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Simultaneous detection of thirteen exons of dystrophin gene by optimized multiplex PCR assay to screen Duchenne/Becker muscular dystrophy

Pooja G Trivedi, Jaydeep B Gajera, Fesal I Ghanchi & Gaurang M Sindhav*

Department of Zoology, Biomedical Technology, Human Genetics and Wildlife Biology & Conservation, University School of Sciences, Gujarat University, Ahmedabad-380 009, Gujarat, India

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Advancements in Polymerase Chain Reaction (PCR) technology and other techniques like Deoxyribonucleic acid (DNA) signal and target amplification have become key procedures in molecular diagnostics. PCR enables the synthesis of nucleic acids *in vitro* through which a DNA segment can be specifically replicated in a semiconservative way that sets forth deletion and mutation analysis. Multiplex PCR (M-PCR) is beneficial over standard and long PCR as this can amplify more than one locus using the respective primer sets. In harmony with this, the present study aimed to optimize M-PCR followed by its chemistry and condition to screen Duchenne Muscular Dystrophy (DMD) [OMIM #310200] and Becker Muscular Dystrophy (BMD) [OMIM #300376]. Muscular Dystrophies (MDs) are a broad group of hereditary, progressive, and degenerative disorders of muscles. X-linked recessive D/BMD are caused by mutation/s in the dystrophin gene [OMIM #300377] that encodes for dystrophin protein [UniProt#P11532]. As dystrophin is the human metagene with 79 exons, mutational analysis is very challenging. Chamberlain set (10 plex), Beggs set (9 Plex), and Kunkel set (7 Plex) is used for many years to diagnose this condition. However, in this study, Beggs set is customized with 13 exons to screen DMD gene mutation in a single reaction. Optimization of M-PCR was designed with many physicochemical parameters. According to the literature and after many appraisals the present study demonstrated the most sufficient concentration of various chemical components and optimal cycling conditions to optimize the modified Beggs set (13 Plex). 50 µL PCR reaction includes primer(s) (0.3–0.5 µM each), dNTP mixture (160 µM each), Dream *Taq* buffer (1X), *Taq* DNA polymerase (6U/50 µL), DNA template (250 ng/50 µL), BSA (0.4 µg/µL), and $MgCl_2$ (1.4 mM). To get the most effective results cyclic conditions obtained were 10 min initial denaturation at 94°C, 62°C annealing temperature, and 35 PCR cycles at 72°C extending temperature. Consequently, the study successfully formulated a less expensive and simple approach for >3000 bp that was used to screen D/BMD. Finally, a developed M-PCR mix with a unique combination of specificity and sensitivity coupled with great flexibility has led to a true revolution in molecular diagnostics.

Keywords: Electrophoresis, Multiplex PCR, Optimization, Physico-chemical indices

Polymerase Chain Reaction (PCR) is used in laboratories in Deoxyribonucleic acid (DNA) cloning procedures, Southern blotting, DNA sequencing, and Recombinant DNA technology[1]. PCR is a conceptually simple, highly sensitive, very specific, and fully automated procedure hence it is called People's Choice Reaction also. To diagnose Duchenne Muscular Dystrophy (DMD) [OMIM #310200] PCR was first stated by Chamberlain in 1988[2]. The use of PCR is limited by cost and sometimes the availability of test sample volume hence, to increase the detection efficiency of PCR, multiplex PCR (M-PCR) methods are established to simultaneously detect and differentiate multiple DNA or RNA in a single sample based on amplicon size in a single reaction[3-5]. This method has been accomplished in many areas likewise deletion analysis, mutation and polymorphism analysis, and quantitative analysis.

While the concept is simple, PCR is a complicated process with many reactants[6]. It is somehow important to optimize M-PCR that will be used for repetitive diagnostic or analytical procedures where optimal amplification is required[7,8]. During the optimization of M-PCR, there can be several difficulties, including less sensitivity and specificity, or preferential amplification of certain specific targets. The amplification of more than one primer pair in the M-PCR increases the chance of obtaining fraudulent amplification because of the formation of primer dimers hence, it is necessary to adjust the

*Correspondence:
E-mail: drsindhavlab@gmail.com

primer to template ratio[5,9]. Primer design for some multiplexed targets can be complex, in that case *in silico* PCR is advantageous as it will calculate theoretical PCR results[10].

Optimization of a particular PCR can be time-consuming and complicated since various reactants are involved. These include the design and concentration of primers, the concentration of the four deoxynucleotides (dNTPs), PCR buffer, selection and concentration of DNA polymerase, quality and concentration of DNA template, addition, and concentrations of PCR additives/cosolvents, the concentration of magnesium ions, thermal cycling conditions, and use of the hot start technique. Optimization of PCR may be affected individually by each of these parameters as well as the combined interdependent effects of any of this indices[11]. A major consideration for the successful accomplishment of M-PCR assays is the time, cost, and accuracy[12].

However, globally at least 7.6 million children are conceived yearly with serious hereditary malady[13]. Genetic disorders are a consequence of changes in human DNAand can be acquired from parent to offspring. Exact predominance is hard to gather, particularly in a country like India where ethnic variety is high. Gujarat, a state with more than 6.5 crore populace likewise needs a listing of exact data for the pervasiveness of genetic diseases. Muscular dystrophies (MDs) are inherited and heterogeneous disorders that are portrayed by the debasement of skeletal muscles and progressive weakness. These are classified by the clinical manifestations, rate of progression, age of manifestation, and mode of inheritance. Many of these conditions are caused by fierce disturbances of diverse components of the dystrophin-glycoprotein complex (DGC) that is vital for the integrity of skeletal muscle fibers[14,15]. Hence, in developing countries like India pocket-friendly M-PCR is advantageous for screening cum diagnostics.

Dystrophin [UniProt#P11532], 427 kDa protein product of the DMD gene, is one of the largest components of the DGC and serves as a molecular shock absorber, it is absent or severely shortened in a devastating DMD, while diminished levels are found in the milder allelic form of Becker muscular dystrophy (BMD) [OMIM #300376]. DMD and the allelic BMD are the most common types of MDs in humans and are together named dystrophinopathies[16]. DMD alone accounts for approximately 80% of all MDs, with an occurrence of around 1 in 3,500 live-born males[17]. To date 34 clinically distinguished MDs have been accounted for with 29 genes[18]. X-linked recessive D/BMD caused by mutations of the DMD gene [OMIM #300377], located at locus Xp21.2. The DMD gene is the only known human metagene, spanning 2.4 Mb, transcript into a 14 kb mRNA, and contains 79 exons.

DMD subjects are usually identified by difficulty in walking, difficulty in climbing stairs, Gower's sign, and gait difficulty from the age of 2-5 years. DMD is mainly characterized by progressive muscle weakness and muscle wasting originating from proximal limbs followed by distal limbs[19], and further shows spinal scoliosis, joint contractures, and pseudohypertrophy of muscles. They get eventually wheelchair-bound by the age of 12 years due to severe lower limb weakness. Respiratory muscle weakness and cardiac failure are very predominant in DMD and often suffer central and obstructive sleep apnea. Consequently, to reduce this genetic burden a prospective, affordable, convenient, and specific molecular diagnosis is very vital.

In D/BMD large deletions encompassing one or more than one exon is common. It accounts for 60-65% of mutations in DMD and approximately 80% in BMD while exonic duplication is 5-15%[20]. In Gujarat, the overall deletion rate of 74% has been reported by Rao *et al.* (2014)[21].

Multiplexes for deletion of X-linked human disease, are designed to give a positive or negative indication of the presence of an exon. As timely and accurate diagnosis is the most important step in D/BMD management and care, it is very relevant to develop a diagnostics method to capture the mutational landscape of the Indian population. M-PCR is the first-line genetic diagnostic and exploring this, the study has been proposed to customize a comprehensive M-PCR method for D/BMD that is rapid, precise, and cost-effective.

Materials and Methods

All the standard chemicals and reagents were imported from Merck, Himedia, SRL, and Qualigens; India. It includes Tris ($C_4H_{11}NO_3$), Tris-HCl ($NH_2C(CH_2OH)_3.HCl$), Potassium chloride (KCl), Magnesium chloride ($MgCl_2$), Sodium chloride (NaCl), NonidetP40 (NP^{40}), Chloroform ($CHCl_3$),

Ammonium sulphate ($(NH_4)_2SO_4$), Isoamyl alcohol ($C_5H_{12}O$), Isopropyl alcohol (C_3H_8O), Distilled saturated phenol, Sodium dodecyl sulphate (SDS), Ethylene diamine tetraacetic acid (EDTA), Agarose, Glacial acetic acid, Ethidium bromide (EtBr), Bromophenol blue, Xylene cyanol, Glycerol.

The standard plastic wares were purchased from Tarson. The standard glassware was purchased from Borosil.

Standard reagents for the M-PCR were as follow: 2X PCR Master Mix (Thermo Scientific, #K0171) (0.05 U/µL *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP), Dream *Taq* DNA polymerase (5U/ µL, Thermo Scientific, #EP0702), 10X Dream *Taq* Buffer (Thermo Scientific, #EP0702), $MgCl_2$ (25 mM, Thermo Scientific, #R0971), BSA (20 mg/mL, Thermo Scientific, #B14), Template DNA, Water, nuclease-free (Thermo Scientific, #K0171), Forward and Reverse Primers (Sigma).

Ethical approval

This study was approved by the Institutional ethics committee (GUJIEC/20/2019IEC) as per the guidelines provided by ICMR.

Sample collection

After taking detailed history and consent, 5 mL of intravenous blood was collected into EDTA.K_2 vacutainer under sterile conditions and stored at 4°C.

DNA isolation

Genomic DNA from peripheral blood leukocytes was isolated by the modified method of phenol-chloroform extraction[22]. The dried DNA pellet was diluted in an appropriate volume of 0.3X TE buffer.

Determination of quality and quantity of DNA by absorbance method

After isolation, genomic DNA was studied for its qualification and quantification by using the absorbance method. DNA concentration was determined at 260 nm and 280 nm (OD 260/280) using Nanophotometer (NanoPhotometer®P-Class Model P300, Implen).

***In silico* PCR**

Primer Blast and UCSC Genome browser were used for this study to scrutinize primers, to optimize annealing and melting point (Tm), and other factors as such GC content, the efficiency of binding, complementarity, and secondary structure. Primer selection followed simple rules: primer length of 19-25 bp or higher and a GC content of 40%–65%, thus having an annealing temperature of 58°C to 62°C or higher. Longer primers (DMD primers, 30-32 bp) allowed the reaction to be performed at a very high annealing temperature and yielded lesser unspecific products.

Selection of primers for M-PCR

In this study, the gene DMD was amplified with the set of Chamberlain *et al.* (1988), Beggs *et al.* (1991), and Kunkel *et al.* (1991)[23-25]. Further, this study is also intended to modify and optimize the Beggs set to cover 13 exons in a single reaction as illustrated in (Fig. 1). All primers were diluted with nuclease-free water and stored at –20°C until use.

Design and development of Uniplex and M-PCR

PCR is an iterative process, consisting of three basic steps: denaturation of the template by heat, annealing of the oligonucleotide primers to the single-stranded target sequence(s), and extension of the annealed primers by a thermostable DNA polymerase. Unlike standard PCR, M-PCR is a powerful laboratory technique for fast *in vitro* enzymatic amplification of target DNA sequences in which more than one locus is simultaneously amplified in the same reaction. In this appraisal, we optimized Uniplex and M-PCR conditions for Chamberlain, Beggs, and Kunkel setas per Rao *et al*. (2014) with some modifications[21] (Tables 1-3 and Fig. 2). Along with these, we modified and optimized Beggs set (Table 1) followed by their sensitivity and specificity. A thermal cycler (Veriti® thermal cycler PCR, ABI) was used for amplification.

Optimization of reaction mix for Uniplex and M-PCR

Agarose gel electrophoresis is the heart of molecular cloning and is used to separate, identify, and purify DNA fragments of varying sizes. These biomolecules are separated on their chargesand size under the influence of the electrical field. After successful amplification of the DMD gene, loading dye was added to PCR products, and the mixture was run on 2.5% agarose gel stained with 1 mg/mL Et Brat 100 V for 2 to 3 h. in a 1X TAE buffer. The gel was observed and photographed by the Gel documentation system (BioDoc-it™ imaging system, UVP) after electrophoresis.

Interpretation of bands

The presence of an amplicon or band in the gel can be scored as successful amplification of the exon. The

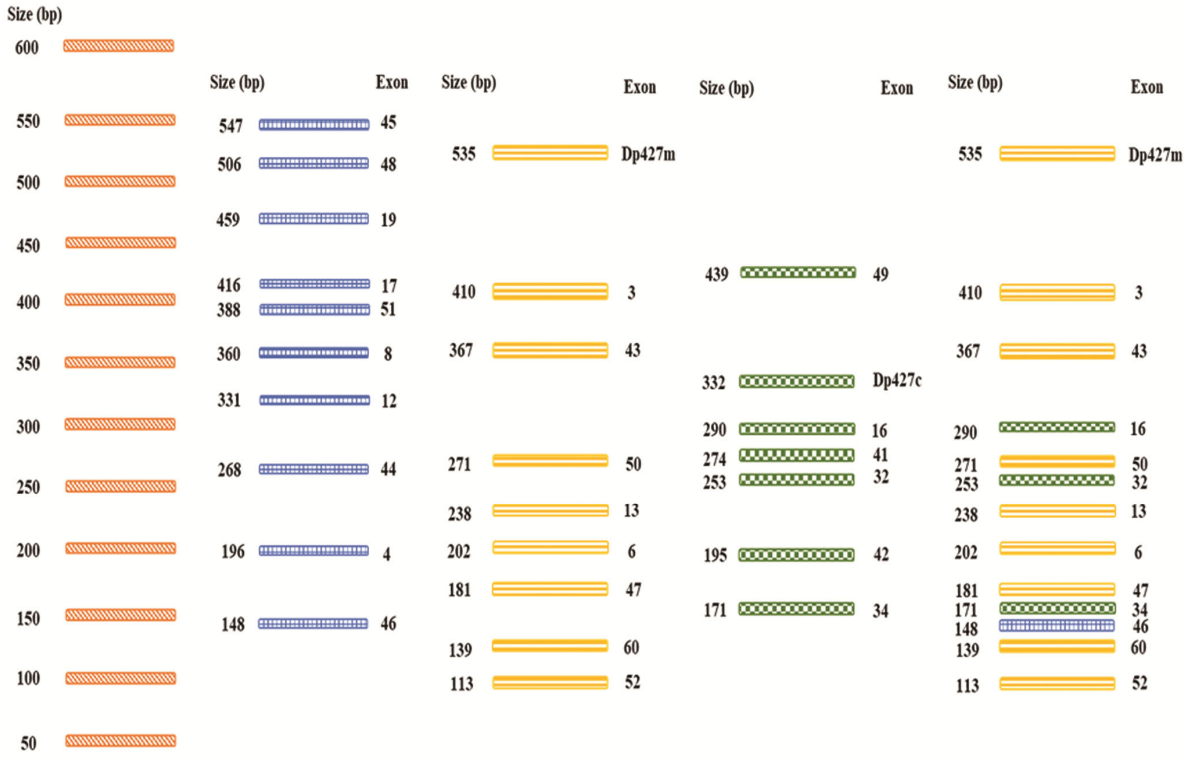

Fig. 1 — Schematic representation of the Chamberlain, Beggs, Kunkel, and modified Beggs primer set for dystrophin gene

Table 1 — Targeted primer sequences (modified Beggs set) for optimization of M-PCR

Exon No.	Primer Sequence	Size (in bp)	Chamberlain, Beggs.and Kunkel set
1	FP: 5′GAAGATCTAGACAGTGGATACATAACAAATGCATG-3′ RP: 5′TTCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC-3′	535	Beggs
3	FP:5′TCATCCA TCATCTTCGGCAGATTAA-3′ RP: 5′CAGGCGGTAGAGTATGCCAAATGAAAATCA-3′	410	Beggs
43	FP:5′GAACATGTCAAAGTCACTGGACTTCATGG -3′ RP: 5′ATATATGTGTTACCTACCCTTGTCGGTCC-3′	357	Beggs
16	FP:5′TCTATGCAAATGAGCAAATACACGC -3′ RP:5′GGTATCACTAACCTGTGCTGTACTC-3′	290	Kunkel
50	FP: 5′CACCAAATGGATTAAGATGTTCATGAAT-3′ RP: 5′′TCTCTCTCACCCAGTCATCACTTCATAG-3′	271	Beggs
32	FP:5′GACCAGTTATTGTTTGAAAGGCAAA -3′ RP:5′TTGCCACCAGAAATACATACCACACAATG-3′	253	Kunkel
13	FP: 5′AATAGGAGTACCTGAGATGTAGCAGAAAT-3′ RP: 5′CTGACCTTAAGTTGTTCTTCCAAAGCAG-3′	238	Beggs
6	FP: 5′CCACATGTAGGTCAAAAATGTAATGAA-3′ RP: 5′GTCTCAGTAATCTTCTTACCTATGACTATGG -3′	202	Beggs
47	FP: 5′CGTTGTTGCATTTGTCTGTTTCAGTTAC-3′ RP: 5′GTCTAACCTTTATCCACTGGAGATTTG-3′	181	Beggs
34	FP:5′GTAACAGAAAGAAAGCAACAGTTGGAGAA-3′ RP:5′CTTTCCCCAGGCAACTTCAGAATCCAAA-3′	171	Kunkel
46	FP:5′GCTAGAAGAACAAAAGAATATCTTGTC-3′ RP:5′CTTGACTTGCTCAAGCTTTTCTTTTAG-3′	148	Chamberlain
60	FP: 5′AGGAGAAATTGCGCCTCTGAAAGAGAACG-3′ RP: 5′CTGCAGAAGCTTCCATCTGGTGTTCAGG-3′	139	Beggs
52	FP: 5′AATGCAGGATTTGGAACAGAGGCGTCC-3′ RP: 5′TTCGATCCGTAATGATTGTTCTAGCCTC-3′	113	Beggs

Optimization of reaction mix for Uniplex and M-PCR

Table 2 —PCR reaction mix for selected primer sets of the dystrophin gene

Reaction sets	2X master mix	Forward primer	Reverse primer	10X *Taq* buffer	*Taq* polymerase (5U/ µL)	Template DNA (50 ng/ µL)	BSA (20 mg/mL)	$MgCl_2$ (25 mM)	Water	Total
Chamberlain (Uniplex)	12.5 µL	1 µL (All primers are with 0.2 µM final con.)	1 µL (All primers are with 0.2 µM final con.)	-	-	2 µL	-	-	8.5 µL	25 µL
Beggs (Uniplex)	12.5 µL	1 µL (Dp427m, 3, 43, 50, 13, 6, 60, 52 with 0.2 µM final con. and 47 with 0.8 µM final con.)	1 µL (Dp427m, 3, 43, 50, 13, 6, 60, 52 with 0.2 µM final con. and 47 with 0.8 µM final con.)	-	-	2 µL	-	-	8.5 µL	25 µL
Kunkel (Uniplex)	12.5 µL	1 µL (49, Dp427c, 16, 41, 32 with 0.4 µM final con. and 42, 34 with 0.8 µM final con.)	1 µL (49, Dp427c, 16, 41, 32 with 0.4 µM final con. and 42, 34 with 0.8 µM final con.)	-	-	2 µL	-	-	8.5 µL	25 µL
Chamberlain (10-plex)	20 µL	10 µL (All primers are with 0.1 µM final con.)	10 µL (All primers are with 0.1 µM final con.)	7 µL	2 µL	5 µL	-	-	6 µL	60 µL
Beggs (9-plex)	20 µL	9 µL (Dp427m, 3, 43, 50, 13, 6, 60, 52 with 0.1 µM final con. and 47 with 0.4 µM final con.)	9 µL (Dp427m, 3, 43, 50, 13, 6, 60, 52 with 0.1 µM final con. and 47 with 0.4 µM final con.)	5 µL	0.8 µL	5 µL	-	-	1.2 µL	50 µL
Kunkel (7-plex)	18 µL	7 µL (49, Dp427c, 16, 41, 32 with 0.2 µM final con. and 42, 34 with 0.4 µM final con.)	7 µL (49, Dp427c, 16, 41, 32 with 0.2 µM final con. and 42, 34 with 0.4 µM final con.)	5 µL	0.8 µL	5 µL	-	-	7.2 µL	50 µL
Modified Beggs (Uniplex)	12.5 µL	1 µL (Dp427m, 3, 43, 50, 13, 6, 60, 52, 46 with 0.2 µM final con., 16, 32 with 0.4 µM final con. and 34, 47 with 0.8 µM final con.)	1 µL (Dp427m, 3, 43, 50, 13, 6, 60, 52, 46 with 0.2 µM final con., 16, 32 with 0.4 µM final con. and 34, 47 with 0.8 µM final con.)	-	-	2 µL	-	-	8.5 µL	25 µL
Modified Beggs (13-plex)	20 µL	6.5 µL (1, 43, 52 with 0.5 µM final con. and 3, 16, 50, 32, 13, 6, 47, 34, 46, 60 with 0.3 µM final con.)	6.5 µL (1, 43, 52 with 0.5 µM final con. and 3, 16, 50, 32, 13, 6, 47, 34, 46, 60 with 0.3 µM final con.)	5 µL	1.2 µL	5 µL	1 µL	2.8 µL	2 µL	50 µL

Optimization of amplification conditions for Uniplex and M-PCR

Table 3 —PCR amplification conditions for selected primer sets of the dystrophin gene

Reaction set	Initial denaturation		35 Cycles						Final elongation	
			Denaturation		Annealing		Extension			
	Temp.	Time	Temp.	Time	Temp.	Time	Temp.	Time	Temp.	Time
Chamberlain (Uniplex)	94°C	10 min	94°C	30 sec	53°C	90 sec	72°C	60 sec	72°C	10 min
Beggs (Uniplex)	94°C	10 min	94°C	30 sec	62°C	90 sec	72°C	60 sec	72°C	10 min
Kunkel (Uniplex)	95°C	10 min	94°C	30 sec	60°C	90 sec	72°C	60 sec	72°C	10 min
Chamberlain (10-plex)	94°C	10 min	94°C	30 sec	53°C	90 sec	72°C	60 sec	72°C	10 min
Beggs (9-plex)	94°C	10 min	94°C	30 sec	62°C	90 sec	72°C	60 sec	72°C	10 min
Kunkel (7-plex)	95°C	10 min	94°C	30 sec	60°C	90 sec	72°C	60 sec	72°C	10 min
Modified Beggs (Uniplex)	94°C	10 min	94°C	30 sec	62°C	90 sec	72°C	60 sec	72°C	10 min
Modified Beggs (13-plex)	94°C	10 min	94°C	30 sec	62°C	90 sec	72°C	60 sec	72°C	10 min

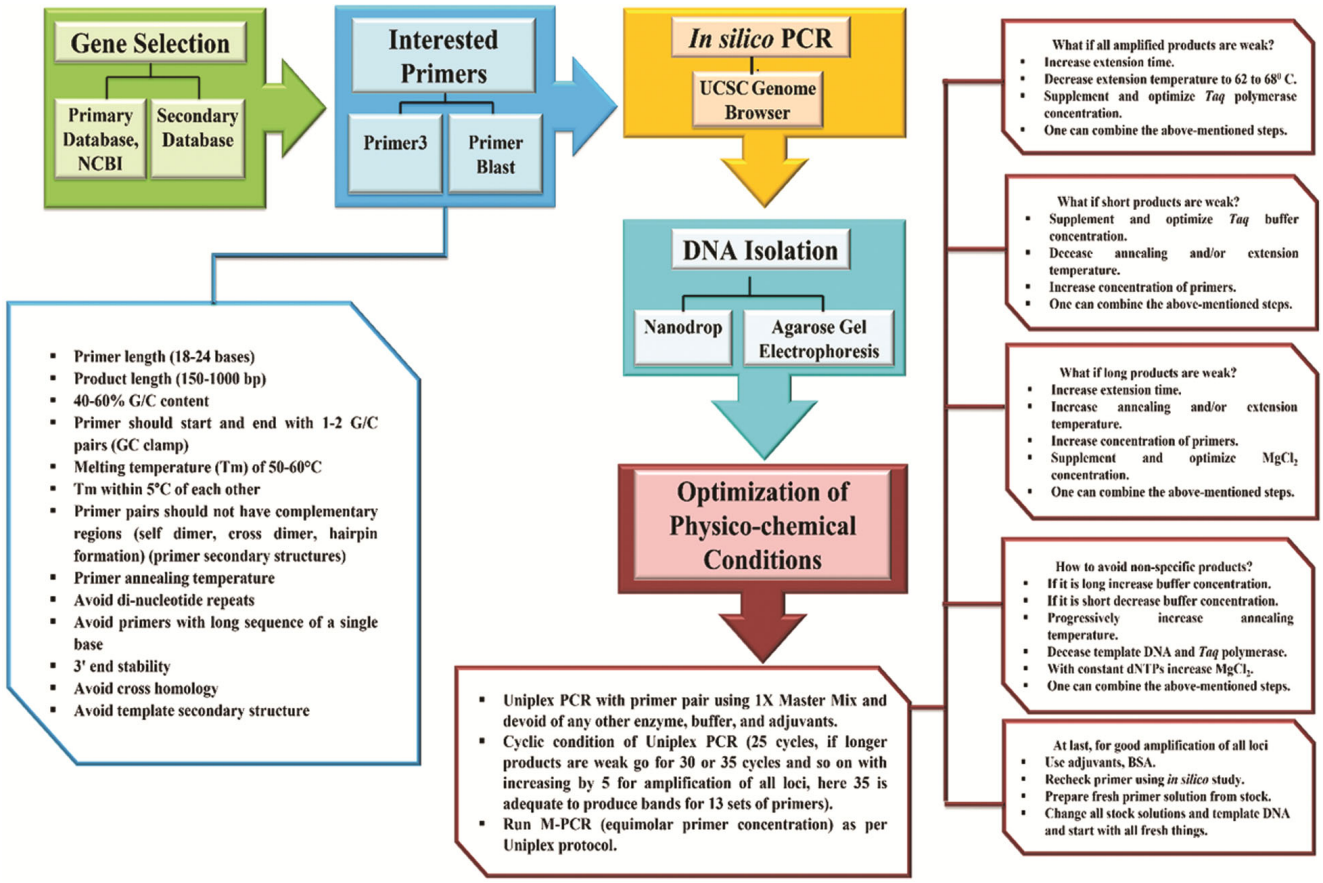

Fig. 2 — Flowchart to optimize M-PCR condition[26]

absence of an amplicon or light band was scored as unsuccessful amplification of the exon.

Results and Discussion

In the present study, an attempt has been made to understand, optimize, and evaluate various essential biochemical and physical indices associated with the M-PCR. The crucial indices presented in this data will provide shed a light on the common problems of M-PCR. In this study, the selected exons for the modified Beggs set were Dp427m, 3, 43, 16, 50, 32, 13, 6, 47, 34, 46, 60, and 52.

To establish reproducible M-PCR for the DMD gene, optimization for our modified Beggs set is carried out by a sequential investigation of each reaction mixture. The most essential parameter for optimizing the M-PCR was to evaluate firstly the PCR cyclic condition that allowed amplification of all loci individually and related to that study divulges 35 cycles are most appropriate.Corresponding to many experiments, the produced results are shown as follows.

Uniplex PCR

To obtain the accurate amplification of each primer of the modified Beggs set, PCR was undertaken. In this experiment, there was successful amplification of all the single loci at the same temperature which is 62°C for 90 sec Other reactants were set as per Table 2 and 0.2 μM to 0.8 μM primer concentration showed proper amplification of each exon. The design and concentration of these primers are very important as they play an important role in the outcome of a PCR assay. Primer concentrations should be between 0.1-0.5 μM in optimal reactions. Higher primer concentrations (>0.5 μM) may cause the accumulation of nonspecific products which promote priming at nonspecific sites on the template. The primers should nevertheless be in excess to avoid their exhaustion before the completion of the reaction which would compromise the yield of the desired amplicon[27]. The study depicts that for individually amplified locus, the annealing time (40 to 110 sec) and the extension time (30 to 90 sec) did not visibly influence the results. But the specificity and yield of PCR products were increased or decreased by changes in annealing temperature[28].

M-PCR for 3 sets and modified Beggs set

Optimization of M-PCR is very precise and complex because of gene similarity, a difference in primer, and PCR condition[12]. Initially, there was some variation from test to test for M-PCR. To solve these reproducibility problems adjustments of PCR components are very essential. As per Table 2 concentrations of each primer and reaction mix were optimized for the 3 sets. For the modified Beggs set there was uneven amplification with some of the products barely visible even after the reaction was optimized for the cycling conditions. Overcoming such a problem, required changing the concentration of various primers in the reaction, with an increase in the concentration of primers for the weak loci and a decrease for the strong loci. After various experiments, the outcomes considered that the final concentration of the primers (0.3–0.5 μM) is most appropriate for the reaction. Figure 3B shows the successful amplification of 3 sets of M-PCR; 10 plex Chamberlain set, 9 plex Beggs set, and 7 plex Kunkel set, along with amplification of our modified Beggs set that is 13 plex.

Standardization of *Taq* buffer concentration

Most reaction buffers consist of a buffering agent, most often a Tris-base buffer, and salt, commonly KCl. The buffer regulates the pH of the reaction, which is necessary for DNA polymerase activity and fidelity. Modest concentrations of KCl will increase DNA polymerase activity by 50–60% over activities in the absence of KCl; 50 mM KCl is considered optimal[29]. Most suppliers of thermostable DNA polymerases provide a 10X reaction buffer with the enzyme. For the modified Beggs set, increasing the buffer concentration from 0.4X to 2.8X, shorter products were amplified more efficiently, whereas the longer products gradually decrease as increasing concentration. After various experiments data showed that the most appropriate buffer concentration for the reaction was 1X. Generally, primer pairs with longer amplification products worked better at low salt concentrations, whereas, primer pairs with short amplification products worked better at higher salt concentrations, where longer products become harder to denature.

Standardization of *Taq* polymerase

In PCR, *Taq* DNA polymerase at high temperatures allows repeated amplification cycles and has an optimum temperature for DNA synthesis of around 72-75°C. As high temperatures (55–72°C) can be used during the primer annealing steps, this bonus improved

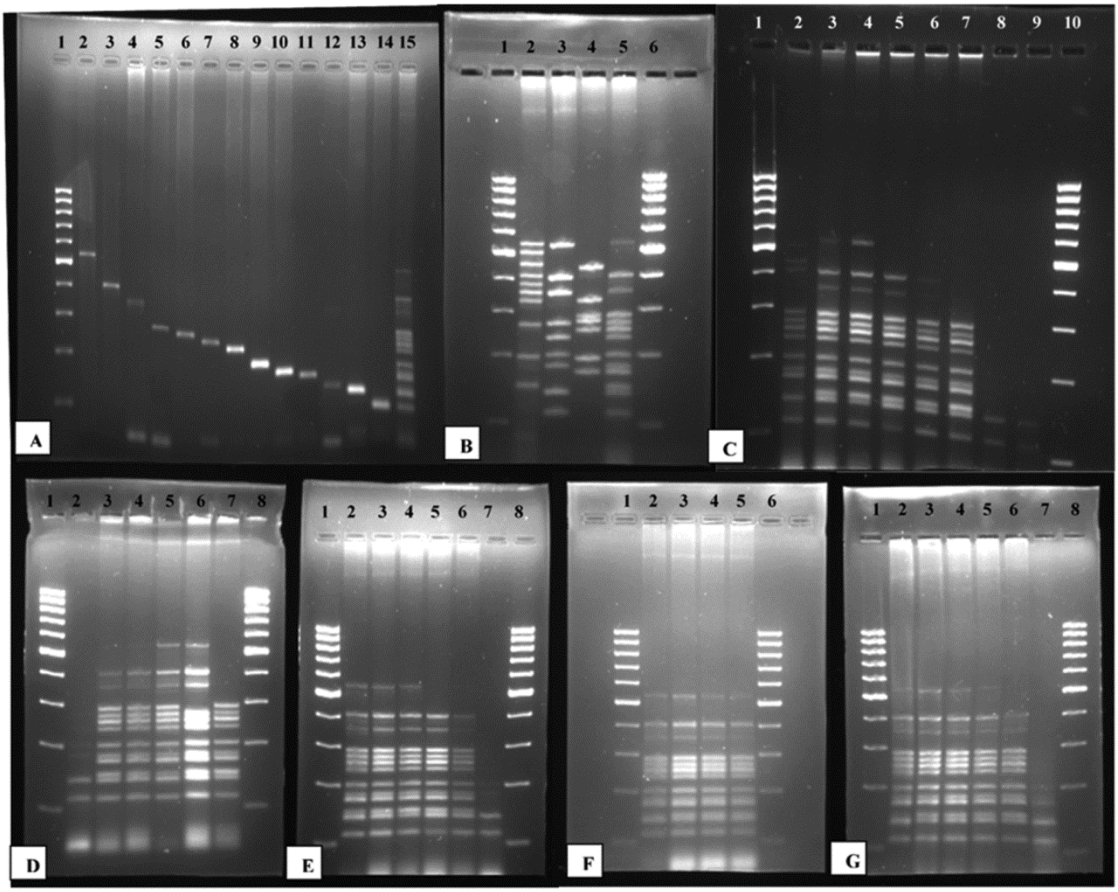

Fig. 3 — Optimization of chemical parameters for Uniplex and M-PCR of selected primers

the specificity of primer annealing leading to greater amplification of targeted sequences[6]. Amplification of modified Beggs set was done using different concentrations of *Taq* DNA polymerase as 3, 4, 5, 6, 7, and 8U/50 µL reaction. While increasing the concentration, a longer product has less amplification. The most efficient enzyme concentration seemed to be around 6U/50 µL reaction volume. Good specificity was encountered at 7U *Taq* DNA polymerase but missing some specific loci (Fig. 3D). Many enzymes in the reaction, possibly because of the high glycerol concentration in the stock solution, resulted in an insufficient amplification of various loci[30].

Sensitivity of M-PCR

In the case of mammalian genomic DNA, up to 1.0 µg of DNA is utilized per reaction an amount that contains ~3 × 10^5 copies of a single-copy gene.Variousamounts of template DNA were used with the same *Taq* polymerase concentration. DNA templates between 500 ng to 1.0 ng/50 µL were tested in this reaction, results showed no significant differences from 500 to 100 ng however in a further decrease in the concentration from 20 ng to 1.0 ng/50 µL the amount of some longer products decreased. As data shows the optimal concentration of DNA was 250 ng/50 µL.

Standardization of BSA concentration

In the current study, BSA was used as an additive,and amplification was done with different concentrations from 0.2 to 0.8 µg/µL for M-PCR of the modified Beggs set. Increasing the concentration of BSA yields less specificity and amplification. As per findings, excessive BSA concentration will stop reaction amplification and specificity. So, results depicted 0.4 µg/µL was usually sufficient for a reaction. In any PCR, DMSO and glycerol are used to improveamplification efficiency and specificity. Therefore, the advantages of these adjuvants need to be tested in each case. Conferring to the literature, BSA in concentrations up to 0.8 µg/µL increases the efficiency and specificity of the PCR much more rather than DMSO or glycerol. BSA does not have an inhibitory effect on amplified products[26].

Standardization of $MgCl_2$ concentration

Magnesium is one of the most crucial factors in the PCR and serves as a cofactor for thermostable DNA polymerases, its concentration can affect the specificity and efficiency of the reaction. Optimization of $MgCl_2$ is most important since *Taq* DNA polymerase depends onit[31]. Magnesium concentration may influence primer annealing, dissociation temperatures, product specificity, formation of primer-dimer artifacts, and enzyme activity/fidelity[27]. Amplification by increasing the concentration of $MgCl_2$ from 0.9 to 6.4 mM at the same *Taq* polymerase concentration portrayed that excessive concentration would inhibit the amplification of longer products. The most efficient $MgCl_2$ concentration was observed to be around 1.4 mM/ 50 µL reaction volume. In addition, *Taq* DNA polymerase, primers, and dNTPs bind with$MgCl_2$. Therefore, the optimal Mg^{2+} concentration will depend on the dNTPs concentration, template DNA, and buffer composition[32]. The present study found that 1.4 mM $MgCl_2$ works well in 160 µM of each dNTP. According to many experiments, the results of the study achieved that excessive $MgCl_2$ concentration stabilizes the double-strand DNA and inhibits the denaturation of DNA, which will decrease specificity.

Standardization of cycle number

The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction, primer extension, and amplification. At least 25 cycles are required to achieve acceptable levels of amplification of single-copy target sequences in mammalian DNA templates. Amplification with increasing numbers of cycles by five was carried out for the modified set. There was no major difference between 30 and 35 cycles. At 35 cycles, the yield of all bands had better resolution than at 30 cycles. So, results suggested that 35 cycles were usually sufficient for a reaction. Increasing the number of cycles up to 45 yields less amplification of longer amplicon.

Standardization of extension time

Results of the analysis showed that primers gave shorter amplification products with less extension time, whereas a decrease in the long products was found with increasing the extension time. The present study stated the optimal extension time is 60 sec In the M-PCR, as more loci are simultaneously amplified, the pool of enzymes and nucleotides becomes a limiting factor and more time is required for the polymerase to complete the amplification of all the products.

Figure 3A shows Agarose gel electrophoresis of the uniplex and M-PCR products amplified with the optimum reagent concentrations and conditions with DNA ladder at the same annealing temperature (62°C for 90 sec for 35 cycles where Lane 1 is Ladder (50 bp), Lane 2 to 14 is for selected single-locus amplification and Lane 15 shows targeted M-Plex of the 13 exons. Figure 3B Agarose gel electrophoresis shows successful amplification of the M-PCR products after optimizing the concentration of each primer set with the optimum reagent mix and conditions. Where indication of the lane is: Lane 1 is Ladder (50 bp), Lane 2: Control- Chamberlain set (exon: 45, 48, 19, 17, 51, 8, 12, 44, 4 and 46), Lane 3: Control-Beggs set (exon: Dp427m, 3, 43, 50, 13, 6, 47, 60 and 52), Lane 4: Control-Kunkel set (exon: 49, Dp427c, 16, 41, 32, 42 and 34), Lane 5: Modified Beggs set (exon: Dp427m, 3, 43, 16, 50, 32, 13, 6, 47, 34,46, 60 and 52), Lane 6: Ladder (50 bp). Figure 3C Agarose gel electrophoresis shows amplification of the M-PCR products with different concentrations of Taq buffer. Where each lane indicates concentration: Lane 1: Ladder (50 bp), Lane 2: 0.4X, Lane 3: 0.8X, Lane 4: 1.0 X, Lane 5: 1.4X, Lane 6: 1.8X, Lane 7: 2.2X, Lane 8: 2.4X, Lane 9: 2.8X, Lane 10: Ladder (50 bp). At the lowest concentration visibility of bands are poor while increasing concentration illustrates prominent amplification. Further increased concentration (lanes 5 to 9) hinders the amplification of long products. Figure 3D Agarose gel electrophoresis shows amplification of the M-PCR products with different concentrations of Taq polymerase. Where each lane indicates concentration: Lane 1: Ladder (50 bp), Lane 2: 3 U/50 µL, Lane 3: 4 U/50 µL, Lane 4: 5 U/50 µL, Lane 5: 6 U/50 µL, Lane 6: 7 U/50 µL, Lane 7: 8 U/50 µL, Lane 8: Ladder (50 bp). At the lowest concentration bands are weakly amplified while increasing concentration illustrates obvious amplification of all loci. Further, the highest concentration (8 U/50 µL) obstructs the amplification of long products. Figure 3E Sensitivity of M-PCR. Agarose gel electrophoresis shows amplification of the M-PCR products with different concentrations of DNA. Where each lane implies: Lane 1: Ladder (50 bp), Lane 2: 500 ng/50 µL, Lane 3: 250 ng/50 µL, Lane 4: 100 ng/50 µL, Lane 5: 20 ng/50 µL, Lane 6: 10 ng/50 µL, Lane 7: 1.0 ng/50 µL, Lane 8: Ladder (50 bp). Along with decreasing concentration (lanes 5 to 7), longer bands are amplified with lesser specificity while at the lowest concentration only 2 loci were amplified. Figure 3F

Agarose gel electrophoresis shows amplification of the M-PCR products with different concentrations of BSA as additives. Where each lane implies various concentrations: Lane 1: Ladder (50 bp), Lane 2: 0.2 µg/µL, Lane 3: 0.4 µg/µL, Lane 4: 0.6 µg/µL, Lane 5: 0.8 µg/µL, Lane 6: Ladder (50 bp). With higher concentrations, (lanes 4 to 5) specificity of the reaction is minimized. Hence, the optimum found concentration is 0.4 µg/µL for a 50 µL reaction. Figure 3G Agarose gel electrophoresis shows amplification with different concentrations of MgCl2. Where each lane implies various concentrations: Lane 1: Ladder (50 bp), Lane 2: 0.90 mM/50 µL, Lane 3: 1.4 mM/50 µL, Lane 4: 2.4 mM/50 µL, Lane 5: 3.4 mM/50 µL, Lane 6: 4.9 mM/50 µL, Lane 7: 6.4 mM/50 µL, Lane 8: Ladder (50 bp). At the lowest concentration (lane 2) all loci were amplified with poor specificity whereas an increase in concentration (lanes 4 to 7) demonstrated poor amplification of long products.

Figure 4A shows Agarose gel electrophoresis shows optimization of cycle numbers. Where each lane implies a number of cycles: Lane 1: Ladder (50 bp), Lane 2: 20 cycles, Lane 3: 25 cycles, Lane 4: 30 cycles, Lane 5: 35 cycles, Lane 6: 40 cycles, Lane 7: 45 cycles, Lane 8: Ladder (50 bp). 20 and 25 cycles were able to amplify only 4 loci with weak specificity. While the increase in cycles impedes the amplification of long amplicons. Hence, we found 35 cycles are ideal with good visibility of bands. Figure 4B Agarose gel electrophoresis shows optimization of extension time. Where indication of each lane is: Lane 1: Ladder (50 bp), Lane 2: 30 sec, Lane 3: 60 sec, Lane 4: 90 sec, Lane 5: Ladder (50 bp) 30 sec was not enough for all 13 loci amplification. 90 sec yields no specificity for 11 loci. While 60 sec is optimal as it yields good specificity for all loci.

Application of the optimized M-PCR assay for clinically suspected D/BMD subject

A subject who visited the Department of Zoology, Gujarat University, Ahmedabad and the Indian Muscular Dystrophy Society & Research Centre (IMDS), Ahmedabad had elevated creatine phosphokinase (CPK), calf hypertrophy, and scoliosis. Subsequently, the standardized M-PCR assay was used to screen the D/BMD condition by evaluating its deletion pattern. After successful amplification, the subject's DNA showed deletion for many targeted loci (Fig. 5).

Figure 5 shows Agarose gel electrophoresis reveals deletion patterns to screen D/BMD. Lane 1: Ladder

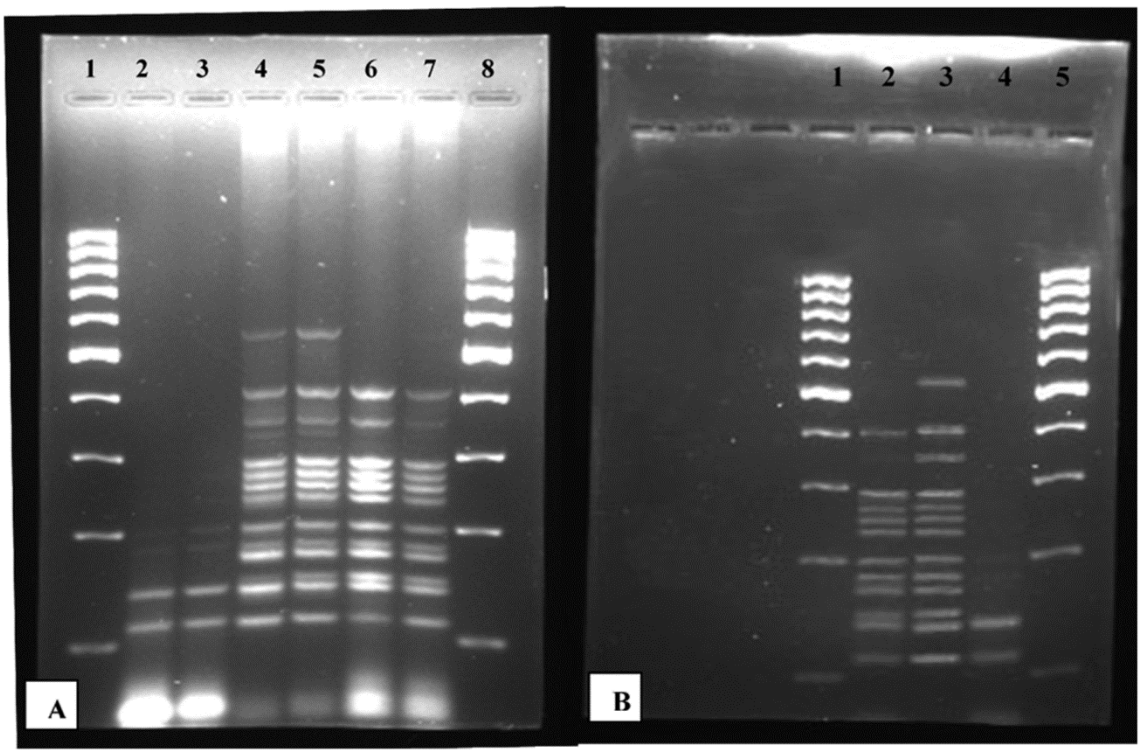

Fig. 4 — Optimization of physical parameters for Uniplex and M-PCR of selected primers

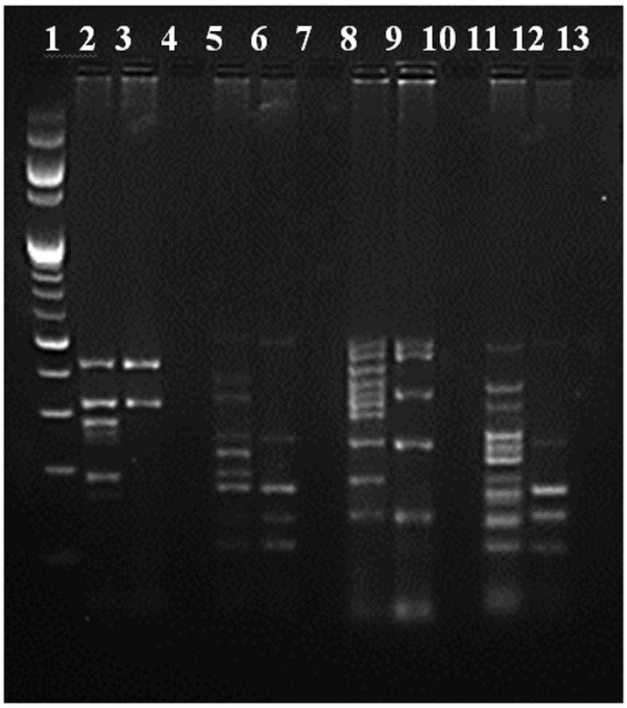

Fig. 5 — M-PCR assay for clinically suspected D/BMD subject

(50 bp), Lane 2: Positive control of Kunkel set, Lane 3: Kunkel set for the subject (presence of exon 49, Dp427c), Lane 4: Negative control of Kunkel set, Lane 5: Positive control of Beggs set, Lane 6: Beggs set for the subject (presence of exon Dp427m, 50, 47, 60, 52), Lane 7: Negative control of Beggs set, Lane 8: Positive control of Chamberlain set, Lane 9: Chamberlain set for the subject (presence of exon 45, 48, 51, 44, 46), Lane 10: Negative control of Chamberlain set, Lane 11: Positive control of Modified Beggs set, Lane 12: Modified Beggs set for the subject (presence of exon Dp427m, 50, 47, 46, 60, 52), Lane 13: Negative control of Modified Beggs set.

Conclusion

DMD has been diagnosed with various assays for many decades that are mostly based on multiplex sets, those are Chamberlain set (10 plex), Beggs set (9 Plex), and Kunkel set (7 Plex). This study uncovers high specificity and sensitivity in D/BMD screening after the successful optimization of the modified Beggs M-PCR set. The devised M-PCR set can amplify 13 exons (>3000 bp), which will establish a new approach to diagnosis. Since, this newly optimized M-PCR set is a mixture of Chamberlain, Beggs, and Kunkel, there will be no requirement to perform the test for different exon mutations for D/BMD separately which is generally found in the Gujarat population. Moreover, the proposed customized set is rapid, precise, and cost-effective thus the study opens new areas for potential research in M-PCR chemistry development. The great boom of this approach empowered a favorable platform for biological enigma and ensured a wealth of data for the researcher. We are also establishing new protocols to cover more exons to investigate this metagene.

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