps. To move to the rear, front, right, left, up, down and half steps, the Up arrow, Down arrow, Right arrow, Left arrow, Page Up, Page down and the Shift key in combination with the afore mentioned keys were used. The calibration dot was aligned on the front of the tray platform with the capillary. The end of the capillary was centered on the x, y calibration point. The z-calibration point was almost touched with the end of the capillary. To save the calibration value Save was clicked. The procedure was repeated for the rear calibration point and Set was clicked.

3.3.3.8 Loading Buffers in cathode and anode end

Cathode end: The two containers holding the buffer were well washed and supplied with fresh buffer before the run. The anode buffer reservoir was filled to the red line with 1X Genetic Analyzer Buffer with EDTA and installed on the pump block.

Anode end: One of the buffer vials was labeled as buffer, and filled to the black lining with 1X Genetic Analyzer with EDTA buffer. The vial was capped, the septum was inserted, and placed in position one on the Genetic Analyzer autosampler. This vial forms the cathode end. The other glass buffer vial was labeled as ‘H_2O’, and filled to the line with filter-sterilized, deionized water. The vial was capped; the septum was inserted, and placed in position two on the Genetic Analyzer autosampler. The tube was completely filled with filter-sterilized, deionized water, and placed in position three on the Genetic Analyzer autosampler.

3.3.3.9 Filling the capillary

The Seq. Fill Capillary module was used to fill the capillary with polymer. When the module was started the instrument pumped the polymer to the capillary for 5 seconds, paused for 10 seconds, and then filled the capillary for 10 minutes. There were no anomalies early in the pumping operation so the run was not paused or cancelled during the programmed 10-second delay. Initially, the glass syringe plunger moved rapidly 1–2 mm before stopping. As indicated by the slow movement of the plunger there were no leak. The 310 Data Collection software and the Manual Control window were opened. The Tray button was pressed and the autosampler tray moved forward. The instrument doors were opened and a small tube containing 0.5 mL water in tube was placed in

position 6 in the front of the autosampler. The Tray button was pressed again to move the autosampler back to its original position. The Seq Fill Capillary was selected from the Module pop-up menu on the Manual Control screen. The position of the end of the glass syringe plunger was noted. The Start was clicked. After the module had run the position of the plunger was noted again. The difference of the position of the plunger was found to be four units (Gel pump: 294 to 298). The pump consumed 4 μL of polymer per 10-minute fill. If more than 7-μL of polymer is consumed it indicates the leaks.
The capillary containing the POP-4 was made entirely free of bubbles or any other contaminants. The presences of bubbles were removed with the adjustment of the three valves located at the left (anode buffer valve), bottom (waste valve) and the upper (luer valve) part of the gel block region.

3.3.4 Preparing the Formamide-Size Standard Mix

To ensure reproducibility of results for all samples, the formamide-size standard mix was prepared as recommended by MRC-Holland. In a reaction tubes the standard mixture was prepared such that for each reaction the mixture contained 0.5-μL ROX labeled Size Standard Liz, 13.5-μL deionized HiDi formamide and 0.75 µl dH_2O. The vial was labeled and the mixture was gently vortexed for 3-5 seconds. The mixture was spun down. The master mixes so prepared were then distributed in 55 aliquots in MicroAmp reaction tubes such that each of the tube contained 14.75 µl of the mixture. The mix so prepared and aliquotted were then stored at 2–6 °C until ready to use. Later on 0.75µl of the PCR products were mixed to each of the formamide-Size standard mix so formed.

Table 3.3.4: Preparation of the master mix for 26 samples

	µl/rxn	**µl/55 rxn**
Distilled water	0.75	0.75 X 55 = 41.25
$HiDi formamide	13.5	13.5 X 55 = 742.5
***Liz**	0.5	0.5 X 55 = 27.5
Total (excluding sample)	*14.75*	*811.25*
Sample	0.75	
Total (including sample)	15.5	

3.3.5 Preparation of the sample

The samples (i.e. MLPA products) were arranged in the exact order in which the samples were to be inserted into the autosampler tray. The master mixes that were divided into 55 aliquots and kept in the freeze were taken out. To 52 of the 55 aliquots, 0.75 µl of the

sample was added corresponding to 26 samples (probe mix A and probe mix B) in order so that each of the mixture contained the final volume of 15.5 μl.

3.3.6 Denaturing the Samples

To denature the sample the PCR product was pooled with the master mix prepared containing the formamide, distilled water and internal standard as Liz.
The sample was denatured at 80°C for 2 minutes to denature the DNA in the MLPA product. The samples were removed from the thermal cycler, and cooled in the refrigerator.

3.3.8 Preparing the autosampler tray

The MicroAmp reaction tubes containing the denatured PCR products that were arranged in the MicroAmp tray were then covered with the MicroAmp retainer. The Genetic analyzer septa strips were applied to seal each of the tubes. Then the Genetic Analyzer retainer clip was applied such that the tubes were fixed in a position.

3.3.9 Transferring the Samples in the autosampler tray

The denatured samples were transferred to a 96-well tray according to the suggested tube arrangements. The tube arrangement and order of the samples in the tray and on the Sample Sheet was maintained to be the same. The modes of the tube arrangement were noted, so that the sample sheet could be prepared correctly. Each tube was sealed with a septum, and the tray was placed into the autosampler.

3.3.10 Starting the Run

In the GeneScan analysis software the initial setting prior to run was arranged as recommended by MRC-Holland. The injection voltage was maintained at 1.6 kV, the temperature was maintained at 60°C, the injection time was 15 secounds and the filter D was used. The run time was set as 25 minutes for each sample. The run was started by selecting or creating a Sample Sheet. It could also be performed by filling out an Injection List. The Run button was clicked in the Injection List to start the run.

In the data collection software excel sheet the data was entered exactly in the order as the samples were arranged in the autosampler tray. The last value was entered by choosing any of the samples (here sample 2.1 was taken). The run time was maintained at 1 min and the Sequence Fill Capillary Sec. was maintained such that after the completion of the sample run, the last sample will be used to wash the capillary. The

monitor displaying the peak pattern had five buttons colored Blue, Green, Black, Red and Orange at the base. The FAM displays blue colored peak whereas the ROX labeled internal size standard Liz displays orange colored peak. So, the other color peaked display besides blue and orange colored peaks were inactivated by clicking the respective buttons. After about half an hour, the screen began to display the blue colored peak pattern beginning from the peak of 60-64 nt, followed by the peaks of higher nucleotide fragments.

3.3.11 DATA ANALYSIS

The novel software Coffalyser.net was used to analyze the data obtained after the capillary electrophoresis run which was further confirmed by visual assessment by overlaying two fragment profiles and comparing the relative intensities of fragments.

CHAPTER VI: RESULTS

4.1 DNA extraction

4.1.1 Gel Electrophoresis of the Extracted DNA

The genomic DNA obtained using the extraction technique (QIAamp® DNA Mini spin column) was found to be below 1000 bp as indicated by the comparative analysis with the molecular marker of 1kb.

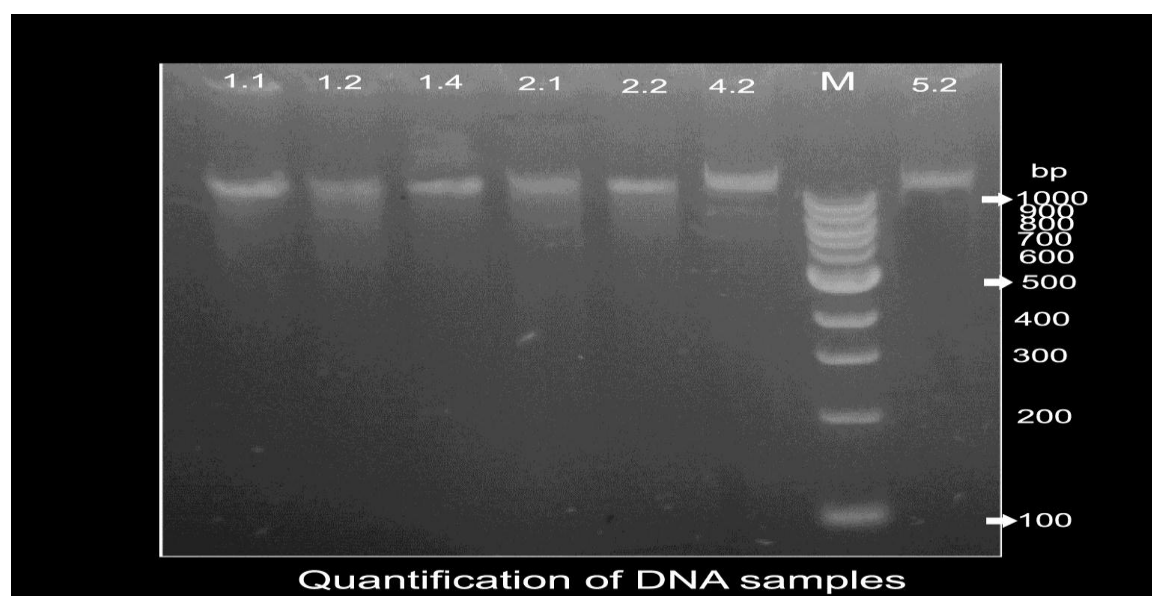


Plate 4.1 (a): Gel electrophoresis of the extracted DNA in the Agarose gel (1.5%). The Lane marked 'M' is 100 bp plus molecular weight marker.

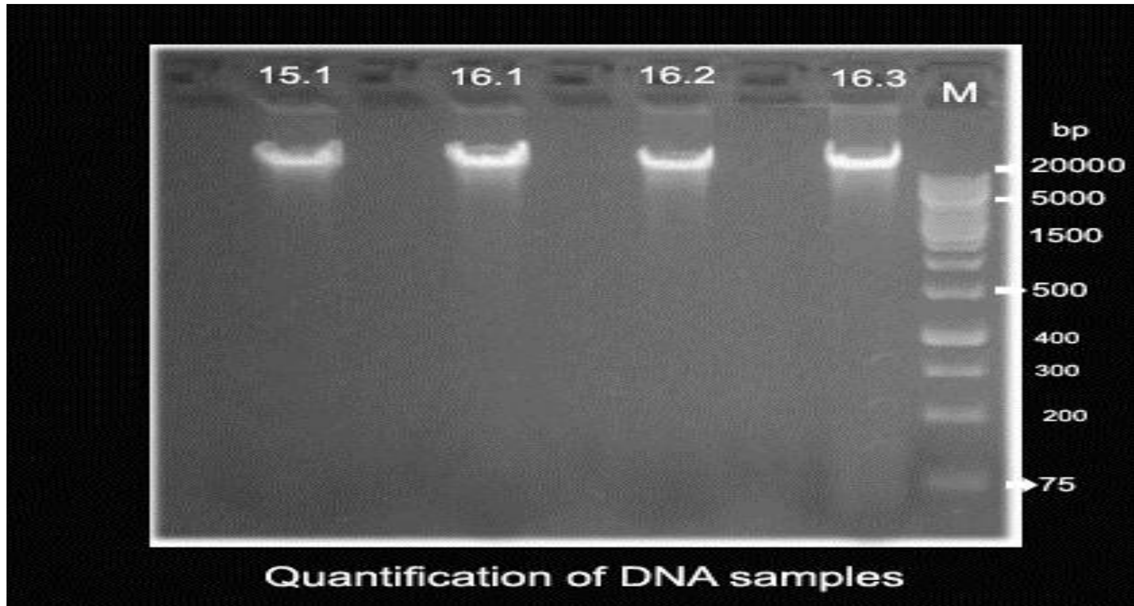


Plate 4.1 (b): Gel electrophoresis of the extracted DNA in the Agarose gel (1.5%). The Lane marked 'M' is 1000 bp plus molecular weight marker.

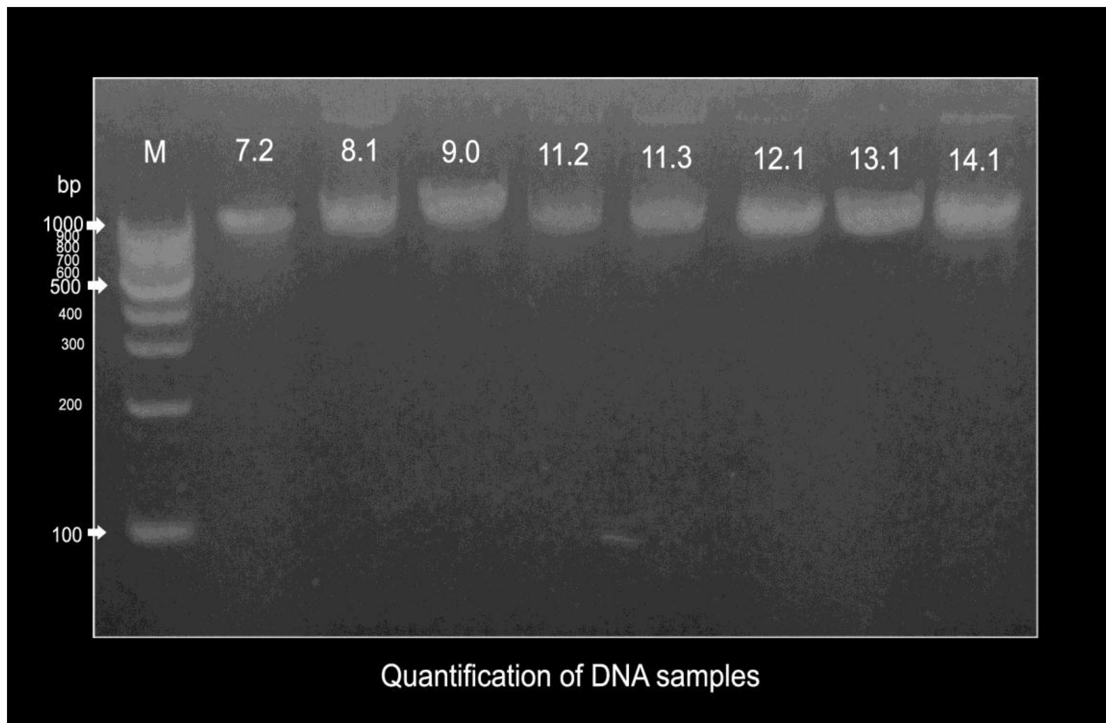


Plate 4.1 (c): Gel electrophoresis of the extracted DNA in the Agarose gel (1.5%). The Lane marked 'M' is 1000 bp plus molecular weight marker.

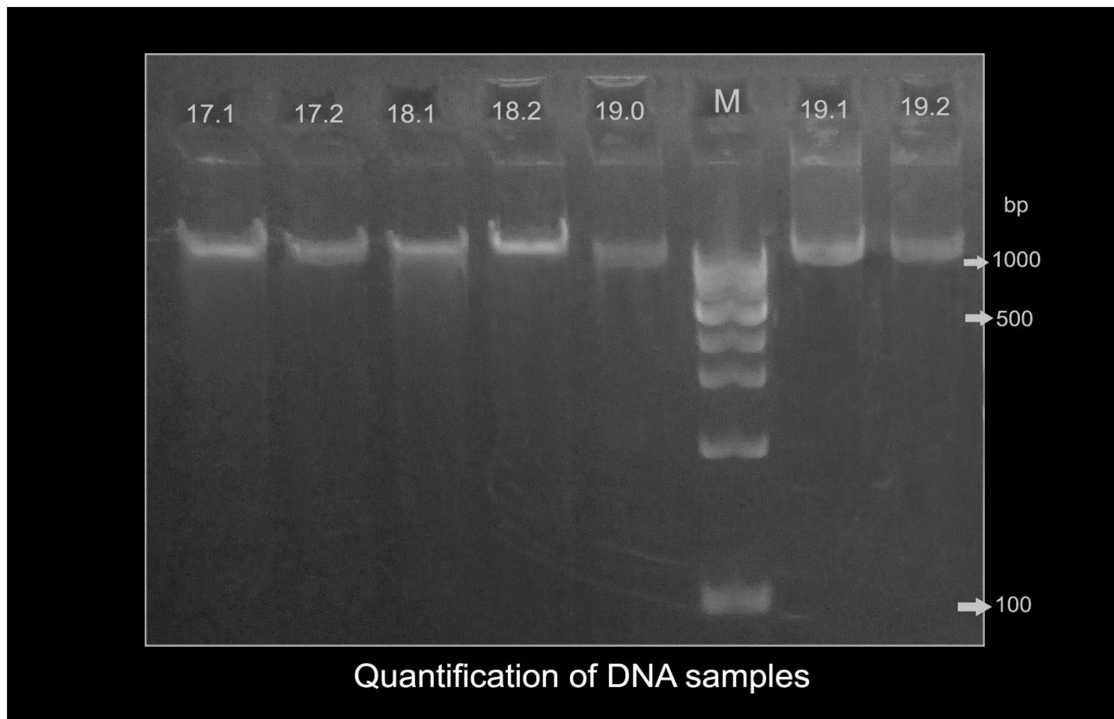


Plate 4.1 (d): Gel electrophoresis of the extracted DNA in the Agarose gel (1.5%). The Lane marked 'M' is 100 bp plus molecular weight marker.

4.1.2 DNA Quantification Results

The DNA estimation by UV spectrophotometer of 26 samples used in this investigation gave a DNA concentration range within 27 µg/ml to 59 µg/ml. It corresponds to the DNA concentration range as suggested by the Qiagen kit (15-60µg/ml), which was used to extract the DNA. Majority of the ratio of the optical density (OD) of 260 nm and 280 nm ranged from 1.5-2.0. The DNA extracted from the samples were of acceptable and adequate quality for the MLPA experiment as the DNA was pure enough (Average OD260/280 =1.76) for MLPA.

4.1.3 DNA Concentration calculation

For example in sample: 2.2

Dilution factor used = 5X

(For the quartz cuvette used, 100 µl of the content was required. To meet the need, 5 µl of sample DNA was added to 95 µl of the elution buffer to make the final volume of 100 µl. Thus the dilution of the DNA concentration was obtained to be 5X.)

The diluted sample gave a reading of 0.154 on a spectrophotometer at OD_{260}. To determine the concentration of DNA in the original sample, the following calculation was performed:

ds DNA concentration = 50 μg/mL × OD_{260} × dilution factor

(1 OD at 260 nm (or Ab260 nm) for double stranded DNA = 50 ug/ ul of ds DNA)

ds DNA concentration = 50 μg/mL × 0.171 × 5

(Note: the value of OD_{260} is within the accuracy range as the absorbance reading at 260 nm should fall between 0.1 and 1.0 to be accurate.)

ds DNA concentration = 42.812 μg/mL

(The concentration is within the range of 15-60 ug/ml as given by the Qiagen kit.

As the Qiagen protocol suggests, 200 ul of blood yields 3- 12 ug/ml of DNA.

So, 200 ul of blood yields 3-12 ug/ml of DNA

Or, 1 ul of blood yields 3/200 – 12/200 ug/ml of DNA

Or, 1000 ul of blood yields (3/200) X 1000 – (12/200) X 1000 ug/ml of DNA

Or, 1 ml of blood yields 15 – 60 ug/ml of DNA. (To be used to estimate the conc. of DNA in quartz cuvette of 1 ml))

The dsDNA concentration were measured accordingly for all the DNA samples which were found to be within the range as suggested by the Qiagen kit protocol and thus were considered to be purely acceptable and adequate for the MLPA experiment.

Table 4.1.3: DNA Quantification Results

SN.	Sample No.	Dilution Factor (DNA:AE)	Absorbance (nm)			Ratio 230/260	Ratio 260/280	Concentration
			230	260	280			(μg/mL)
1	1.1	5 : 95	0.109	0.158	0.097	0.69	1.63	39.5
2	1.2	5 : 95	0.125	0.227	0.117	0.55	1.93	56.8
3	1.4	5 : 95	0.072	0.125	0.067	0.58	1.85	31.4
4	2.1	5 : 95	0.058	0.1092	0.058	0.54	1.89	27.3
5	2.2	5 : 95	0.097	0.171	0.092	0.57	1.86	42.7
6	4.2	5 : 95	0.094	0.2008	0.111	0.47	1.80	50.2

7	5.2	5 : 95	0.152	0.2452	0.165	0.62	1.48	61.3
8	7.2	5 : 95	0.132	0.194	0.098	0.68	1.96	48.6
9	8.1	5 : 95	0.0432	0.173	0.1047	0.25	1.65	43.2
10	9.0	5 : 95	0.104	0.189	0.103	0.55	1.83	47.4
11	11.2	5 : 95	0.094	0.131	0.064	0.67	2.04	32.8
12	11.3	5 : 95	0.086	0.201	0.101	0.43	1.98	50.4
13	12.1	5 : 95	0.130	0.240	0.118	0.54	2.03	60.2
14	13.1	5 : 95	0.076	0.142	0.075	0.56	1.88	35.4
15	14.1	5 : 95	0.074	0.234	0.115	0.32	2.03	58.6
16	15.1	5 : 95	0.085	0.194	0.099	0.44	1.94	48.4
17	16.1	5 : 95	0.088	0.197	0.104	0.45	1.89	49.3
18	16.2	5 : 95	0.073	0.222	0.116	0.33	1.92	55.6
19	16.3	5:95	0.183	0.332	0.168	0.55	1.97	42.13
20	17.1	5 : 95	0.184	0.340	0.180	0.54	1.89	85.2
21	17.2	5:95	0.133	0.196	0.099	0.68	1.97	49.0
22	18.1	5:95	0.121	0.224	0.116	0.54	1.92	56.01
23	18.2	5:95	0.073	0.166	0.086	0.44	1.91	41.5
24	19.0	5:95	0.133	0.205	0.1043	0.65	1.96	51.25
25	19.1	5:95	0.125	0.231	0.120	0.54	1.92	57.75
26	19.2	5:95	0.130	0.236	0.115	0.55	2.05	59.0

4.2 Multiplex ligation dependent probe amplification (MLPA)

4.2.1 Testing of the evaporation problem during hybridization

During the hybridization reaction performed on day 1 in the MLPA assay, an extra tube containing 8ul of water was added to detect the probable evaporation problem during the hybridization. The tube containing the distilled water was found to contain 6-8 µl of water after the overnight incubation at 60°C.

4.2.2 Gel Electrophoresis of the MLPA product

The gel electrophoresis of the products of the MLPA assay was performed on 4% agarose gel. The gel electrophoresis of the MLPA products showed that the distinct fragments of the probe amplified are within the range of 100 to 500 bp.

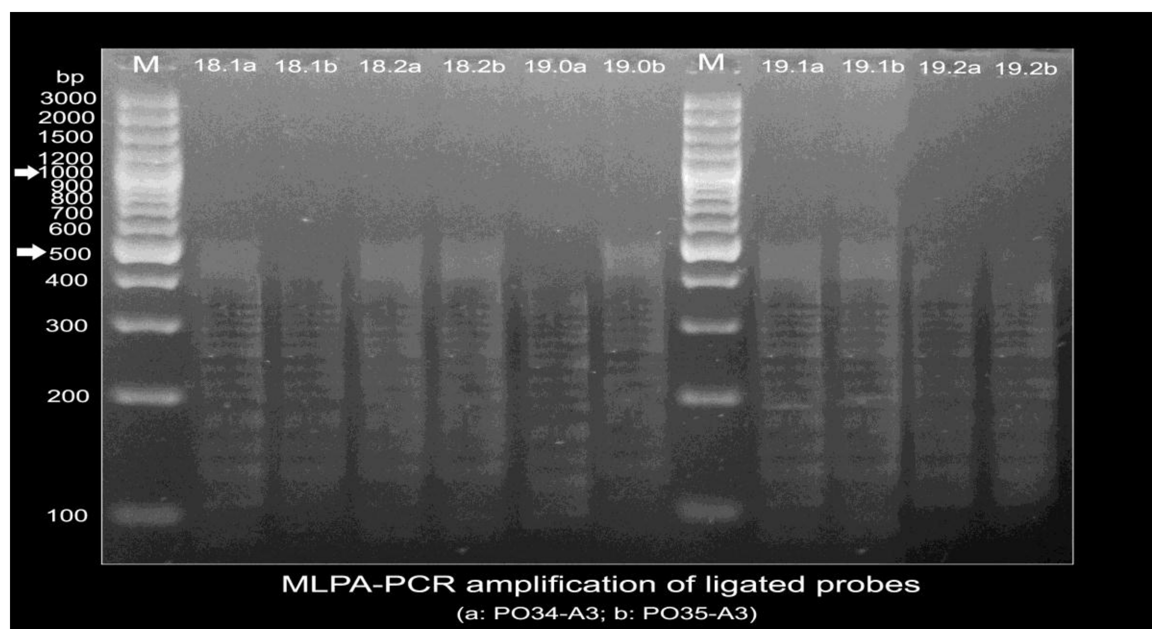


Plate 4.2: Gel electrophoresis of the MLPA-PCR product in the Agarose gel (4%). The Lane marked 'M' is 100 bp plus molecular weight marker.

4.3 CAPILLARY ELECTROPHORESIS (CE)

4.3.1 POLYMER LOADING AND CAPILLARY SET UP

During the polymer loading in syringe, pump block and the capillary no air bubbles were observed and the site was entirely free of contamination. While connecting the capillary to the pump block the end of the capillary was protruded well beyond the opening at the tip of the capillary fitting. Thus the capillary end was directly below the opening to the glass syringe. During the positioning of the capillary in the detector the green dots and the red dots in laser device was found to be exactly overlapped with the corresponding dots in the capillary. And the laser detector was exactly overlapped with the faded part of the capillary tube. After positioning the capillary near the electrode the distance between the tip and the electrode was maintained at about 0.5 mm. Besides, during the filling of the capillary there were no anomalies early in the pumping operation so the run was not paused or cancelled during the programmed 10-second delay. The fill didn't stop earlier than the recommended time and there was the slow

movement of the plunger which also ensured that there was no leak during the loading of the capillary.

To sum up, during the preparation of the genetic analyzer for the GeneScan run with POP-4 and setting up of the reagents no complications hereabout were found.

4.3.2 SAMPLE RUN

After the sample run, the screen began to display the blue colored peaks after about half an hour beginning from the peak of 60-64 nt, followed by the peaks of higher nucleotide fragments. The following day, the entire electropherograms of the samples were found to have generated in the form of the blue colored peak patterns which is the color displayed by the FAM dye.

4.3.3 GeneScan™ –500 LIZ® Size Standard Peak analysis

The peak pattern of the size standard GeneScan 500 LIZ indicates that there are sixteen different significant peaks as established by the Applied Biosystem. The peaks are within the range of 35-500 base pairs providing the 16 single stranded labeled fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bases.

4.3.4 ELECTROPHEROGRAM ANALYSIS OF THE MALE REFERENCE SAMPLE (SAMPLE 16.2)

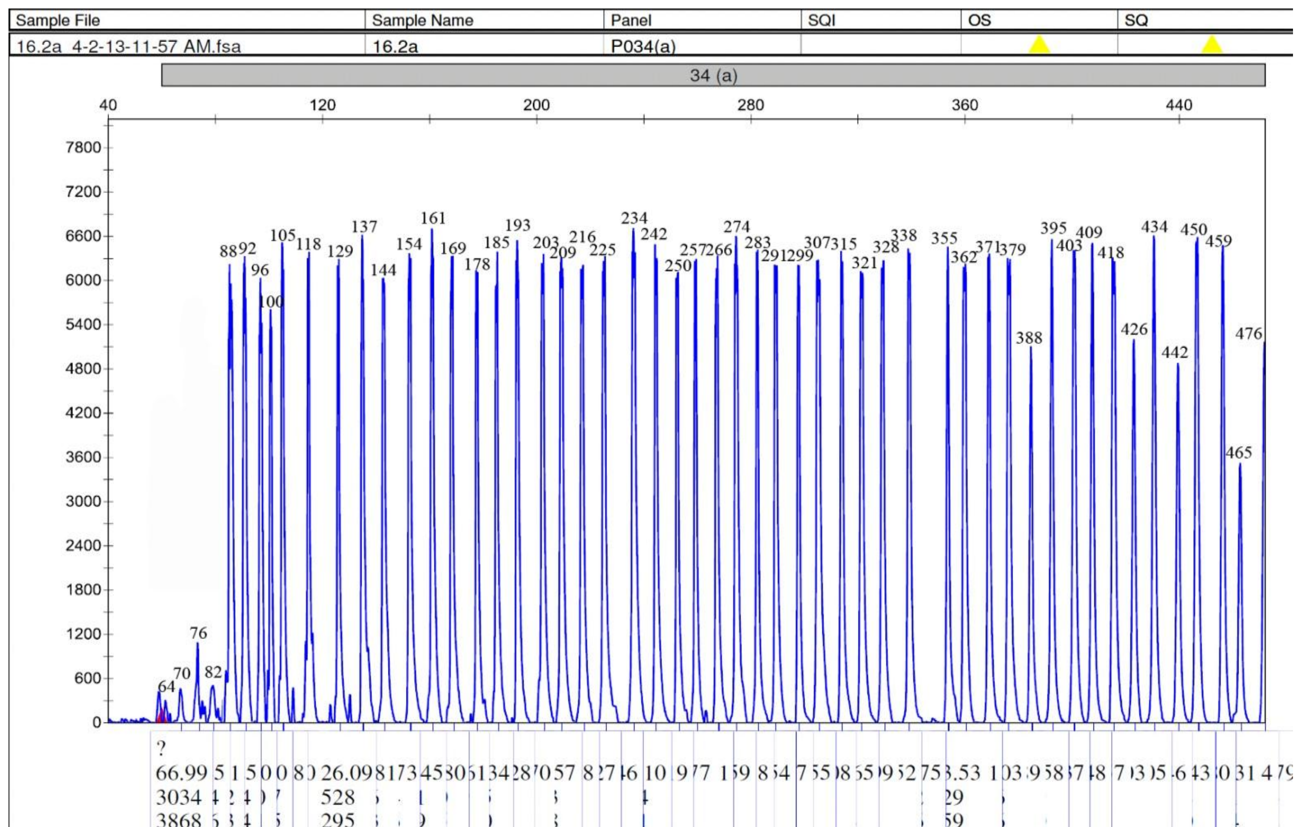


Fig4.3.4 (a): Electropherogram of probe set PO34 (a) of the male reference sample 16.2

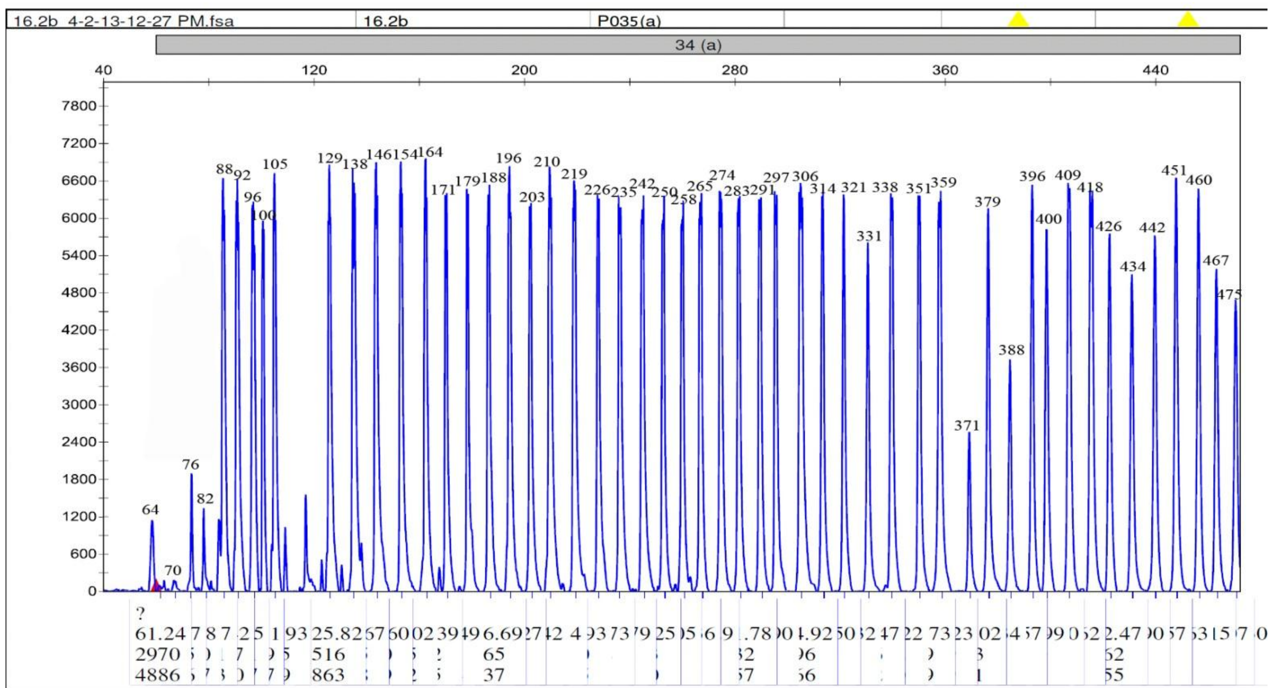


Fig4.3.4 (b): Electropherogram of probe set PO35 (a) of the male reference sample 16.2

The electropherogram of both the probe set (PO34 (a) and PO35 (b)) of the male reference sample 16.2 displayed that there is no severe (unusual) sloping in the MLPA peak pattern in which the peaks of longer MLPA probe may appear more than 3 times lower than those of the shorter MLPA probes. There are no very high baselines present in the peak pattern and there are no any signal bleeding in other channels or spectral pull up or pull down patterns in the dyes. There are no irregular current patterns or spike peaks that could interfere with the peak detection. There is no random decrement of some signals and increment of the others. No split peaks are visible in the MLPA probe signals.

Evaluation of the peak pattern also reveals that the internal size standard patterns are not unusual. There is the presence of the 92 nt control fragment and all the necessary probe amplicons. All the four peaks of the Q-fragments (64 nt, 70 nt, 76 nt and 82 nt) are found to be lower than 1/3 peak of 92 nt control fragment in the electropherogram. There is no presence of primer peaks (20 to 60 nt) or excessive primer dimer in the electropherogram as indicated by the diminished peak patterns preceding the peak of the four Q-fragments. The peak of the D-fragments (88 nt and 96 nt) are also higher than 40% of the 92 nt control fragment in both the probe set. There is the significant presence of the peaks corresponding to the peak of 100 nt (X-fragment), 105 nt (Y-fragment) and 118 nt (Y-fragment) in the electropherogram of probe set PO34, and 100

nt (X-fragment) and 105 nt (Y-fragment) in the electropherogram of probe set PO35, which is the strong indication that the electropherogram is that of the male sample.

4.3.5 ELECTROPHEROGRAM ANALYSIS OF THE FEMALE REFERENCE SAMPLE (SAMPLE 11.2)

Fig4.3.5 (a): Electropherogram of probe set PO34 (a) of the female reference sample 11.2

Fig4.3.5 (b): Electropherogram of probe set PO35 (a) of the female reference sample 11.2

The electropherogram of both the probe sets of the female reference sample 11.2 also shows that there is no severe (unusual) sloping in the MLPA peak pattern, no very high baseline present in the peak pattern, and there are no any signal bleeding in other channels or spectral pull up or pull down patterns in the dyes. Besides, there is no irregular current patterns or spike peaks that could interfere with the peak detection; there is no random decrement of some signals and increment of the others, no split peaks are visible in the MLPA probe signals or off scale peaks and there are no large differences in relative peak heights between the samples.

The internal size standard patterns are not unusual. There is the presence of the 92 nt control fragment that forms a bench mark to compare other control fragments and all the necessary probe amplicons. There is no presence of prime peaks (20 to 60 nt) or excessive primer dimer in the electropherogram as indicated by the diminished peak patterns preceding the peak of the four Q-fragments. All the four peaks of the Q-fragments (64 nt, 70 nt, 76 nt and 82 nt) are lower than 1/3 peak of 92 nt control fragment in the electropherogram. The peaks of the D-fragments (88 nt and 96 nt) are also higher than 40% of the 92 nt control fragment.

The essential peaks to consider in the electropherograms of the sample 11.2 are the peaks corresponding to the Y-fragments i.e. 105 nt and 118 nt in the electropherogram of probe set PO34. There is the presence of the peak of 100 nt (X-fragment) and the reduced form of the other two peaks of 105 nt (Y-fragment) and 118 nt (Y-fragment) in probe set PO34 (a). In addition, there is the presence of the peak of 100 nt (X-fragment) and the absence of the peak of 105 nt (Y-fragment) in the electropherogram of the probe set PO35. These evidences support the fact that it is a female sample.

Table 4.3.5: Electropherogram analysis of Male and female reference samples

SN	Sample	Clinically diagnosed as	PO34-A3		PO35-A3		Duplicated or deleted exon
			Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon	Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon	
1	16.2 (male reference)	Normal	None	None	None	None	None
2	11.2 (female reference)	Normal (Female)	105 (del) 118 (del)	Y-fragment (del) Y-fragment (del)	105(del)	Y-fragment (del)	None

4.3.6 ELECTROPHEROGRAM ANALYSIS OF THE TEST SAMPLE (SAMPLE 2.2)

Fig 4.3.6 (a): Electropherogram of probe set PO34 (a) of the test sample (2.2)

Fig 4.3.6 (b): Electropherogram of probe set PO34 (a) of the test sample (2.2)

There is the absence of random decrement of some signals and increment of the others, splitting of the peaks, large differences in relative peak heights between the samples, broadening of peak and the off scale peaks in both the electropherograms. But there was some very slight sloping in the peak pattern in the latter half of the electropherogram.

In the electropherograms of the two probe sets of the sample 2.2, the peak heights of the Q-fragments are very low compared to the 92 nt control fragments. And there are no peaks of primer dimer formation either. The peak of the D-fragments (88 nt and 96 nt) are also higher than 40% of the 92 nt control fragment. There is the presence of the peaks corresponding to the 100 nt (X-fragment), 105 nt (Y-fragment) and 118 nt (Y-fragment) in the electropherogram of probe set OP34 and 100 nt (X-fragment) and 105 nt (Y-fragment) in the electropherogram of probe set PO35, indicating the male sample and absence of sample swapping.

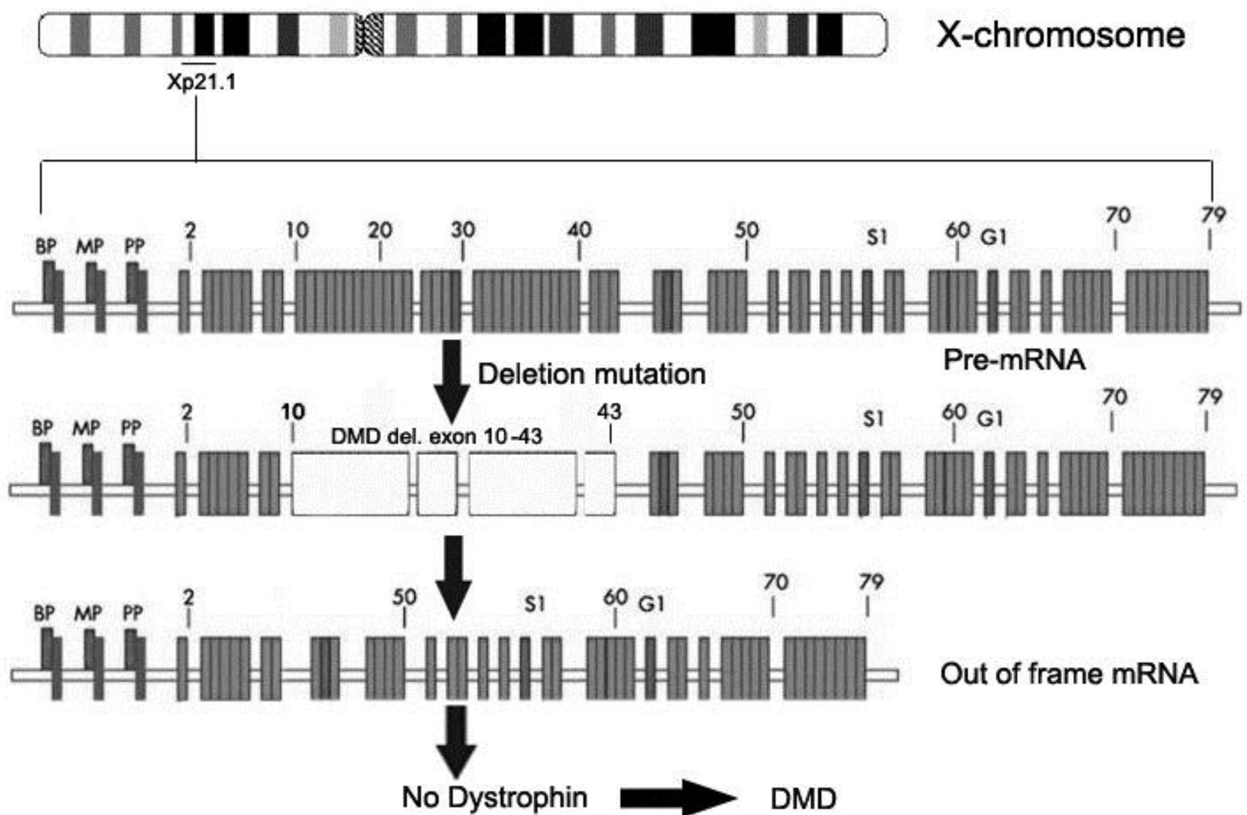


Fig 4.3.6 (c) Deletion mutation in sample 2.2

When we compare the electropherograms of the probe set PO34 of the test sample and the corresponding reference sample, it reveals that there is lack of the peaks of probes of 144 nt, 154 nt, 178 nt, 185 nt, 216 nt, 225 nt, 257 nt, 299 nt, 328 nt, 371 nt, 403 nt,

442 nt, 459 nt and 476 nt. Similarly, when we compare the electropherograms of the probe set PO35 of the test sample and the corresponding reference sample, it reveals that there is lack of the peaks of probes of 138 nt, 154 nt, 171 nt, 188 nt, 210 nt, 226 nt, 242 nt, 258 nt, 283 nt, 297 nt, 314 nt, 331 nt, 351 nt, 371 nt, 388 nt, 400 nt, 426 nt, 442 nt, 460 nt and 475 nt. The absence of these peaks helps us to determine the deleted exons and the framedness of the sample which are tabulated below.

Table 4.3.6: Electropherogram analysis of the test sample 2.2

Sample	Clinically diagnosed as	PO34-A3		PO35-A3		Duplicated or deleted exon	Framed - ness
		Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon	Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon		
2.2	DMD	(144, 154, 178, 185, 216, 225, 257, 299, 328, 371, 403, 442, 459, 476) del	(41, 21, 42, 22, 43, 23, 24, 25, 26, 27, 28, 29, 10, 30) del **i.e.** (10, 21-30, 41-43) del	(138, 154, 171, 188, 210, 226, 242, 258, 283, 297, 314, 331, 351, 371, 388, 400, 426, 442, 460, 475) del	(11, 31, 12, 32, 13, 33, 14, 34, 15, 35, 16, 36, 17, 37, 18, 38, 19, 39, 20, 40) del **i.e.** (11-20, 31-40) del	DEL Ex10-43	Out-of-frame

4.3.7 ELECTROPHEROGRAM ANALYSIS OF OTHER TEST SAMPLES

The electropherograms of the other test samples were also analysed by comparing with the peak pattern of the male and female reference samples. There were 24 samples in total among which 3 of them were normal samples (a normal male sample, a female parent and a sister of a DMD client) and the remaining 21 samples were test samples. The capillary electrophoresis run (ABI-310 genetic analyzer) demonstrated that it could pick up the deletions in 14 of the 21test samples considered. Thus MLPA was efficient in accurately confirming mutations in about 67% of all cases. Besides the most prevalent exonic deletion regions were found to be confined in the exon 7-14, the proximal zone and 45-53, the first half of C-terminal domain. The DMD exonic deletions/duplications reading frame checker 1.9 was used to determine the reading frame (in-frame or out-frame).No novel mutations were identified in this study. To sum up, the MLPA assay confirmed mutations in 67% of the patients in our study.

Table 4.3.7 (a): Electropherogram analysis of the MLPA negative test samples

SN	Sample	Clinically diagnosed as	PO34-A3		PO35-A3		Duplicated or deleted exon	Framed-ness
			Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon	Duplicated/deleted probe length (nt)	Duplicated/deleted corresponding exon		
1	4.2	DMD	None	None	None	None	None	None
2	5.2	DMD	None	None	None	None	None	None
3	8.1	BMD	None	None	None	None	None	None
4	13.1	DMD	None	None	None	None	None	None
5	15.1	DMD	None	None	None	None	None	None
6	18.2	DMD	None	None	None	None	None	None
7	19.2	DMD	None	None	None	None	None	None

Table 4.3.7 (b): Electropherogram analysis of MLPA positive test samples

SN	Sample	Clinically diagnosed as	PO34-A3		PO35-A3		Duplicated or deleted exon	Framedness
			Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon	Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon		
1	1.1	DMD	(395, 434, 465)del	(48, 49, 50) del	None	None	**DEL EX48-50**	OoF
2	1.4	DMD	None	None	179del	52del	**DEL52**	OoF
3	2.2	DMD	(144, 154, 178, 185, 216, 225, 257, 299, 328, 371, 403, 442, 459, 476) del	(41, 21, 42, 22, 43, 23, 24, 25, 26, 27, 28, 29, 10, 30) del i.e. (10, 21-30, 41-43) del	(138, 154, 171, 188, 210, 226, 242, 258, 283, 297, 314, 331, 351, 371, 388, 400, 426, 442, 460, 475) del	(11, 31, 12, 32, 13, 33, 14, 34, 15, 35, 16, 36, 17, 37, 18, 38, 19, 39, 20, 40) del i.e. (11-20, 31-40) del	**DEL Ex10-43**	OoF
4	7.2	DMD	None	None	179del	52del	**DEL Ex52**	OoF
5	9.0	DMD	291del	45del	None	None	**DEL Ex45**	OoF
6	11.3	DMD	None	None	(146, 179, 219) del	(51, 52, 53) del	**DEL Ex51-53**	OoF
7	12.1	DMD	(291, 321,	(45, 46, 47,	None	None	**DEL Ex45-50**	OoF

			362, 395, 434, 465)del	48, 49, 50)del				
8	14.1	DMD	(321, 362, 395, 418, 434)del	(46, 47, 48, Xq13, 49)del	None	None	**DEL Ex46-49**	OoF
9	16.3	DMD	None	None	179del	52del	**DEL Ex52**	OoF
10	17.1	DMD	None	None	146del	51del	**DEL Ex51**	OoF
11	17.2	DMD	None	None	(171, 210, 242)del	(12, 13, 14) del	**DEL Ex 12-14**	OoF
12	18.1	DMD	None	None	146del	51del	**DEL Ex51**	OoF
13	19.0	DMD	291del	45del	None	None	**DEL Ex45**	OoF
14	19.1	DMD	(355, 388, 426)del	(7, 8, 9) del	None	None	**DEL EX7-9**	OoF

Note:- OoF: Out of Frame

4.3.8 ELECTROPHEROGRAM ANALYSIS OF THE NORMAL SAMPLES

Among the three normal samples (2 female samples and 1 male sample) run along with the test samples the electropherograms of the two normal samples 1.2 and 2.1 displayed the electropherograms characteristic to the female reference sample. And the electropherogram of one of the normal sample 16.1 displayed the electropherogram characteristic to the male reference sample.

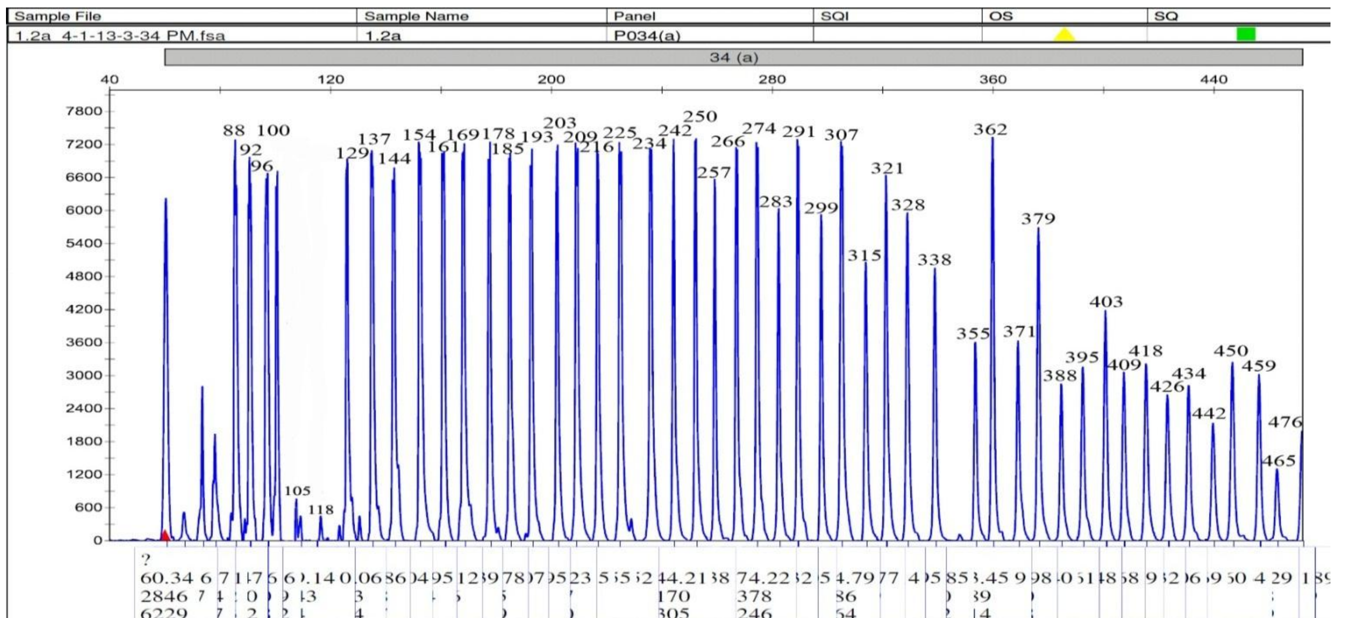


Fig 4.3.8 (a): Electropherogram of probe set PO35 (a) of the normal sample 1.2

Fig 4.3.8 (b): Electropherogram of probe set PO35 (a) of the normal sample 1.2

Fig 4.3.8 (c): Electropherogram of probe set PO34 (a) of the normal sample 16.1

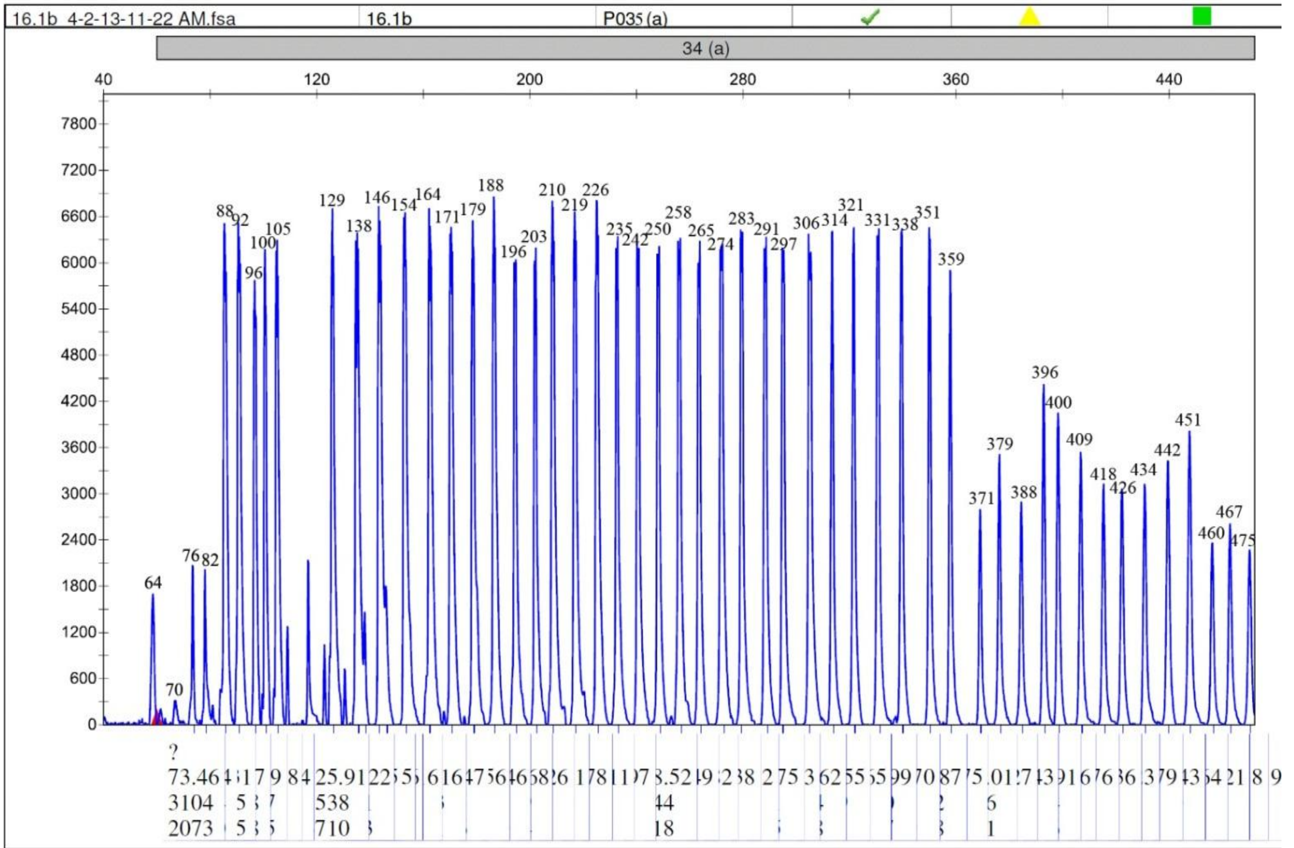


Fig 4.3.8 (d): Electropherogram of probe set PO35 (a) of the normal sample 16.1

Table 4.3.8: Electropherogram analysis of the normal samples

SN	Sample	Clinically diagnosed as	PO34-A3		PO35-A3		Duplicated or deleted exon	Framedness
			Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon	Duplicated or deleted probe length (nt)	Duplicated or deleted corresponding exon		
1	1.2	Normal (sister)	105(del) 118(del)	Y-fragment (del) Y-fragment (del)	105(del)	Y-fragment (del)	None	None
2	2.1	Normal (female parent)	105(del) 118(del)	Y-fragment (del) Y-fragment (del)	105(del)	Y-fragment (del)	None	None
3	16.1	Normal (male)	None	None	None	None	None	None

CHAPTER V: DISCUSSION

5.1 DNA extraction: Optimization and quantification

The DNA extraction was performed using the kit manual (QIAamp® DNA Mini and Blood Mini Handbook Cat no 51104, Third Edition, 2010, Qiagen, Sample and Assay technologies techniques) as provided by the QIAGEN. The kit provides the optimized protocol for the high quality DNA extraction. But during the experiments it was found that the slight modification in some of the steps of the protocol helps in improving the result.

After the application of the mixture from the micro-centrifuge tube to the mini spin column, containing the silica based membrane, it was centrifuged at 6000 X g (8000 rpm) for 1 minute instead of the centrifugation at the full speed for 1 minute in order to reduce the noise. The QIAamp Mini spin column was then placed in a clean 2 ml collection tube provided in the kit, and the tube containing the filtrate was discarded. When the lysate did not completely pass through the column after the centrifugation, it was centrifuged again at higher speed until the QIAamp Mini spin column is empty. In the final step of washing by the Buffer AW2 the cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The buffer AW2 contains the sodium azide that acts as the preservative. Only the filtrate was discarded and the QIAamp Mini spin column was placed in the same 2 ml collection tube again. The content was then centrifuged at full speed for 1 minute. It was the additional step that was applied during the extraction to ensure the elimination of the chance of possible AW2 carry over. During the elution of the DNA in Buffer AE (10 mM Tris HCl; 0.5 mM EDTA; pH 9.0)) 150 μl of the buffer was used instead of 200μl to yield the higher concentration of DNA.

The QIAGEN kit protocol for the DNA extraction is also based on the general steps of DNA extraction i.e. Lyse, Bind, Wash, and Elute. The Proteinase K provided in the kit is the modified form of endopeptidase K, also known as *Tritirachium* alkaline proteinase which is a serine proteinase that catalyzes the hydrolysis of peptide bonds more readily in an intact protein than in small peptides. It is the enzyme that catalyzes the hydrolysis of keratin, and of other proteins with subtilisin-like specificity. Thus the proteinase K acts as the sensitizer in the reaction. The proteinase K is completely free of DNase and RNase activity. The activity of the proteinase K solution is 600 mAU/ml solution (or 40 mAU/mg proteins). The AL buffer used in the experiment is the lysis buffer that lyses the blood cells and also helps in the denaturation of proteins. The mini spin column consists of the silica based membrane on it which helps in the binding of DNA. It has been known since the 1950s that DNA binds in a reversible manner in silica in the presence of chaotropic salts as guanidine salts. The interaction between double-stranded DNA and the silicate matrix is thought to be due to the dehydration of the phosphodiester backbone, which

allows the exposed phosphate residue to adsorb to the silica. The adsorbed double-stranded DNA remains in either a native or partially denatured (single stranded) state and cannot be eluted from the matrix by solvent as 50% ethanol. However when the immobilized DNA is rehydrated by washing with aqueous buffer as EA provided in the kit it can be quantitatively recovered in column effluent (Sambrook J and Russel DW, 2001).The kit combines the selective binding properties of a silica-based membrane with flexible elution volumes. The buffer AL and the washing buffers AW1 contain the chaotropic salt guanidine hydrochloride that helps in the binding of DNA and precipitation of DNA in the presence of alcohol. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. The use of 2 different wash buffers, Buffer AW1 and Buffer AW2, significantly improves the purity of the eluted DNA and also helps in its preservation as buffer AW2 contain sodium azide as the preservative. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding. The pure DNA bound to the silica membrane is then eluted out using the buffer AE after the subsequent washing by buffer AW1 and AW2 that helps in DNA binding and preservation and also in the removal of the impurities.

The procedures during the extraction are designed such that there is no sample to sample cross contamination and allow safe handling of potentially infectious samples. The QIAGEN kit manual was found to be a good protocol for the extraction of a pure and quality DNA that was suitable for MLPA.

The DNA extracted from this technique was assessed by quantifying the DNA taking the absorbance at 230 nm, 260 nm and 280 nm and by testing the quality of DNA produced on Agarose Gel Electrophoresis (1%).Nucleotides, RNA, ssDNA and dsDNA all will absorb at 260nm and contribute to the total absorbance. OD of value 1 at 260nm = 50µg/ml. The ratio of absorbance at 260nm and 280nm is used to assess the purity of DNA and RNA. A ratio of 1.7- 1.8 is generally accepted as "pure" for DNA; a ratio of ∼ 2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of proteins, phenol or other contaminants that absorb strongly at or near 280nm.And the absorbance readings at 260 nm should lie between 0.1 and 1.0 to be accurate. The possible contamination due to proteins and other chemical substances as phenolates and thiocynates are removed with the help of the chemicals as proteinase K and washing buffers used during the experimentation. Correspondingly the contamination of protein was found to be negligible as given by the absorbance ratio at 260 nm and 280 nm. The majority of the ratio of the optical density (OD) of 260 nm and 280 nm ranged from 1.5-2.0 suggesting the purity of DNA. The purity of DNA was determined by calculating the ratio of absorbance at260 nm to

absorbance at 280 nm. The DNA estimation by UV spectrophotometer of the samples used in this investigation gave a DNA concentration range within 15µg/mL to 60 µg/mL. It corresponds to the DNA concentration range as suggested by the Qiagen kit, which was used to extract the DNA. The DNA extracted from the samples were of acceptable and adequate quality for the MLPA experiment as the DNA was pure enough (OD260/280 =1.76) for MLPA.

For a pure DNA sample the A230/260 should be around 0.556.If the ratio is appreciably higher than expected, it may indicate the presence of contaminants which absorb at 230 nm (e.g. the phenolate ions and thiocynates. The ratio 230/260 which was used as a secondary measure of nucleic acid purity also showed that the DNA is not contaminated with phenolate ion, thiocyanates and other organic compounds.

Hence, the quantitation and quality check of investigated DNA in present study was observed adequate for the MLPA reaction. The QIAamp procedure was found to be suitable for use with the whole blood which has been treated with EDTA. There was no need of the prior separation of leukocytes and the purification required no phenol/chloroform extraction or alcohol precipitation. The DNA eluted in Buffer AE could then direct be added to the enzymatic reaction as MLPA. The purified DNA was found to be free of protein, nucleases and other contaminants or in inhibitors. The DNA purified using the QIAamp kit is up to 50 kb in size, with the fragments of approximately 20-30 kb predominating which is suitable for direct use in PCR or Southern-blotting applications. DNA of this length denatures completely during thermal cycling and can be amplified with high efficiency.

5.2 Multiplex ligation dependent probe amplification (MLPA) assay

The MLPA reactions make us of two probe sets viz. PO34-A3 and PO35-A3 and a single PCR primer pair for all its probes that makes the method very robust. Each of the MLPA probe consist of oligonucleotides that gets bind to adjacent DNA target sequences in order to be ligated. After the ligation, the probe becomes a single molecule that will be amplified exponentially during the PCR reaction. Each of the distinct probes in an MLPA probemix generates a PCR amplicon of unique length. The generation of these unique lengths appears in the gel electrophoresis (4%) as barred fragments.
MLPA being a relative technique, it only detects the relative difference. And hence the use of reference sample is an essence. The reference sample chosen for the reaction is that of the healthy individuals who voluntarily donated the blood for the test. These samples are expected to have a normal copy number for the reference probes and the dystrophin gene, so that we can detect the abnormal probe signals that indicate the deletions and/or duplication of sequences detected by the MLPA probes.

The DNA samples diluted with the elution buffer AE are heated at 98°C in a thermocycler with the heated lid to ensure the complete denaturation of the DNA. After the denaturation the single stranded DNA are stabilized at 25°C.

The denaturation step is followed by the hybridization step in which the hybridisation master mix are prepared containing 1.5 µl of MLPA buffer (yellow cap) and 1.5 µl of probe mix (black cap) for each reaction. The MLPA buffer is composed of potassium chloride (KCl), Tris-HCl and PEG-6000 maintained at slightly alkaline pH (pH 8.5).
In general, the standard PCR buffer contains 50 mM of monovalent cation as KCl that works well for the amplification of segment of DNA >500 bp in length. Raising the KCl concentration to about 70-100 mM often improves the yield of shorter DNA segments. Since the amplifiable products range from 130 to 490 bp in the probe set, the concentration of KCl in MLPA buffer is maintained at 1.5M. Washing of the DNA with TE buffer (300 mM tris-HCl and 1 mM EDTA) in a slightly alkaline condition, pH 8.5 helps in maintaining the denaturation stage of DNA. In addition, the EDTA also chelates the divalent cations such as Mg^{2+} and thereby inhibits the action of any residual nucleases that degrade DNA.
PEG-6000 is a modified form of polyethylene glycol that helps in the further purification and precipitation of DNA molecule according to its size. It increases the efficiency of re-association of complementary chain of nucleic acid during hybridization and blunt end ligation of DNA molecule. It is suggested that the addition of PEG to the hybridization solution increases the rate of hybridization about tenfold (Sambrook J and Russell DW,2001). It also acts as an enhancer in suboptimal extension of annealed primers. If the target DNA is blunt ended inclusion of PEG in the reaction increases the efficiency of ligation.

The MLPA reaction consists of two probe sets viz. PO34-A3 and PO35-A3 containing the probes that alternatively target each of the exons of the DMD gene (79 exons) on Xp21.1 chromosome.
The PO34-A3 probemix contains 45 different probes with amplification products between 129 and 490 nt. Out of these 45 probes, 5 probes are the reference probes whereas the remaining 40 probes are the probes targeting the alternative 40 exons of the DMD gene (Ex1-10, Ex21-30, Ex41-50 and Ex61-70). It also contains the 10 control fragments generating an amplification product smaller than 120 nt. The control fragments includes 4 Q-fragments (64-70-76-82 nt), 3 D-fragments (88-92-96 nt), 1 chromosome X-specific fragment (100 nt) and 2 (105-118 nt) chromosome Y-specific probes.

The PO35-A3 probemix contains 45 different probes with amplification products between 129 and 490 nt. Out of these 45 probes, 5 probes are the reference probes

whereas the remaining 40 probes are the probes targeting the remaining alternative 40 exons of the DMD gene (Ex11-20, Ex31-40, Ex51-60, Ex71-79 and 1DP427c of DMD). It also contains the 9 control fragments generating an amplification product smaller than 120 nt. The control fragments includes 4 Q-fragments (64-70-76-82 nt), 3 D-fragments (88-92-96 nt), 1 chromosome X-specific probe (100 nt) and 1 chromosome Y-specific probes (105 nt).

These specific probe mixes (1-4 fmol of each synthetic probe oligonucleotide and each M13-derived oligonucleotide in TE) are mixed with the MLPA buffer (salt solution containing 1.5 M KCl, 300 mM Tris-HCl pH 8.5, 1mM EDTA) to make the master mix and are allowed to hybridise with the targeted exons in the DNA samples at 60°C overnight (16-20 hours) which is the optimized temperature and time for the hybridization of the probes to the targeted exons.

The fragments so hybridized to the targeted gene fragments are ligated using the ligase enzymes Ligase-65 and the ligase buffers A (transparent cap) and B (white cap). The ligase buffer A consists of NAD of the bacterial origin at pH 3.5 while the ligase buffer B consists of Tris-HCl, non-ionic detergents and $MgCl_2$ at pH 8.5. Thus the mixture of ligase buffer A and B acts as a dilution buffer with 2.6 mM $MgCl_2$, 5mM Tris-HCl pH 8.5, 0.0013% non-ionic detergents and 0.2 mM NAD. The ligase-65 enzyme used for the ligation is of the bacterial origin. Ligase-65 is an enzyme that required NAD to create the phosphodiester bond. It catalyzes the formation of phosphodiester bonds between the directly adjacent 3' hydroxyl and 5' phosphoryl termini of nucleic acid molecules as DNA. And the cofactor generating high energy intermediates in the reaction may is NAD^+. During the first step of ligation reaction the diphosphate linkage of NAD^+ is used as a phosphoanhydride and the adenyl group is transferred to the e-amino group of a lysine residue. It works preferentially at nicks in double stranded DNA. Ligase-65 cannot ligate single stranded molecules to each other and are also unable to ligate double stranded molecules with blunt ends or with short (5 nt or less) sticky ends. The presence of non-ionic detergent leads to the effective dissociation of enzyme-product complex. The solution of PEG and $MgCl_2$ is generally used to precipitate DNA and are useful in suboptimal extension of annealed primer. The optimum temperature for the ligation of the probes and the targeted DNA fragments is 54°C. After the ligation, the 5 minute incubation at 98°C leads to the heat inactivation of ligase-65 enzyme followed by the lowering of the temperature to 20°C. At this temperature the tubes can be removed from the thermocycler and the reaction product can be stored at room temperature for several hours or at 4°C for up to a week. The BRIJ used in the Ligase-65, PCR primer mix and polymerase is a non-ionic polyoxyethylene detergent solution (surfactant) that provides unsurpassed purity, quality and stability of the products. The β-

mercaptoethanol (0.1%) is the reducing agent of choice to maintain the biological/protein function as that of the enzymes as it has the lower interference.

The ligated products are then treated with the polymerase master mix for the general polymerase chain reaction. The PCR primer mix (brown cap) is composed of the synthetic oligonucleotides (one of which is fluorescently labeled with FAM), dNTPs, Tris-HCl, KCl, EDTA and BRIJ (0.04%) at pH 8.5. And the polymerase (orange cap) is composed of polymerase enzyme of bacterial origin along with its supplements Glycerol, BRIJ (0.5%), EDTA, DTT (0/1%), KCl and Tris-HCl at pH 7.5. The polymerase is warmed in hand for about 10 secounds to reduce the viscosity. The MLPA uses a single primer set (10 pmol). The sequence of the labeled primer is 5'GGTTCCTAAGGGTTGGA3' and that of the unlabeled primer is 5'GTGCCAGCAAGATCCAATCTAGA3'.Polymerase enzyme (bacterial enzyme) helps in the extension of the annealed primers catalyzing the addition of deoxynucleotide triphosphate to a recessed 3'-hydroxyl group and in the amplification of DNA fragments. DNA polymerase require free divalent cations usually Mg^{+2} for activity. The dNTPs (all four dioxynucleoside triphosphates) used are at the concentration of 2.5 nmol whereas the reaction mix consists of 2.5 U polymerase. The high concentration of dNTPs is inhibitory which may be due to sequestering of Mg^{+2}.

Glycerol is a monophasic reagent that is generally used to optimize the speed and extent of RNase inactivation. It helps in polymerase enzyme stabilization and also acts as adjuvants in reaction mixture in amplification. It is an additional component reported by Ford and Rose (1994) to improve the efficiency of long PCR at a concentration of 5% v/v in the final reaction mixture to promote separation of DNA strands at lower temperature and EDTA at a concentration of 0.75 mM in the final reaction mixture to chelate divalent cations as Mn^{+2} that might promote scission of DNA strands. The glycerol also allows the enzyme preparation to be stored at -20°C without freezing. Because glycerol and other vicinal dials also stabilize protein-protein interactions (including inter-domain interactions in single-subunit proteins) it protects the enzymes against denaturation.

Each probe consists of two oligonucleotides, one synthetic (short) and one M13 derived (long) that hybridise to adjacent sites of the target sequence. The short synthetic oligonucleotide of each probe contains a target specific sequence (21-30nt) at the 3' end and a common 19 nt sequence, identical to the labeled PCR primer, at the 5' end. DTT is a strong reducing agent that helps in the primer extension.

The MLPA products were satisfactory as the fragments of DNA amplified were found to be within the range of 60 to 490 nucleotides, which is the desired length of the probes used in the reaction.

The amplification showed that the product so obtained after the MLPA is suitable for the sequence type capillary electrophoresis.

5.3 Capillary electrophoresis (CE)

5.3.1 PREPARATION OF THE 310-GENETIC ANALYZER FOR GENE SCAN RUN WITH POP-4 AND SETTING UP THE REAGENTS

6-FAM is the most commonly used fluorescent dye for the attachment to oligonucleotides and is compatible with most fluorescence detection equipment. FAM becomes protonated below pH 7 which results in decreased fluorescence. Thus run condition is maintained at pH range 7.5-8.5. The FAM dye has its absorbance maxima at 495 nm (Ab_{max} 495 nm) and the emission maxima at 520 nm (Em_{max}520 nm).

POP-4 and POP-6 are the polymers that are generally used in capillary electrophoresis. POP-6 is the polymer that is recommended for sequencing whereas polymer POP-4 is recommended for gene mutation analysis as in case of DMD gene mutation analysis. Polymers POP-4 and POP-6 differ in their resolutions. The POP polymers dynamically coat the capillary wall to control the electro-osmotic flow.

The protocol uses formamide as a sample preparation reagent. Fresh formamide were deionized and aliquotted into smaller volumes for storage. During the preparation of formamide care was taken that the formamide used is not stored for longer periods as they become acidic due to the formation of formic acid on longer storage. It may result in depurination and fragmentation of the DNA upon heating and also contribute to the degradation of fluorescent dyes. The Hi-Di formamide that doesn't solidify at -20°C are discarded.

During the preparation of the genetic analyzer for the GeneScan run necessary precautions were taken to avoid any possible hazards. Consequently, no complications were seen during the sample run.
Besides, during the processes associated with polymer loading like loading the syringe with the polymer, filling the pump block channel with the polymer and filling the capillary with the polymer care were taken to prevent the formation of air bubbles and it was ensured that all the air bubbles are removed from the site and it is entirely free of contamination. It is because the presence of the bubbles or even a droplet prevents the electric conduction hampering the entire capillary run.

During the filling of the capillary there were no anomalies early in the pumping operation so the run was not paused or cancelled during the programmed 10-second delay. During the 10-minute fill, pumping automatically stops if the instrument detects

movement of the plunger corresponding to 25-μL or more of polymer. The leaks had to be checked if the fill stopped early. After the module had run the position of the plunger is again noted. The difference of the position of the plunger was found to be four units (Gel pump: 294 to 298). It indicates that the pump consumed 4μL of polymer per 10-minute fill. The capillary syringe consists of 70 divisions. As the gel pump number increases by 4 units (say, from 294 to 298), the syringe pumps by four divisions and it is considered that the capillary is filled. If more than 7-μL of polymer is consumed it indicates the leaks. There were no any complications of the kind.

100% Methanol is used as the cleansing agent to clean the laser port. Methanol is usually preferred than other alcohols being highly and readily volatile.

During the preparation of the formamide size standard mix, Liz is used as the internal standard. Liz is the ROX labeled DNA fragment that gives an orange colored peak patterns. Based on this internal standard the peak pattern of the probe lengths obtained from the sample run can be determined. So, Liz acts as a ladder during the capillary run.

To prevent PCR carry-over contamination, it is recommended to work with amplified PCR products in an area separate from where reaction trays are loaded prior to PCR during the preparation of the sample.

After the denaturation of the sample the samples were removed from the thermocycler and cooled in the refrigerator so that the two strands of the DNA so denatured remain as such. HiDi formamide is the chemical agent that maintains the single stranded form of the DNA after the denaturation. Formamide causes dissociation of the duplex in a manner that depends on sequence domains. The resulting single stranded DNA has a much lower electrophoretic mobility than dsDNA. The tendency of ssDNA to fold back on itself with the formation of intramuscular base pairs can be overcome by using formamide (which denatures short base pair regions). Applied biosystem recommends using Hi-Di to re-suspend the purified sequencing products (Cammarack R, 2006).
Before loading the samples in the autosampler tray for the run the tube arrangement and order of the samples in the tray and on the Sample Sheet was maintained to be the same. In the data collection software excel sheet the data was entered exactly in the order as the samples were arranged in the autosampler tray. The last value was entered by choosing any of the samples (here sample 2.1 was taken) such that after the completion of the sample run, the last sample will be used to wash the capillary.

5.3.2 ANALYSIS OF THE MALE REFERENCE SAMPLE (SAMPLE 16.2)

The MLPA peak pattern of the male reference sample 16.2 shows that there is no severe (unusual) sloping in the MLPA peak pattern. There are no very high baseline present in

the peak pattern, the internal size standard pattern are not unusual and there are no any signal bleeding in other channels or spectral pull up or pull down patterns in the dyes. Besides, no irregular current patterns or spike peaks that could interfere with the peak detection. So, the male reference sample fulfills all the necessary criteria of the raw data checklist, indicating that it is suitable to be used as the control sample.

Evaluation of the peak pattern reveals that there is the presence of the 92 nt control fragment and all the necessary probe amplicons. The 92 nt control fragment is one of the internal quality control fragments that is ligation dependent, and behaves similarly to other MLPA probes. If there is the absence of the primer peaks or the primer dimers (20 to 60 nt region) and the 92 nt control fragment it suggests the defect in the fluorescent dye. But if there is the presence of all four Q fragments (64, 70, 76 and 82 nt fragments) along with the primer peaks and the absence of 92 nt control fragment, it indicates the PCR failure or failure in the ligation reaction. So, the 92 nt control fragment forms a bench mark to compare other control fragments.

All the four peaks of the Q-fragments (64 nt, 70 nt, 76 nt and 82 nt) are lower than 1/3 peak of 92 nt control fragment in the electropherogram. The four Q-fragments are the oligonucleotides present in both MLPA probemixes. As they contain both the MLPA PCR primer sequences in a single molecule they do not need to hybridise to the target DNA or be ligated in order to be amplified during the PCR. These Q-fragments are present in such small quantities that their amplicons are completely out competed by the amplicons of the MLPA probes when enough DNA sample is used. So, when the peaks of the Q-fragments are low or invisible, it suggests that sufficient DNA was present and the ligation reaction was successful. But when the peaks of all four Q-fragments are higher than 1/3 of the height of the 92 nt control fragment and all the probes, it means that the amount of DNA sample was insufficient or the ligation reaction failed. Thus the reduced peaks of all the four Q-fragments indicate the presence of enough DNA sample in the run and the successful ligation reaction. Thus, although the optical density (260 nm) measurements may overestimate the DNA concentration, e.g. due to contamination with RNA, the sufficiency of DNA quantity can be estimated on the basis of the Q-fragments.

The significant presence of the peaks indicating the corresponding peaks of 100 nt (X-fragment), 105 nt (Y-fragment) and 118 nt (Y-fragment) in the electropherogram of probe set OP34 and 100 nt (X-fragment) and 105 nt (Y-fragment) in the electropherogram of probe set PO35 indicate that there is no sample swapping and it hence affirms that the sample is that of the male client.

The peak of the D-fragments (88 nt and 96 nt) are also higher than 40% of the 92 nt control fragment. These D-fragments are the two DNA denaturation fragments which are the synthetic MLPA probes, detecting a sequence within a strong CpG island (which

is the chromosomal regions with very high CG content that makes them difficult to denature). The absence or reduced form of these two D-fragments indicate the incomplete denaturation of the sample DNA, resulting in unreliable results for probes detecting sequences in or near (<5kb) CpG islands. This can be due to salt concentration of the DNA sample as the melting temperature of DNA is increased at higher concentrations. As CpG islands are often located near mRNA transcription start locations, exon 1 probe (of 137 nt in PO34)is most commonly affected by incomplete denaturation. The significant presence of peaks of D-fragments suggests the complete denaturation of the DNA sample.

There is no presence of prime peaks (20 to 60 nt) or excessive primer dimer in the electropherogram as indicated by the diminished peak patterns preceding the peak of the four Q-fragments. It indicates the successful PCR reaction. There is no random decrement of some signals and increment of the others that could be the result of incomplete hybridisation. No split peaks are visible in the MLPA probe signals that could be the result of low temperature of the capillary electrophoresis or the degradation of PCR products. The absence of the off scale peaks indicate the sufficient amount of PCR product injected during the capillary electrophoresis run. There is no case of the appearance of the sloping in which the peaks of longer MLPA probe may appear more than 3 times lower than those of the shorter MLPA probes. So, the formamide, polymer or the capillaries used in the capillary electrophoresis are in good state. There are no large differences in relative peak heights between the samples which could arise due to contaminants in DNA sample, use of extremely large DNA, saturation of the fluorescent detection device due to overloading of capillaries or peak broadening due to deteriorated gel or capillaries. The reference sample 16.2 has overcome all the possible defects that could be present in the PCR products and capillary electrophoresis run. So, the sample 16.2 can be taken as the male reference sample and proceeded with data analysis.

5.3.3 ANALYSIS OF THE FEMALE REFERENCE SAMPLE (SAMPLE 11.2)

The MLPA peak pattern of the female reference sample 11.2 shows that there is no severe (unusual) sloping in the MLPA peak pattern, no very high baseline present in the peak pattern, the internal size standard pattern are not unusual and there are no any signal bleeding in other channels or spectral pull up or pull down patterns in the dyes. Besides, no irregular current patterns or spike peaks that could interfere with the peak detection. So, the female reference sample fulfills all the necessary criteria of the raw data checklist, indicating that it is suitable to be used as the control sample.

Evaluation of the peak pattern reveals that there is the presence of the 92 nt control fragment that forms a bench mark to compare other control fragments and all the

necessary probe amplicons. All the four peaks of the Q-fragments (64 nt, 70 nt, 76 nt and 82 nt) are lower than 1/3 peak of 92 nt control fragment in the electropherogram suggesting that sufficient DNA was present and the ligation reaction was successful.

The essential peaks to consider in the electropherograms of the sample 11.2 are the peaks corresponding to the Y-fragments i.e. 105 nt and 118 nt in the electropherogram of probe set PO34. The presence of the peak of 100 nt (X-fragment) and the reduced form of the other two peaks of 105 nt (Y-fragment) and 118 nt (Y-fragment) indicate that the sample considered is that of the female client. In addition, the absence of the peak of 105 nt (Y-fragment) in the electropherogram of the probe set PO35 also support the fact that it is a female sample.

The peaks of the D-fragments (88 nt and 96 nt) are also higher than 40% of the 92 nt control fragment. This significant presence of peaks of D-fragments suggests the complete denaturation of the DNA sample.

There is no presence of prime peaks (20 to 60 nt) or excessive primer dimer in the electropherogram as indicated by the diminished peak patterns preceding the peak of the four Q-fragments indicating the successful PCR reaction. There is no random decrement of some signals and increment of the others that could be the result of incomplete hybridisation. No split peaks are visible in the MLPA probe signals that could be the result of low temperature of the capillary electrophoresis or the degradation of PCR products. The absence of the off scale peaks indicate the sufficient amount of PCR product injected during the capillary electrophoresis run. There is no case of the appearance of the sloping. So, the formamide, polymer or the capillaries used in the capillary electrophoresis are in good state. There are no large differences in relative peak heights between the samples which could arise due to contaminants in DNA sample, use of extremely large DNA, saturation of the fluorescent detection device due to overloading of capillaries or peak broadening due to deteriorated gel or capillaries. Thus to sum up the sample 11.2 can be taken as the female reference sample and proceeded with the data analysis.

5.3.4 ANALYSIS OF THE NORMAL SAMPLE (SAMPLE 1.2, 2.1 and 16.1)

When we compare the electropherograms of the two probe sets of the samples 1.2, 2.1 and 16.1, the peak heights of Q-fragments in all samples are found to be low as compared to the control fragment of 92 nt. It indicates the presence of sufficient DNA and the successful ligation reaction during the performance of MLPA. The successful complete denaturation of the sample DNA is confirmed by the presence of the D-fragments which are higher than 40% of the 92 nt control fragments. And it also fulfills other criteria of a good electropherogram as the absence of random decrement of some

signals and increment of the others, splitting of the peaks, large differences in relative peak heights between the samples, broadening of peak, random sloping and the off scale peaks in both the electropherograms.

In case of the samples 1.2 and 2.1, the presence of the peak corresponding to the 100 nt (X-fragment) but the absence of the peaks corresponding to the 105 nt and 118 nt (Y-fragments) in the electropherograms of the probes set PO34 indicate that the samples are that of female client. The presence of the peak corresponding to 100 nt (X-fragment) but the absence of the peak corresponding to 105 nt (Y-fragment) in the electropherograms of the probe set PO35 also suggest the same.

Similarly, the presence of the peaks corresponding to the 100 nt (X-fragment), 105 nt (Y-fragment) and 118 nt (Y-fragment) in the electropherogram of probe set PO34 and 100 nt (X-fragment) and 105 nt (Y-fragment) in the electropherogram of probe set PO35 in sample 16.1 indicate that the sample is that of the male client. The presence of these X- and Y-fragment peaks in the electropherograms also indicate that there is no sample swapping.

Comparing the electropherograms of the probe set PO34/35 of the normal samples and the corresponding reference it was revealed that there are no any significant variations in between the electropherograms of the corresponding references and the normal samples. And the comparison also suggest that there is the presence of all the peaks indicating the presence of the intact exons present in the probe set in the concerned sample.

So all the seventy nine exons targeted by these probes are well amplified as in case of the reference sample. Thus the electropherograms of the probe sets indicate that the sample is normal as per the genetic analysis by the ABI-310 genetic analyzer.

5.3.5 ANALYSIS OF THE TEST SAMPLE (SAMPLE 2.2)

In the electropherograms of the two probe sets of the sample 2.2, the peak heights of the Q-fragments are very low compared to the 92 nt control fragments. It indicates that the sufficient DNA was present and the ligation reaction was successful. And there are no peak of primer dimer formation either which also suggests the successful PCR. The peak of the D-fragments (88 nt and 96 nt) are also higher than 40% of the 92 nt control fragment indicating the complete denaturation of the sample DNA.

Besides the absence of random decrement of some signals and increment of the others, splitting of the peaks, large differences in relative peak heights between the samples, broadening of peak and the off scale peaks in both the electropherograms confirm that it fulfills all the criteria that a good electropherogram should have. Though the latter half

of the electropherogram shows very slight sloping in the peak, the sloping doesn't affect the interpretation.

The sample is that of the male client. It is also indicated by the presence of the peaks corresponding to the 100 nt (X-fragment), 105 nt (Y-fragment) and 118 nt (Y-fragment) in the electropherogram of probe set OP34 and 100 nt (X-fragment) and 105 nt (Y-fragment) in the electropherogram of probe set PO35. The presence of these X-and Y-fragment peaks in the electropherograms also indicate that there is no sample swapping.

When we compare the electropherograms of the probe set PO34 of the test sample and the corresponding reference sample, it reveals that there is lack of the peaks of probes of 144 nt, 154 nt, 178 nt, 185 nt, 216 nt, 225 nt, 257 nt, 299 nt, 328 nt, 371 nt, 403 nt, 442 nt, 459 nt and 476 nt. It means the corresponding exons (exons 41, 21, 42, 22, 43, 23, 24, 25, 26, 27, 28, 29, 10 and 30 respectively) targeted by these probes are not amplified. Thus the mentioned exons are deleted as indicated by the electropherogram.

Similarly, when we compare the electropherograms of the probe set PO35 of the test sample and the corresponding reference sample, it reveals that there is lack of the peaks of probes of 138 nt, 154 nt, 171 nt, 188 nt, 210 nt, 226 nt, 242 nt, 258 nt, 283 nt, 297 nt, 314 nt, 331 nt, 351 nt, 371 nt, 388 nt, 400 nt, 426 nt, 442 nt, 460 nt and 475 nt. It means the corresponding exons (exons 11, 31, 12, 32, 13, 33, 14, 34, 15, 35, 16, 36, 17, 37, 18, 38, 19, 39, 20 and 40 respectively) targeted by these probes are not amplified. Thus these exons are deleted as indicated by electropherogram.

Combining the results depicted by the two electropherograms of the test sample 2.2, it is found that there is the deletion of the exons 10 to 43 in the sample. There is the deletion in the sample covering exon 10 to 43 (34 of the 79 exons) where 43% of the exons are deleted spanning almost 1MB of the gene. This deletion of a large part of the dystrophin gene indicates that the dystrophin protein encoded by the mutated dystrophin gene is not intact and the sample is likely to be that of the patient suffering from DMD.

5.3.6 GeneScan™ –500 LIZ® Size Standard Peak analysis

The presence of the all the sixteen peaks affirm that there were no faults in the experiment performed.

To ensure the reproducibility of results for all samples, the formamide-size standard mix is prepared as recommended by MRC-Holland. In a reaction tubes the standard mixture is prepared such that for each reaction the mixture contained 0.5-μL GeneScan™-500-LIZ® as the Size Standard. The result of a run performed with fragments of known length is contained in the Size Standard Files. The file can be used to analyze other samples run

with the same size standard under the same conditions to determine the size of fragments of unknown length.

The GeneScan™-500-LIZ® Size Standard is a product of the Applied Biosystem (Product P/N 4322682) containing the DNA fragments of various known lengths labeled with the ROX dye. Due to the presence of the ROX dye the peak patters appear orange-red in color. It is an internal lane size standard developed for use with the Applied Biosystems fluorescence-based DNA electrophoresis systems. The use of an internal lane size standard enables automated data analysis and is also essential for achieving high run to run precision in sizing DNA fragments by electrophoresis. GeneScan™ -500 LIZ Size Standard is designed for sizing DNA fragments in the 35-500 bp range as revealed in the result and as claimed by the Applied Biosystems. Each of the DNA fragments is labeled with a proprietary fluorophore, which results in a single peak when run under denaturing or native conditions.

In the GeneScan 500 LIZ size standard the 250 bp peak is sensitive to small temperature variations on capillary electrophoresis instruments. So, the 250 bp fragment is not used when defining the size standard in GeneScan™ or GeneMapper® Software. Besides, the 340 bp peak is also subjected to large temperature variations. Fragment analysis primer peaks can often interfere with the detection of the 35 bp peak. The analysis are also analysed based on this information.
The GeneScan® Analysis Software analyzes raw data to quantify the DNA fragments and determine the size of the fragments by comparing them to fragments contained in the size standard. The samples and a size standard can be electrophoresed simultaneously, as dyes come in sets of four colors.

This size standard is used to analyze other samples run with the same size standard under the same conditions to determine the size of fragments of unknown length. So, LIZ acts as a ladder during the capillary run. Besides, in case the size standard peaks are low and broad, it is almost certain that the problem lies in the capillaries or polymer.

Though the entire research work was satisfactory, there are certain areas where further improvements could be made which were hindered by various factors during the study. It is always recommended to compare different MLPA analyse by using DNA extracted from the same tissue and with the same method. The present study fulfils the former mentioned criteria as DNA was extracted from the blood sample in all the cases using the same protocol. But during the hybridization step in MLPA, the time period of hybridization couldn't be maintained exactly to 16 hours for all the samples due to the lack of continuous supply of electricity. The hybridization period in the samples ranged from 13 hours to 16 hours. Regarding the samples in which the hybridization period couldn't be attained continuously for 16 hours, the tubes were again placed at 60°C and continued until the total

hybridization is up to 16 hours. But as suggested by MRC-Holland, hybridisation of probes to their targets is nearly complete after 12 hours. And in case the hybridisation reaction is interrupted due to power failure, the tubes could just be placed again at 60°C and continue until the total hybridization is between 16 and 24 hours (MLPA, General protocol, Version 31; 2011). But still, it could make some difference (though negligible) in the result provided the time period of hybridization could be maintained continuously for 16 hours in all the samples concerned. And when the large numbers of samples are run simultaneously, the ligation incubation period of the first tubes will obviously be longer. But this has no influence on the result either (MLPA, General protocol, Version MDP-v002, 2012).

A crucial point in the interpretation of MLPA results is represented by the detection of deletions involving a single exon. In these cases, in fact, the apparent deletion could actually consist of a change in the exon sequence hampering the correct hybridization of the specific probe. This sequence variation can be represented either by a pathogenic point mutation or by a polymorphism not affecting gene function. Thus, apparent single exon deletions detected by MLPA was supposed to be checked by an independent method as gene sequencing. By adding common point-mutation-specific (PMS)-MLPA probes to dosage MLPA multiplexes, full dosage analysis and limited point mutation analysis could be performed simultaneously without any significant increase in labor (Stern, R.F. et al.; 2004). But due to the unavailability of such probes during the research work, the full dosage analysis and limited point mutation analysis couldn't be performed. It is also recommended that the standard deviation of each probe of the control sample be analyzed to be sure that it is not significant. But it couldn't be done due to the presence of limited probes and considering the cost that it bears.
The generation of pedigree of individual patient suffering from DMD/BMD and its analysis is another task that couldn't be done during the research work due to the time constrain. Inclusion of all these factors could strengthen the specific analysis of the mutation in dystrophin gene and help in determining the pattern of inheritance of the disease or to reveal either the mutation is de novo. These factors can be considered during the follow up of the concerned study.

CHAPTER VI: SUMMARY

Duchenne muscular dystrophy (DMD) and its less severe allelic form Becker muscular dystrophy (BMD) are X-linked progressive muscle wasting genetic diseases caused due to the mutation (deletion, duplication and point mutation) in any of the 79 exons of the dystrophin gene located in Xp21.2 chromosome. Dystrophin gene is the largest known gene (2.4 Mb) encoding the dystrophin protein expressed as multiple isoforms including the skeletal muscle protein (427 kDa). Muscular dystrophin protein is an actin binding protein acting as an anchoring protein in muscle sarcolemma that keeps the muscle coordination intact. The in-frame mutation of this gene leads to the less severe form (BMD) whereas the out-of-frame mutation leads to the severe form (DMD) of dystrophinopathies. But it was found that the gene rule is that loss of 36 or more exons is usually associated with a severe phenotype, regardless of the reading frame suggesting the minimum size requirement for the functional dystrophin. The deletions involved in cysteine-rich domain or ABD1 (actin binding domain-1) is more deleterious than mutation involving the central rod domain. The finding of eDystrophin database shows that duplications are generally more deleterious than deletions.

Death is certain in most case of DMD at 20-22 years of early age (usually below 25 years of age). But the recent advances in DMD treatment have shown that methods like exon skipping are powerful techniques that can help to cure the disease. The determination of the mutation in the dystrophin gene is the basis of the treatment of this disease. Even if the condition is not entirely curable, knowing the range of mutation helps a lot in determining the severity of the cases and hence necessary precautions and supportive treatment procedures can be employed.

Among the two major dystrophinopathies, Becker muscular dystrophy (BMD) has often been overlooked or misdiagnosed as LGMD or spinal muscular atrophy (SMA). So, it's important to have the precise genetic test before assuming that the problem is actually BMD. The location of deletion in dystrophin gene are apparently non-random with a preponderance found in two hot spot regions at the 5' terminus and in the distal half of the central rod domain around exon 44-53 (include 75% of the deletion). But the condition may vary in various population or ethnic group. Besides, determination of the carrier or the client and the mutation pattern in the family helps in the pedigree analysis which is the basis of genetic counseling. The concerned study of the kind has been an essence in our country to equate the data with the worldwide scenario.

The mutation pattern of the dystrophin gene began when Chamberlain et al. described a series of primers that can amplify 6 exons (8, 17, 19, 44, 45, 48) that are deleted in many DMD/BMD patients. Since then, various improvements were made in the multiplex PCR

assay that gave rise to the advanced modified form of multiplex PCR called multiplex ligation dependent probe amplification (MLPA). This method developed by JP Schouten et al. is a relative technique that is used to determine the copy number variation of DNA sequences in a single multiplex PCR based reaction. In this technique the MLPA probes hybridised to the sample DNA targeting all the 79 exons of the dystrophin gene are amplified. The hybridisation of the probes targeting the exons depends upon the presence of the targeted site in the gene. The PCR amplicons thus obtained are separated and quantified by capillary electrophoresis. The peak pattern (electropherogram) of the test sample obtained from the capillary electrophoresis is compared with the reference sample that helps to determine the mutation (deletion, duplication, point mutation) of the exon(s) and hence the framedness. The result further helps in determining the severity of the disorder.

With the aim to introduce this powerful and reliable assay of genetic disease diagnosis in Nepal, the research was performed in collaboration with the Muscular Dystrophy Foundation Nepal (MDF-Nepal) and Central Department of Biotechnology (CDBT), Kirtipur, Nepal. The fresh blood samples were collected from the DMD/BMD patients in MDF-Nepal by trained personnel. DNA were extracted and the MLPA assay was performed. The PCR products of the MLPA assay were then run on the capillary electrophoresis (ABI-310 genetic analyzer) in Center for Molecular Diagnostic, Nepal (CMDN) and the electropherograms of each sample were evaluated.

There were 26 samples in total that were assayed. 2 of the samples were reference samples (one male reference sample and one female reference sample), 3 of them were normal samples (a normal male sample, a female parent and a sister of a DMD client) and 21 of them were test samples. The capillary electrophoresis run (ABI-310 genetic analyzer) demonstrated the deletions pick up in 14 of the 21 test samples considered. Thus during the research, MLPA assay was found to be efficient in accurately confirming the mutations in about 67% of all cases. The percentage of deletion pick up in the study is compatible with the recent studies in Chinese and Indian population. The peak pattern and the corresponding exonic deletion shows that the most prevalent exonic deletion regions are confined in the exon 7-14, the proximal zone and in the exon 45-53, the first half of C-terminal domain. The framedness (in-frame or out-frame) were determined by using the DMD exonic deletions/duplications reading frame checker 1.9 as recommended by MRC-Holland which showed that 14 of the samples that are clinically diagnosed as DMD have out-of-frame deletion. But the exact framedness can only be confirmed by sequencing. No novel mutations and duplication were identified during the study.

These results suggest that despite some limitations, the optimized MLPA assay can be used as a reliable genetic tool in genetic disease diagnosis as DMD/BMD in countries like Nepal.

CHAPTER VII: CONCLUSION

Although there are many other molecular tools that are applied in the diagnosis of genetic disease there is always a need of a technique that is precise, cost effective, reliable and time subsiding. MLPA assay meets these needs to more or less extent. The large application of this approach is the result of a number of advantages provided by MLPA assay when compared to other techniques. MLPA analysis is a high throughput analysis, allowing up to 96 samples to be handled simultaneously, with results being available within 24 h. It allows the study of several regions of the human genome in a single reaction. Target sequences are very short (50–70nucleotides), allowing MLPA to identify single gene aberrations, too small to be detected by techniques like Fluorescent in-situ hybridization (FISH). Moreover, MLPA results are not influenced by amount of DNA content used and requires only 20 ng human DNA (3000 cells / 0.5 ml amniotic fluid). As compared to some other sophisticated molecular tools like the array CGH, it is a low cost and technically uncomplicated method.

From the present investigation, the use of MLPA assay was observed to be effective to be used as a reliable genetic tool in genetic disease diagnosis. The important finding of this study is that the exonic deletion in the DMD/BMD samples considered during the research show that the locations of deletion are confined around the exon 7-14 (the proximal zone) and the exon 45-53 (the first half of C-terminal domain). The result suggests that the location of deletion in dystrophin gene is apparently non-random with a preponderance found in the hot spot regions in Nepalese population as well. Besides the deletion pick up rate is also found to correspond with the deletion pick up rate of other countries. There has always been a need of extensive study on effective, safe and cheap and reliable molecular diagnostic method in our country. This study aims to fulfill the same objectives though at the preliminary state. This finding encourages us for further investigation and research work on various aspects of genetic disease diagnosis and to develop the trend of genetic counseling.

Although it is not much talked topic death due to genetic disease cannot be ignored. The genetic disease may appear as a result of the de novo mutation but the frequency of many of these diseases is due to the ignorance of the family members regarding the diseases. Thus provided the necessary information the rate of many such genetic diseases can be decreased or even nullified. MLPA assay is an essential tool in determining the mutation of such genetic disorder. It can be used in all laboratories performing diagnostic genetic testing both as a confirmation tool and as a diagnostic system applicable also to the copy number variation analysis in rare genetic conditions.

It has been proven that MLAP assay is very helpful in determining novel mutation and is capable of detecting point mutation as well. Thus due to its various positive aspects as rapidity, technical simplicity, cost effectiveness and wide range of application MLPA assay is a very useful technique in genetic disease diagnosis in our country. This technique can be very useful in the generation of the base line of many other genetic diseases that can be a basis of eradicating or at least reducing the frequency of the occurrence of these genetic diseases. The use of this reliable molecular tool can lead to a whole new wave of academic research in the field of genetic disease diagnosis in Nepal.

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APPENDICES

❖ Thermocycler Program For The MLPA Reaction

SN	Process	Steps		Temperature	Time
1	DNA denaturation	1.		98^0C	5 minutes
		2.		25^0C	Pause
2	Hybridisation reaction	3.		95^0C	1 minute
		4.		60^0C	Pause
3	Ligation reaction	5.		54^0C	Pause
		6.		54^0C	15 minutes
		7.		98^0C	5 minutes
		8.		20^0C	Pause
4	PCR reaction	9.	35 cycles	95^0C	30 seconds
				60^0C	30 seconds
				72^0C	60 seconds
		10.		72^0C	20 minutes
		11.		15^0C	Pause

❖ Materials Provided Per Salsa MLPA Kit

SALSA MLPA KIT COMPONENT	INGREDIENTS
SALSA MLPA Buffer (yellow cap)	KCl, Tris-HCl, EDTA and PEG-6000. pH 8.5
SALSA Ligase-65 (green cap)	Glycerol, BRIJ (0.05 %), EDTA, Beta-Mercaptoethanol (0.1 %), KCl, Tris-HCl. pH 7.5, Ligase-65 enzyme (bacterial origin)
Ligase buffer A (transparent cap) Ligase buffer B (white cap)	NAD (bacterial origin), pH 3.5 Tris-HCl, non-ionic detergents, MgCl2, pH 8.5
SALSA PCR Primer mix (brown cap)	Synthetic oligonucleotides, one of which is fluorescently labeled (FAM), dNTPs, Tris-HCl, KCl, EDTA, Brij (0.04%), pH 8.0
SALSA Polymerase (orange cap)	Glycerol, BRIJ (0.5%), EDTA, DTT (0.1%), KCl, Tris-HCl, Polymerase enzyme (bacterial origin), pH 7.5
Probemix (black cap)	Synthetic oligonucleotide, oligonucleotides purified from bacteria, Tris-HCl, EDTA, pH 8.0

- SALSA MLPA PO34-A3 DMD probe mix

Length (nt)	SALSA MLPA probe control fragments
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA
88-92-96	D-fragment: Low signal of 88 or 96 nt fragment indicates incomplete denaturation
100	X-fragment: Specific for the X-chromosome
105	Y-fragment: Specific for the Y-chromosome
118	Y-fragment: Specific for the Y-chromosome

*The length of all the probe control fragments lies below 120 nt.

Length (nt)	SALSA MLPA	Chromosomal position	
		Reference	DMD
129*	Reference probe	Xq12	
203	Reference probe	Xp22	
274*	Reference probe	Xq28	
418	Reference probe	Xq13	
490	Reference probe	Xq28	
Ranging from 129-490	DMD probe	-	Exon: 1-10, 21-30, 41-50, 61-70

*This probe is located within, or close to, a very strong CpG island. A low signal of this probe can be due to incomplete sample DNA denaturation, e.g: due to the presence of salt in the sample.

Control Fragments	Probes	Number	Total
Q-fragments		4	10 control fragments
D-fragments		3	
X-fragment(s)		1	
Y-fragments		2	
	Reference probe	5	5 reference probes
	DMD probe	40	40 DMD probes

- SALSA MLPA PO35-A3 DMD probe mix

Length (nt)	SALSA MLPA probe control fragments
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA
88-92-96	D-fragment: Low signal of 88 or 96 nt fragment indicates incomplete denaturation
100	X-fragment: Specific for the X-chromosome
105	Y-fragment: Specific for the Y-chromosome

*The length of all the probe control fragments lies below 120 nt.

Length (nt)	SALSA MLPA	Chromosomal position	
		Reference	**DMD**
129*	Reference probe	Xq12	
203	Reference probe	Xp22	
274*	Reference probe	Xq28	
418	Reference probe	Xq13	
490	Reference probe	Xq28	
Ranging from 129-490	DMD probe	-	Exon:11-20, 31-40, 51-60, 71-79, **DP427c**

*This probe is located within, or close to, a very strong CpG island. A low signal of this probe can be due to incomplete sample DNA denaturation, e.g: due to the presence of salt in the sample.

Control Fragments	Probes	Number	Total
Q-fragments		4	9 control fragments
D-fragments		3	
X-fragment(s)		1	
Y-fragment(s)		1	
	Reference probe	5	5 reference probes
	DMD probe	40	40 DMD probes (**including DP427c**)

Probe description: P034/35-A3 DMD-1

Notes

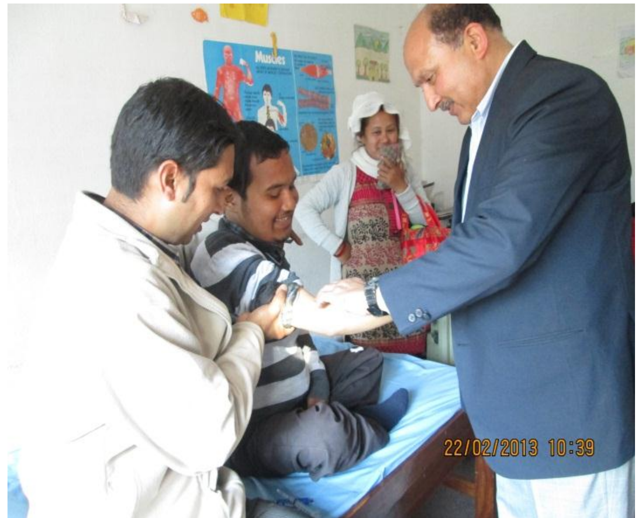
* LPO is the 5' half of the probe, RPO is the 3' half of the probe.
* The two sequences are directly adjacent and together they form the complete hybridising sequence of the probe.
* The first nucleotides of the LPO may not be complementary to the target DNA and is part of the stuffer sequence.
* Locations are based on hg18 / mapview build 36. Mapview location consists of the chromosome no. + Distance (nt) from the p-telomere to the start of the probe sequence.
* Observed length of the fragments depend on the software, sequencer type and molecular weight markers used.

Length	Exon	Chr. pos.	LPO	RPO
137	1	Xp21.1	GGGATCACTCACTTTCCCCCTACAG	GACTCAGATCTGGGAGGCAATTACCTTCGGAGAAAAAC
169	2	Xp21.1	GCATTTTAGATGAAAGAGAAGATGTTCAAAAGAAAA	CATTCACAAAATGGGTAAATGCACAATTTTCTAAGGTAAGAATGGTT
209	3	Xp21.1	GGAAGCAGCATATTGAGAACCTCTTCAGTG	ACCTACAGGATGGGAGGCGCCTCCTAGACCTCCTCGAAGGCC
242	4	Xp21.1	CCACAAGAGTTCATGCCCTGAACAA	TGTCAACAAGGCACTGCGGGTTTTGCAGAACAATAATGTA
283	5	Xp21.1	CGTGAATATTGGAAGTACTGACATCGTAGATGGAAAT	CATAAACTGACTCTTGGTTTGATTTGGAATATAATCCTCCACTGGCA
315	6	Xp21.1	GCTGGATTGCAACAAACCAACAGTGA	AAAGATTCTCCTGAGCTGGGTCCGACAATCAACTCGTAA
355	7	Xp21.1	CTAGGCCAGACCTATTTGACTGGAATAGTGT	GGTTTGCCAGCAGTCAGCCACACAACGACTGGAA
388	8	Xp21.1	CTTGCCTCAACAAGTGAGCATTGAAGCCAT	CCAGGAAGTGGAAATGTTGCCAAGGCCACCTAAAGTGACTA
426	9	Xp21.1	GCAGATCACGGTCAGTCTAGCACAGGGATAT	GAGAGAACTTCTTCCCCTAAGCCTCGATTCAAGAGCTATGCCTA
459	10	Xp21.1	GCAGCATTTGGAAGCTCCTGAAGACAAGTCA	TTTGGCAGTTCATTGATGGAGAGTGAAGTAAACCTGGACCGTT
138	11	Xp21.1	GGGGTACATGATGGATTTGACAGCCC	ATCAGGGCCGGGTTGGTAATATTCTACAATTGGGAAGTAAGCTGAT
171	12	Xp21.1	GGGAAAATGGAGGAAGAGCCTCTTGG	ACCTGATCTTGAAGACCTAAAACGCCAAGTACAACAACATAAGGTAGGT
210	13	Xp21.1	CTCTCACTCACATGGTGGTGGTAGTTGAT	GAATCTAGTGGAGATCACGCAACTGCTGCTTTGGAAGAACAACTTA
242	14	Xp21.1	CGAGGTATTGGGAGATCGATGGGCAAACAT	CTGTAGATGGACAGAAGACCGCTGGGTTCTTTTACAAGACATCCTTC
283	15	Xp21.1	CAGTGCCTTTTTAGTGCATGGCTTTCAG	AAAAAGAAGATGCAGTGAACAAGATTCACACAACTGGCTTTAAAGAT
314	16	Xp21.1	GCGGATCTAGAAAAGAAAAAGCAATCCATG	GGCAAACTGTATTCACTCAAACAAGATCTTCTTTCAACACTGAA
351	17	Xp21.1	CTACGGTGACCACAAGGGAACAGATCCT	GGTAAAGCATGCTCAAGAGGAACTTCCACCACCACCTC
388	18	Xp21.1	GCTGGATTACTCGCTCAGAAGCTGTGTT	GCAGAGTCCTGAATTTGCAATCTTTCGGAAGGAAGGCAACTTCTCA
426	19	Xp21.1	GCCATAGAGCGAGAAAAAGCTGAGAA	GTTCAGAAAACTGCAAGATGCCAGCAGATCAGCTCAGGCCCTG
460	20	Xp21.1	CAACTGAACAGCCGGTGGATCGAA	TTCTGCCAGTTGCTAAGTGAGAGACTTAACTGGCTGGAGTATCAGAA
154	21	Xp21.1	TCATAGCCCTGAAAGAGAAAGGACAAGG	ACCCATGTTCCTGGATGCAGACTTTGTGGCCTTTACAAATCAT
185	22	Xp21.1	GCCACCAATGCGCTATCAGGAGACCAT	GAGTGCCATCAGGACATGGGTCCAGCAGTCAGAAACCAA
225	23	Xp21.1	CTCTGCAAGAGCAACAAAGTGGCCTATACT	ATCTCAGCACCACTGTGAAAGAGATGTCGAAGAAAGCGCC
257	24	Xp21.1	CTCTGAAGGAGGAATGGCCTGCCCT	TGGGGATTCAGAAATTCTAAAAAAGCAGCTGAAACAGTGCAGAG
299	25	Xp21.1	GTGTCAATGAAGGTGGGCAGAAGATAA	AGAATGAAGCAGAGCCAGAGTTTGCTTCGAGACTTGAGAC
328	26	Xp21.1	GGAGGTTTGGAGAAAACTGTAAGCCTCC	AGAAAGATCTATCAGAGATGCACGAATGGATGACACAAGCTGAA
371	27	Xp21.1	CGCGAAAGTGAAACTCCTTACTGAGTCTGTA	AATAGTGTCATAGCTCAAGCTCCACCTGTAGCACAAGAGG
403	28	Xp21.1	CAGGAAGTTTGGGCATGTTGGCATGAGTT	ATTGTCATACTTGGAGAAAGCAAACAAGTGGCTAAATGAAGTAGAA

442	29	Xp21.1	CAGATTCGCATATTGGCACAGACCCTA	ACAGATGGCGGAGTCATGGATGAGCTAATCAATGAGGAAC
476	30	Xp21.1	GGCTGTAAGGAGGCAAAAGTTGCTTG	AACAGAGCATCCAGTCTGCCCAGGAGACTGAAAAATCCTTACA
154	31	Xp21.1	TCAGGGGAAGGAGGCTGCC	CAAAGAGTCCTGTCTCAGATTGATGTTGCACAGGTATATGTTATTTCAG
188	32	Xp21.1	CAGTGAAGATGCACTTGCCTGCATTGGA	AACAAAGAGTGTGGAACAGGAAGTAGTACAGTCACAGCTAAATCATTGT
226	33	Xp21.1	GTCTGAGTGAAGTGAAGTCTGAAGTGG	AAATGGTGATAAAGACTGGACGTCAGATTGTACAGAAAAAGCAGAC
258	34	Xp21.1	TGAAATTGTCCCGTAAGATGCGAAAGGAAAT	GAATGTCTTGACAGAATGGCTGGCAGCTACAGATATGGAATTGACAA
297	35	Xp21.1	GAGAAACAGAAGGTGCACCTGAAGAGT	ATCACAGAGGTAGGAGAGGCCTTGAAAACAGTTTTGGGCAAGAAGGAGA
331	36	Xp21.1	GACCAGAATGTGGACCACATCACAAAGT	GGATCATTCAGGCTGACACACTTTTGGATGAATCAGAGAAAAAGAAA
371	37	Xp21.1	CCAGTCTTAATTCTGTGTGAAATGGCTGCAAA	TCGATGGTTGAGCTCTGAGATTTGGGGCTCTACTAATTTCC
400	38	Xp21.1	CTTGAACCACTGGAGGCTGAAATTCA	GCAGGGGGTGAATCTGAAAGAGGAAGACTTCAATAAAGATATG
442	39	Xp21.1	CAATGAGGGTACTGTAAAAGAATTGTTGCAAAGAGG	AGACAACTTACAACAAAGAATCACAGATGAGAGAAAGCGAGAGGAA
475	40	Xp21.1	GGATTTGAGGTCTCAAAGAAGAAAAAAGGCTCTAG	AAATTTCTCATCAGTGGTATCAGTACAAGAGGCAGGCTGATGATCTCCT
144	41	Xp21.1	TGCGTAGGCAAGCTGAGGGCTTGTCT	GAGGATGGGGCCGCAATGGCAGTGGAGCCAACTCAGATCCAG
178	42	Xp21.1	CCACACTGTCCGTGAAGAAACGATGATGGT	GATGACTGAAGACATGCCTTTGGAAATTTCTTATGTGCCTTCT
216	43	Xp21.1	GCAAGAAGACAGCAGCATTGCAAAGT	GCAACGCCTGTGGAAAGGGTGAAGCTACAGGAAGCTCTCTCCCAG
250	44	Xp21.1	CAGTGGCTAACAGAAGCTGAACAGTTTCTC	AGAAAGACACAAATTCCTGAGAATTGGGAACATGCTAAAT
291	45	Xp21.1	CTCAGCAATCCTCAAAAACAGATGCCAGT	ATTCTACAGGAAAAATTGGGAAGCCTGAATCTGCGGTGGCAGGA
321	46	Xp21.1	GGGTTGGAGGAAGCAGATAACATTGCTAGT	ATCCCACTTGAACCTGGAAAAGAGCAGCAACTAAAAGAAAAG
362	47	Xp21.1	TGCGCCAGGGAATTCTCAAACAATT	AAATGAAACTGGAGGACCCGTGCTTGTAAGTGCTCCCATAAGC
395	48	Xp21.1	CTTGAAGACCTTGAAGAGCAGTTAAATCAT	CTGCTGCTGTGGTTATCTCCTATTAGGAATCAGTTGGAAATTT
434	49	Xp21.1	CAAGCTAAACAACCGGATGTGGAAGAGATTTT	GTCTAAAGGGCAGCATTTGTACAAGGAAAAACCAGCCACTCAGCC
465	50	Xp21.1	CTCTGAGTGGAAGGCGGTAAACCGTTT	ACTTCAAGAGCTGAGGGCAAAGCAGCCTGACCTAGCTCCT
146	51	Xp21.1	GTTGGAGGTACCTGCTCTGGCAGAT	TTCAACCGGGCTTGGACAGAACTTACCGACTGGCTTTCTC
179	52	Xp21.1	CGATCCGTAATGATTGTTCTAGCCTCTTGA	TTGCTGGTCTTGTTTTTCAAATTTTGGGCAGCGGTAATGAGTT
219	53	Xp21.1	GAAGCTAAGGAAGAAGCTGAGCAGGTCT	TAGGACAGGCCAGAGCCAAGCTTGAGTCATGGAAGGAGGGTCCCTA
250	54	Xp21.1	GACCTCCGCCAGTGGCAGACAAAT	GTAGATGTGGCAAATGACTTGGCCCTGAAACTTCTCCGGGATT
291	55	Xp21.1	GAGAGGCTGCTTTGGAAGAAACTCATAGAT	TACTGCAACAGTTCCCCCTGGACCTGGAAAAGTTTCTTGCCTGG
321	56	Xp21.1	GGTTCCGATGATGCAGTCCTGTTACA	AAGACGTTTGGATAACATGAACTTCAAGTGGAGTGAACTTCGGAAAAAGT
359	57	Xp21.1	CTGGTGTGGCTACAGCTGAAAGATGAT	GAATTAAGCCGGCAGGCACCTATTGGAGGCGACTTTCCAGCAGT
396	58	Xp21.2	CCTGTAATCATGAGTACTCTTGAGACTGTACG	AATATTTCTGACAGAGCAGCCTTTGGAAGGACTAGAGAAACTCTACC
434	59	Xp21.2	GACTGGCAGAGAAAAATAGATGAGACCC	TTGAAAGACTCCAGGAACTTCAAGAGGCCACGGATGAGCTGGACCT
467	60	Xp21.2	CACTTCGAGGAGAAATTGCGCCTCTGAAA	GAGAACGTGAGCCACGTCAATGACCTTGCTCGCCAGCTTACCACTT
161	61	Xp21.2	TGGACCGAGTCAGGCAGCTGCAT	GAAGCCCACAGGGACTTTGGTCCAGCATCTCAGC
193	62	Xp21.2	GTCTGTCCAGGGTCCCTGGGAG	AGAGCCATCTCGCCAAACAAAGTGCCCTACTATATCAAGTAAGTTG
234	63	Xp21.2	GTGTTTTAGCCACGAGACTCAAACAACTTG	CTGGGACCATCCCAAAATGACAGAGCTCTACCAGTCTTTAGG
266	64	Xp21.2	GCAAAGGGCCTTCTGCAGTCTT	CGGAGTTTCATGGCAGTCCTATAAGCTGAGAATCTGA
307	65	Xp21.2	GATCTCTTGAGCCTGTCAGCTGCATGTG	ATGCCTTGGACCAGCACAACCTCAAGCAAAATGACCAG
338	66	Xp21.2	GGGAGGATCCGTGTCCTGTCTTTTA	AAACTGGCATCATTTCCCTGTGTAAAGCACATTTGGAAGACAA
379	67	Xp21.2	GGAAGCAGCTCCGGACACTTGGCT	CAATGTTACTGCCCCCAAAGGATGCAACTTCACCCAACTGTC
409	68	Xp21.2	CGAAGCGGCCCTCTTCCTAGACTGGATGA	GACTGGAACCCCAGTCCATGGTGTGGCTGCCCGTC

450	69	Xp21.2	GACATCTGCCAAAGCTGCTTTTTTTCTG	GTCGAGTTGCAAAAGGCCATAAAATGCACTATCCCATGGTGGAA
481	70	Xp21.2	GGGTATTTTGCGAAGCATCCCCGAATGG	GCTACCTGCCAGTGCAGACTGTCTTAGAGGGGGACAACATGGA
164	71	Xp21.2	GTTTTGCAGTCCCGTTACTCTGATCAAC	TTCTGGCCAGTAGATTCTGCGTGAGTACTTTTTTTGCTGAAGGG
196	72	Xp21.2	CTCAGCCTGCCTCGTCCCCTCAGCTTT	CACACGATGATACTCATTCACGCATTGAACATTATGCTAGCAG
235	73	Xp21.2	GTGCTCTCATTAGGAGAGATGCTATCATTTAG	ATAAGATCCATTGCTGTTTTCCATTTCTGCTAGCCTG
265	74	Xp21.2	CTCAGCCTCGTAGTCCTGCCCAGATCTTG	ATTTCCTTAGAGAGTGAGGAAAGAGGGGAGCTAGAGAGAATCCT
306	75	Xp21.2	GTCCCCGGGATGCTGAGCTCATT	GCTGAGGCCAAGCTACTGCGTCAACACAAAGGCCGCCTGGAAG
338	76	Xp21.2	CACGGTGTCCTCTCCTTCTACCTCTCTAC	AGAGGTCCGACAGCAGTCAGCCTATGCTGCTCCGAGTG
379	77	Xp21.2	GCTTCTCAGTCCTCCCCAGGACACA	AGCACAGGGTTAGAGGAGGTGATGGAGCAACTCAACAACTCCTTCCC
409	78	Xp21.2	GGAAGAAATACCCCTGGAAAGCCA	ATGAGAGAGGTTAGTGAGATTCAGGCTCACGGCCATGG
451	79	Xp21.2	GACACAATGTAGGAAGTCTTTTCCACATGGCA	GATGATTTGGGCAGAGCGATGGAGTCCTTAGTATCAGTCATGA
484	DP427c	Xp21.1	CTGGTTCCCAGCAGTCGGCAGTAATAGA	ATGCTTTCAGGAAGATGACAGAATCAGGAGAAAGATGCTGTTT
Additional fragments present in PO34-A3 DMD probemix ONLY				
118	18	Yq11.21	GTTTATTCTAACCTAGGCAAACGGCATGCT	ATCACAAGAAAGGTTTAAAGCTTTGATAAAATGGGGGAGATTTCAT
Reference probes present in PO34/35-A3 DMD probemix				
129	1	Xq12	CGAGGCCAGCACCATGCAACTCCT	TCAGCAACAGCAGCAGGAAGCAGTATCCGAAGGC
203	10	Xp22.13	CGTTCTTGTCTACCCCAATGACCTGCACTT	GAACAGAGGGAACCACGAAGATTTTATGATGAATCTGAGGT
274	4	Xq28	GTGAGAGCGCAAAGACATTGTTTCATCCTCCA	TGCCAAGGCCAAACAGAGAGGAGCCTGTGGACAG
418	4	Xq13.1	GCAGCTGACTACCAGCAGGCCCA	GCAAGGAGGCAGACAACACTGTCAAGATGGCCACACAGGCC
490	4	Xq28	CCTTCACCATCACGGGCAACAACAGCAACTTT	GCTCAGAGGTTCCAGGGCATCTACCGCTGCTTTGCCAGCAATA
Additional fragments present in PO34/35-A3 DMD probemix				
64-70-76-82	Q-fragments: DNA quantity only visible with less than 100 ng sample DNA			
88-92-96	D-Fragments: Low signal of 88 or 96 nt fragment indicates complete denaturation			
100	X-fragment: Specific for the X chromosome			
105	Y-fragment: Specific for the Y chromosome			

The first reference probe among the five reference probe (indicated at 01690-L00423) used is located within, or close to, a very strong CpG island. A low signal of this probe may be due to incomplete sample DNA denaturation.



Collection of blood sample in MDF-Nepal

From Left to Right

1. SALSA MLPA Buffer

2(a). Probemix PO35-A3

2(b). Probemix PO34-A3

3. SALSA PCR Primer mix FAM

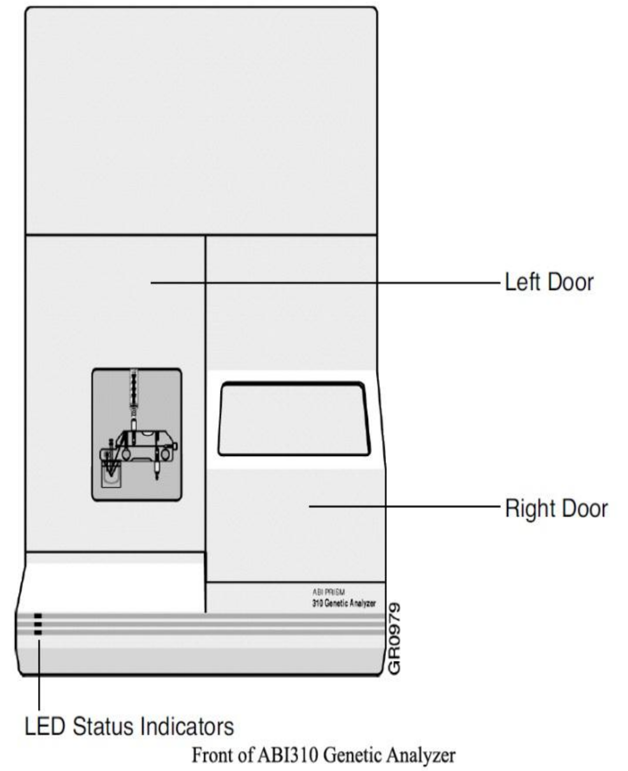
4(a). Ligase Buffer A

4(b). Ligase Buffer B

5. SALSA Polymerase

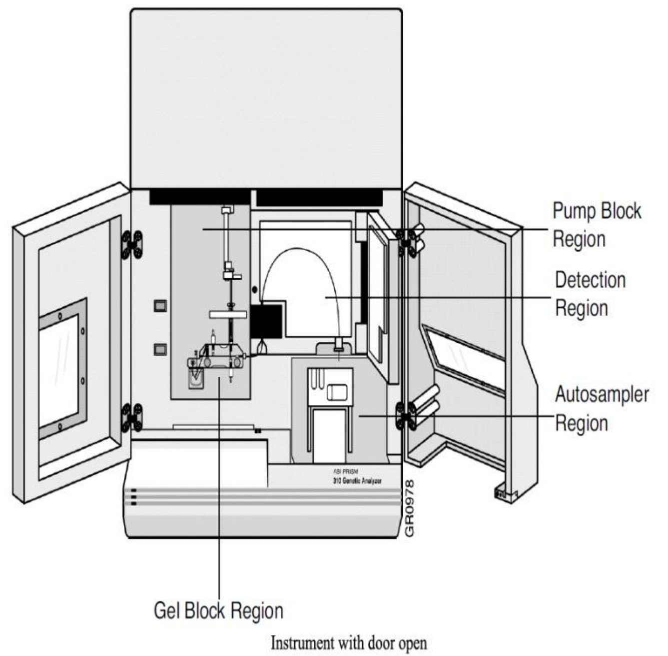
6. SALSA Ligase-65

Different colored capped MLPA reagents



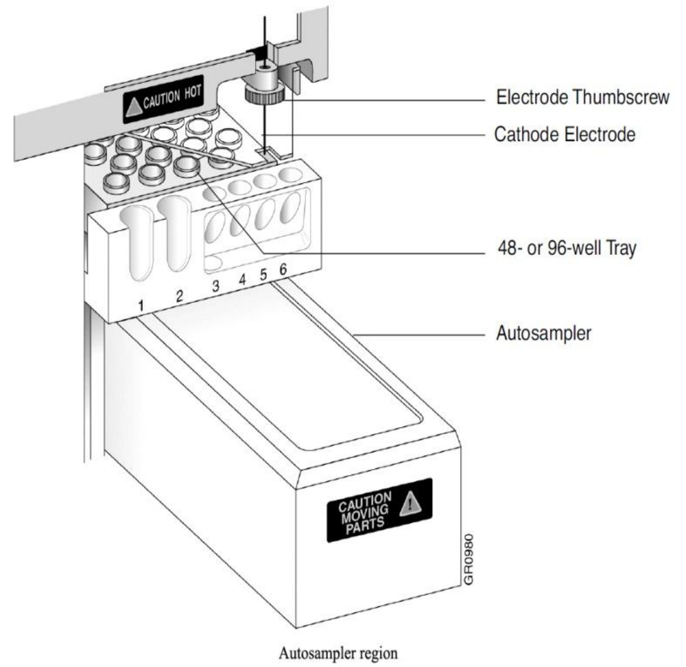
Front of ABI310 Genetic Analyzer

Front of ABI-310 Genetic Analyzer

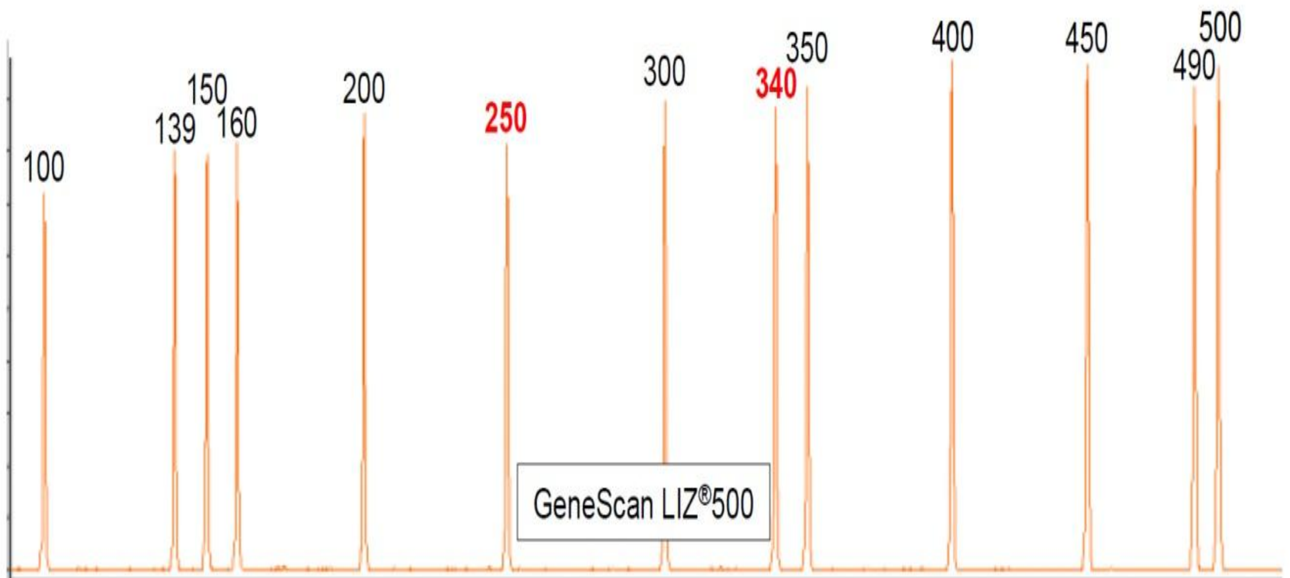


Instrument with door open

ABI-310 Genetic Analyzer with door open



Autosampler region



Standard electropherogram of GeneScan Liz 500