Chapter **2**

Rapid and cost effective detection of small mutations in the DMD gene by high resolution melting curve analysis

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Abstract

Duchenne/Becker muscular dystrophy (DMD/BMD) is caused by large deletions or duplications in two-thirds of the cases. The remaining one-third DMD patients have small mutations in the DMD gene. Screening for such small mutations is a daunting and costly task. High resolution melting curve analysis (HR-MCA) followed by sequencing for amplicons with altered melting profiles can be used to scan DNA for small alterations. We first validated the technique as screening procedure for the DMD gene and then screened a group of unrelated 22 DMD/BMD patients and 11 females. We managed to identify all previously found mutations by means of HR-MCA, which provided its validation. Furthermore, 17 different pathogenic mutations were found in the screening group, of which 10 were novel. Our results provide validation of HR-MCA as a powerful and inexpensive pre-sequencing scanning method. This technology is now ready for routine diagnostic use on DMD/BMD patients and female carriers.

Introduction

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular disorder, characterized by rapidly progressive muscle weakness and wasting. DMD is one of the most common types of muscular dystrophy, with an incidence of one in 3500 newborn boys [1]. The onset of symptoms is generally before the age of 5. Affected individuals are confined to a wheelchair before the age of 12 and usually die in the course of the second or third decade, due to respiratory or heart failure [2].

Becker muscular dystrophy (BMD) shows a milder phenotype and is less common, with an incidence of 1:20,000 newborn males. BMD is characterized by delayed onset of muscle weakness and clinical symptoms. Many BMD patients remain ambulant later in life and have a longer life span than DMD patients [2].

DMD and BMD are allelic X-linked recessive diseases, caused by mutations in one of the largest human genes known to date, the DMD gene, which is distributed over about 2.4 million base pairs [3]. The vast majority of affected individuals are boys. However, a few affected females have been reported, in whom the disease was associated with a translocation with a breakpoint within Xp21 locus [4] or due to skewed X-inactivation, in which the majority of muscle cells used the mutated DMD gene, while the normal gene is inactivated through non-random X-inactivation [5].

The DMD gene has 79 exons [6], coding for a 14 kb mRNA transcript. The 427 kDa cytoskeletal dystrophin protein is localized to the cytoplasmic face of the sarcolemma [7]. Dystrophin protein is an important component of the dystrophin–glycoprotein complex that stabilizes the membrane of striated muscle. The absence of dystrophin leads to sarcolemmal fragility, muscle weakness, and eventually muscle degeneration [8].

The extremely large size of the dystrophin gene makes it vulnerable to structural changes. Many pathogenic mutations have been reported among DMD patients; 60% of these mutations are intragenic deletions ranging from one to several exons, and 5–10% are duplications [3]. The remaining one-third of sequence changes are mutations at the nucleotide level [3, 9].

There is a hypothesis known as the reading frame rule. It predicts that deletions or duplications,

which shift the reading frame of dystrophin messenger RNA, produce premature, truncated, nonfunctional protein and cause the severe DMD phenotype. On the other hand, BMD is caused by inframe deletions/duplications, which allow the generation of partially functional, internally deleted or duplicated protein. The reading frame hypothesis holds true for over 92% of all DMD and BMD patients [10].

The great majority of deletions and duplications cluster in a minor and a major hot spot within the DMD gene. The first one spans exons 2–20, while the second, major one spans exons 45–53 [3] and [11]. These mutations can be detected by a variety of methods including Southern blotting [11], multiplex PCR [12] and [13], multiplex amplifiable probe hybridization (MAPH) [14], and recently multiplex ligation-dependent probe amplification (MLPA). The last allows fast and reliable detection of deletions and duplications throughout the DMD gene [15].

A number of scanning methodologies have been developed to enhance small pathogenic mutation detection in patients without detectable large deletions and duplications. These methods include denaturing gradient gel electrophoresis (DGGE) [16], denaturing high performance liquid chromatography (dHPLC) [17], single strand conformation polymorphism analysis (SSCP) [18], fluorescent multiplex conformation sensitive capillary electrophoresis (FM-CSCE) [19], direct sequencing [20], and the protein truncation test (PTT) [21], each with its particular advantages and disadvantages.

The first aim of our study was to evaluate the HR-MCA as a mutation scanning method in the DMD gene and to minimize the cost of mutation scanning. The second was to implement an effective and convenient diagnostic strategy in BMD/DMD patients and carriers to detect small mutations.

Materials and methods

Patients

HR-MCA was performed on a group of 22 patients (12 DMD and 10 BMD) and a group of 11 females: five obligate carriers, five possible carriers (mothers and sisters of isolated DMD patients) and one young symptomatic female in whom cytogenetic analysis had excluded a translocation with a breakpoint in Xp21. All 12 patients suspected of suffering from DMD

exhibited severe phenotypes and elevated serum CK levels. DMD phenotype was confirmed by absence of dystrophin using immunohistochemical analysis in eight cases, in one case only reduced expression of dystrophin was observed and in three no muscle biopsy had been taken. The diagnosis of BMD was based on clinical criteria and elevated serum CK levels. No muscle biopsy had been taken from the majority of BMD-like patients (7 out of 10). Reduced dystrophin expression on Western blot was detected in one case and a weak patchy dystrophin pattern on muscle sections was observed in the other two. Genomic DNA was isolated from peripheral blood by standard procedures [22]. Large deletions and duplications in the DMD gene had been previously excluded by MLPA in all cases.

Validation

In order to determine the efficiency of HR-MCA for mutation scanning, we tested 40 heterozygous and 34 hemizygous variants in 45 different amplicons. These samples were selected from previous studies. In order to enhance heteroduplex formation for hemizygous variants, each hemizygous variant was tested in three ways: without mixing with wild type DNA, mixing with other male genomic wild type DNA before PCR amplification and post-PCR mixing with other male wild type PCR product.

Primers

Sequencing primers with M13 tails were designed previously by using primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). All 79 exons and adjacent intron/exon junctions were amplified and optimized for high resolution melting curve analysis. To maximize the sensitivity of the technique, exons 3, 23, 48, 53, 61, 67, 68, 76, and 79, which had three melting domains, were split into multiple amplicons. In addition, new primers were designed for exon 19, with smaller fragment size to avoid having three melting domains and exon 65, which failed to give a PCR product.

All new primers were designed using either primer 3 or light scanner primer design software (Idaho Technology). To predict the number of melting domains, these primers were tested using the melting program (version 1.0; INGENY International BV, Goes, The Netherlands). In 10 amplicons, 19, 3-GC, 61A, 61B, 68A, 68B, 79B2, 79C2, 79D1 and 79D2, a short GC stretch was

added to avoid three melting domains. The total number of amplicons was 96 (Supplementary Table 1).

Probes

We designed unlabeled probes (incorporating a 3′ phosphate in order to prevent polymerase extension) that perfectly match the five most frequent variants in five different exons of the DMD gene. The probe sequences, annealing temperatures and primer ratios for genotyping are shown in Table 1.

Table 1: Sequences and PCR conditions for five different probes.

Amplicon	Probe sequences	Annealing temperature (C°)	Primer ratios	No. of cycles	Variant
17	CTGAAGTCTTTCGAGCAATGTCTGACC	61	1 to 5	55	c.1993 -37T>G
37	AAACTTGATGGCAAACCACGGTGAC	61	1 to 10	55	c.5234G>A
48b	AGAAGGACCATTTGACGTTAAGGTAGG	61	1 to 5	55	c.7096C>A
54	GCATTCATAAAAGGTATGAATTATATTAT	61	1 to 5	55	c.8027+11C>T
66	CAGATGTAAGTCGTGTATACTAATGCTG	61	1 to 5	55	c.9649 +15T>C

PCR

PCR was performed in 96-well, non-transparent plates (ABgene) in 10 µl total volume with: 1 × PCR buffer (Roche), 2 mM $MgCl_2$, 2 mM dNTPs, 3 pmol of each primer, 1 × LCGreen Plus (Idaho Technology), 0.5 U of fast start Taq DNA polymerase (Roche) and 20 ng of DNA template. All PCR wells were covered with 15 µl of mineral oil (Sigma), and centrifuged at 2500 RPM for 1 min before PCR.

PCR was carried out in a gradient cycler (Bio-RAD). The thermo-cycling protocol was as follows: 10 min at 95 °C, 40 cycles of 20 s at 95 °C, 30 s at the annealing temperature, 40 s at 72 °C, and 5 min at 72 °C. In order to promote heteroduplex formation, samples were

denaturated by heating to 95 °C for 1 min and cooling down to 15 °C in the thermo-cycler before HR-MCA.

Asymmetric PCR

Asymmetric PCR was performed whenever unlabeled probes were used. Primer asymmetry ratios of 1:5 to 1:10 produced sufficient single stranded product for probe annealing (see Table 1). PCR reactions were performed as described above with some minor modifications as follows: 1 pmol of the forward primer, 5 or 10 pmol of the reverse primer (see Table 1), and 5 pmol of each unlabeled probe. The thermo-cycling protocol was done as described above but with 55 cycles and an annealing temperature of 61 °C.

Post PCR mixing

Samples from hemizygous males were mixed post-PCR. After successful PCR amplification, which was tested by a light scanner (Idaho Technology), post-PCR mixing was performed between amplicons of two different non-related male patients. As males have only one X-chromosome, mixing is necessary to ensure that heteroduplex formation can occur. Post-PCR mixing was done as follows:

Ten microliters of PCR product from each patient was covered with 15 μl of mineral oil (Sigma), centrifuged at 2500 RPM for 1 min, heated to 95 °C for 5 min and cooled down to 15 °C before HR-MCA.

Melting analysis

After PCR, the plates were imaged in a 96-well Light Scanner (Idaho Technology). The fluorescence data were collected from 65 to 98 °C for the amplicon scanning, and from 55 to 98 °C for the unlabeled probe genotyping at a temperature transition rate of 0.1 °C/s. Melting curves were analyzed by using the commercial light scanner software on the high sensitivity setting as previously described [23], [24] and [25]. After exponential background subtraction, fluorescence data were normalized between 0% and 100%. Slight temperature errors or buffer differences between wells or runs were corrected by temperature shifting in regions of low fluorescence and high temperature (2–5% normalized fluorescence). This facilitated clustering of

curves for heterozygous samples. Difference plots of normalized and temperature overlaid curves were obtained by subtracting the fluorescence values of each curve from the mean reference values, which were defined as the most popular genotype (wild type).

Sequencing

Since HR-MCA is a non-destructive method, all amplicons that produced abnormal melting profiles were sequenced from the original PCR reactions for female carrier samples. All altered male patient amplicons were confirmed by sequencing of independent PCR products. PCR was performed in MicroAmp reaction tubes (Applied Biosystems) in 25 µl total volumes containing: 10 × commercial PCR buffer or 5 × STR buffer which contains (0.5 M $(NH4)_2SO4$, 0.5 M Tris–HCL, pH 8.8, 1 M $MgCl_2$, 10 mM EDTA, 14 M β-mercapto-ethanol, and ultra-pure water), 1.5–3 mM $MgCl_2$ (Supplementary Table 2), 2.5 mM dNTPs, 2.5 pmol each primer, 1 U of Taq DNA polymerase (Promega) and 200 ng of DNA template. PCR was carried out in a Biometra T-Professional (Westburg). The thermo-cycling protocol was as follows: 5 min at 95 °C, 35 cycles of 20 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and 5 min at 72 °C. After amplification, the PCR products were purified by the AMPure PCR purifications system using solid-phase paramagnetic bead technology (Agencourt). Sequencing was performed in both sense and antisense direction using uniform BigDye (Terminator v3.1 sequencing reactions, Applied Biosystems) with PAGE purified M13F (−21M13) or M13R (M13REV) sequencing primer. Sequencing reactions were then purified using a column filtration procedure (DTR V3 96-wells plates, Edge Biosystems) and final analysis was done using the ABI 3730 [26]. After electrophoresis, data processing was automated using SeqScape 2.1.1 software (ABI). Base calls with quality values below QV = 25 were checked manually. The primer sequences (with M13 tail) that were used for amplification of DMD amplicons are shown in (Supplementary Table 3).

Results

Data from the HR-MCA

-Validation

DNA samples from patients or female carriers with known sequence changes, were used to optimize parameters for HR-MCA mutation scanning. Scanning for variants relies on differences

in the melting curve profile, which are most apparent in difference plots. The most common genotype, wild type, was selected as a reference to form the baseline, while the variant samples showed clearly distinctive melting curves.

We could not detect the different known sequence variants in exons 3, 23, 48, 53 and 79B. All of these fragments had three melting domains that could mask the presence of the variants. Therefore, new primer sets were designed for all fragments with three melting domains, to reduce the number of melting domains per fragment. Our data show that this approach enhanced the resolution of HR-MCA.

The initial testing correctly identified all 40 heterozygous and 24/34 hemizygous variants. The remaining 10 hemizygous variants were detected only after post-PCR mixing with wild type male DNA, an example is shown in Fig. 1A and B. Panel A shows exon 16 with one aberrant melting profile for a heterozygous variant (c.1961T > C) from a female sample and no aberrant melt profile for the male sample. Whereas panel B shows the result for the same exon after post-PCR mixing for the males samples, with two aberrant melt profiles, which represent the heterozygous (c.1961T > C) and the hemizygous (c.1869C > T) variants.

In several exons there was clustering of different sequence variations, which were readily distinguishable from each other and from the wild type, showing different melting curves.

Variants could also be distinguished in homozygous and heterozygous form. In exon 53, the abnormal curve produced by the same sequence variant (c.7728T > C) in homozygous form differs from that caused by heteroduplex formation in the heterozygous form (Fig. 1C).

In order to reduce the burden of sequencing, five unlabeled probes were designed to identify frequently found (see Table 2) variants (c.1993-37T > G, c.5234G > A, c.7096C > A, c.8027 + 11C > T, c.9649 + 15T > C) in amplicons 17, 37, 48b, 54, and 66, respectively. We successfully detected both heterozygous and homozygous–hemizygous variants. All three possible genotypes within the tested samples set were recognized by a single unlabeled probe. A perfectly matched probe-target hybrid had a higher Tm than the mismatched ones. Heterozygous amplicons, on the other hand, showed two peaks with two different temperatures representing

both genotypes, this is exemplified by sequence variant (c.5234G > A) in exon 37 (Fig. 2A and B).

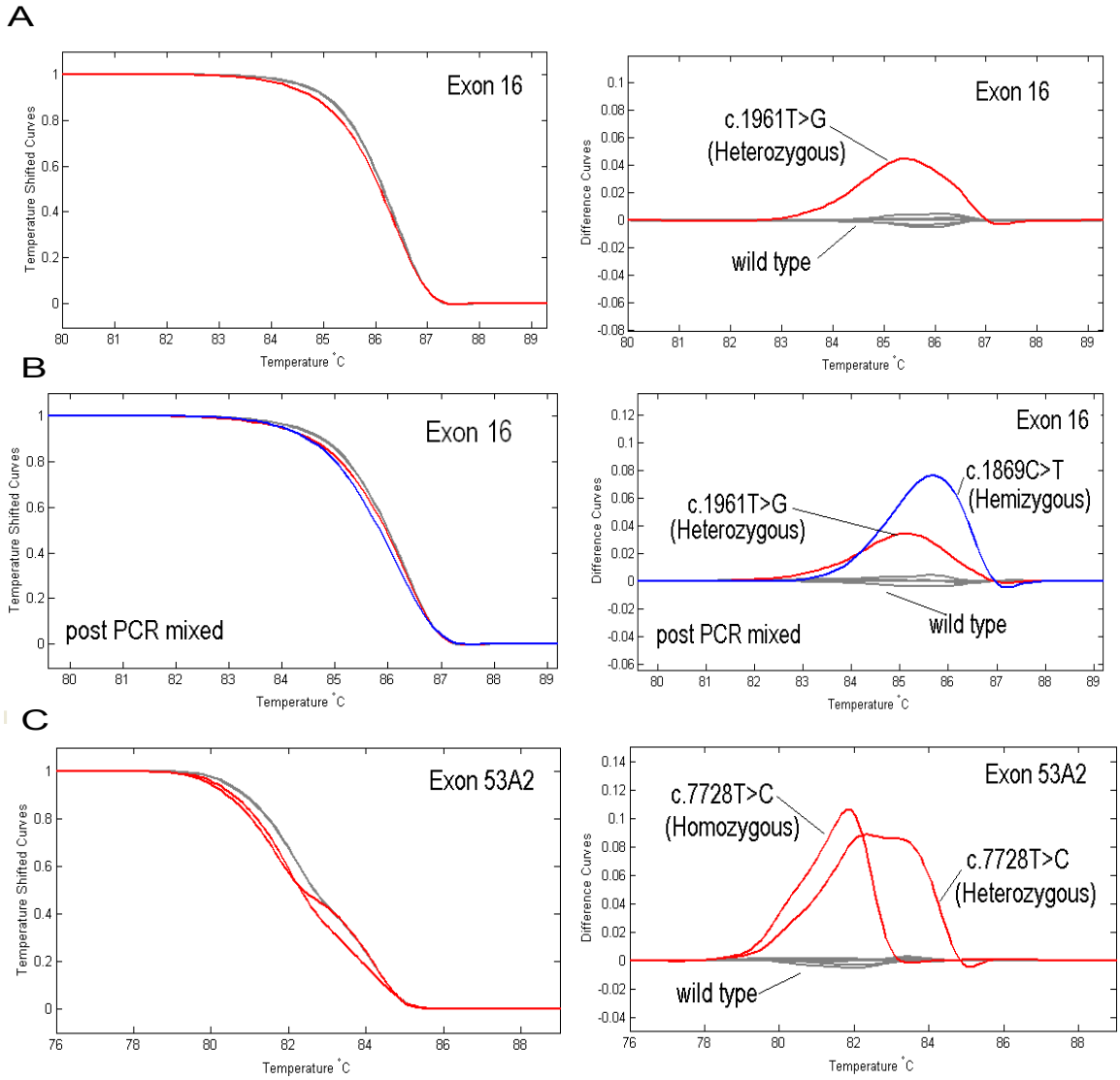

Fig. 1. Temperature shifted (left) and subtractive difference plots (right) of wild type and variants. (A and B) Exon 16, on (A) only the heterozygous variant from female sample is detected, while in (B) the hemizygous variant is only detected after post-PCR mixing. (C) The different melting curve profiles for the same variant in heterozygous and homozygous state.

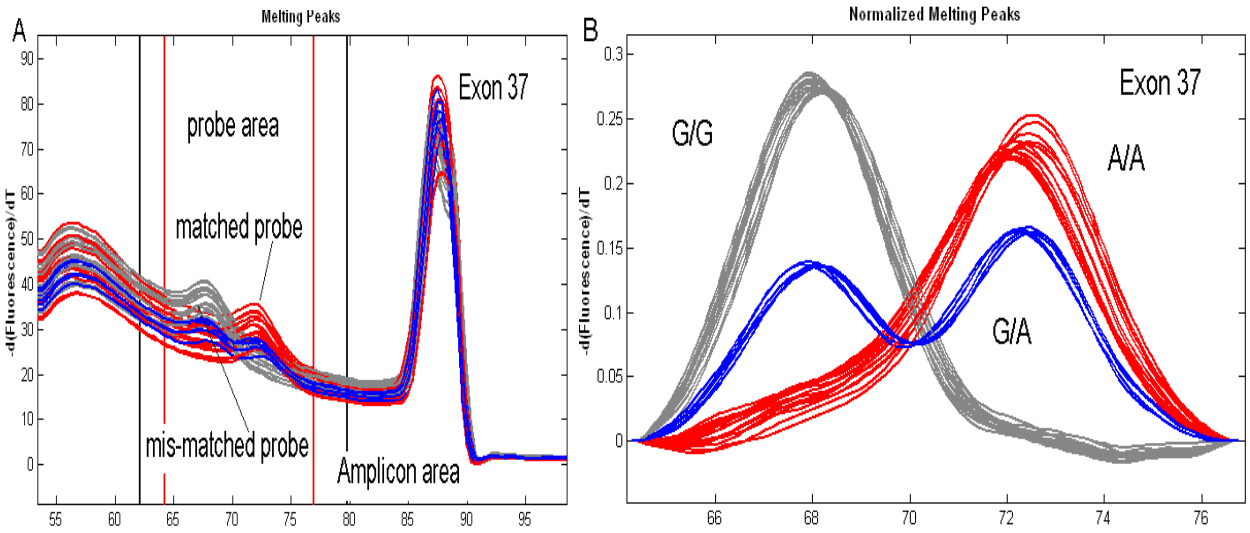

Fig. 2. (A and B) Common DMD gene variants in exon 37 detected by genotyping with unlabeled probes. (A) Both the amplicon and probe area of exon 37. (B) The enlargement of the probe area.

-Patient screening group

After validation of the scanning method, we tested the group of 22 (BMD, DMD) patients and 11 females. Amplicons with abnormal melting curves were sequenced to determine the changed variant. Five different heterozygous pathogenic mutations were detected directly within the female group. Furthermore, seven out of 12 hemizygous pathogenic mutations were found in male patients. Five out of 12 hemizygous pathogenic mutations (c.187-2A > G, c.3097_3098del, c.3603 + 2T > A, c.5771_5772del, c.6611dup) were detected only after post-PCR mixing, because they showed an altered fluorescence curve compared to the wild type profile.

The only deletion/insertion mutation (c.597_614delinsCTAGTTTC), in exon 7 in a DMD male patient, was detected directly without post-PCR mixing (Fig. 3A). However, the abnormal curve produced by the same hemizygous mutation became clearer after post-PCR mixing (Fig. 3B).

The results of genotyping our patients and carriers show that there is a great advantage of having oligonucleotide probes corresponding to the frequently occurring variants, because it reduces the number of sequencing reactions (Table 2).

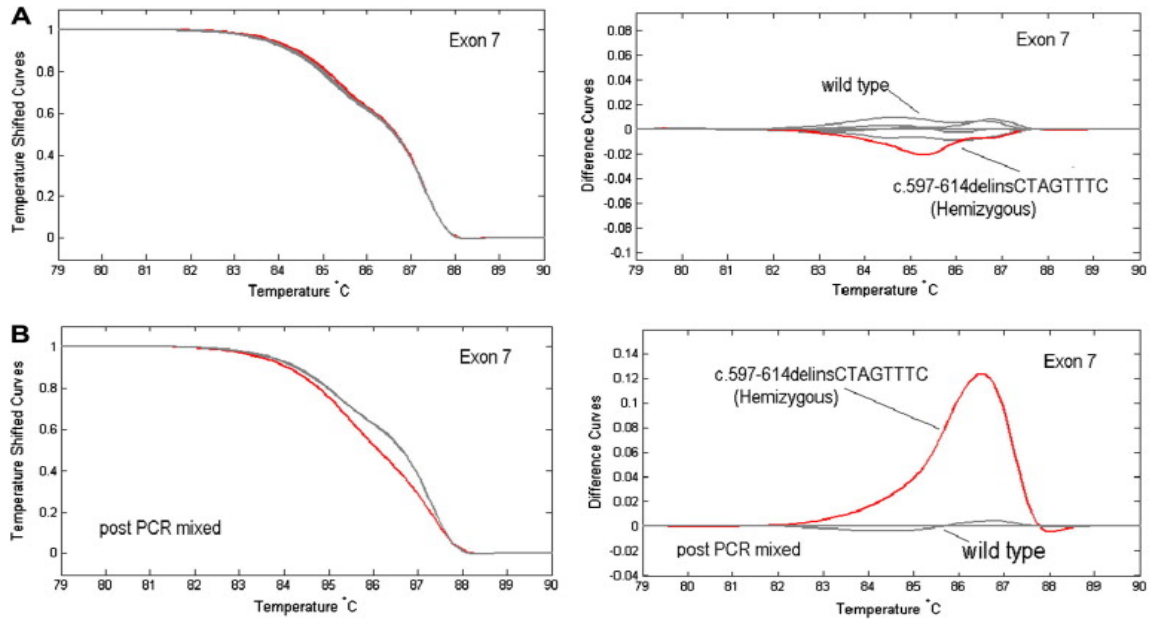

Fig. 3. Temperature shifted (left) and subtractive difference plots (right) of wild type and a mutation in exon 7. (A) Exon 7 from a male patient without post-PCR mixing; the deletion/insertion mutation can be detected. (B) The post-PCR mixing of the same exon, that produces a different and clearer melting curve.

Data from sequencing analysis

In total, 17 different pathogenic mutations were detected in 33 cases (12 DMD, 10 BMD and 11 female carriers) of which 10 were novel (Table 3). Most mutations were identified in obligate carriers (4/5) and DMD patients (10/12). Mutations were identified in all eight DMD patients with absence of dystrophin in the muscle tissue. A mutation was also found in two patients from whom no muscle tissue was available. No mutation was found in the other two, one of whom had reduced dystrophin expression. Seven of the 10 mutations in the DMD patients were novel (see Table 3).

Table 2. variants detected by HR-MCA and sequencing in BMD/DMD patients and carriers.

Exon	DNA change	Protein	Frequency/Remarks
3	c.94-9dupT		4⁄33
3	c.186+35A>T		1⁄33
6	c.530+19C>T		once +patho
9	c.832-18_832-17delinsGA		once +patho
14	c.1635A>G		3⁄33
14	c.1704+51T>C		3⁄33
17	c.2168 +13T>C		1⁄33
17	**c.1993-37T>G**		**23⁄33**
21	c.2645G>A	p.Gly882Asp	12⁄33
23	c.3021G>A		1⁄33
25	c.3406A>T	p.Thr1136Ser	1⁄33
27	c.3734C>T	p.Thr1245Ile	once +patho
31	c.4234-13A>G		2⁄33
33	c.4519-34T>A		once +patho
34	c.4675 -53G>T		2⁄33
37	**c.5234G>A**	**p.Arg1745His**	**19⁄33**
43	c.6290+27T>A		2⁄33
44	c.6291-115G>A		5⁄33
45	c.6463C>T	p.Arg2155Trp3/33	3⁄33
48	c.6913-114A>T		Once
48b	**c.7096C>A**	**p.Gln2366Lys**	**27⁄33**
49	c.7200+53C>G		11⁄33
53	c.7728T>C		8⁄33
54	c.8027+13T>G		once +patho
54	**c.8027+11C>T**		**14⁄33**
59	c.8762A>G	p.His2921Arg	once +patho
59	c.8810A>G	p.Gln2937Arg	3⁄33
64	c.9361+138T>C		7⁄33
66	**c.9649+15T>C**		**28/33**
75	c.10789C>T		2⁄33
75	c.10797+42C>G		1⁄33
79	c.*477_*484del		2⁄33
79	c.*491_*492dupCA		6⁄33
79	c.*1051_*1052ins		1⁄33
79	c.*1447A>G		1⁄33

- + patho, when a variant is found in combination with a pathogenic mutation.
- All variants in bold were detected by HR-MCA/ probes.

Table 3. Pathogenic mutations found in BMD/DMD patients and carriers.

Test Sample #	Phenotype	Sex	Exon	DNA change	Protein	New	Dystrophin (IHC)
21	BMD	male	4	c.187-2A>G		No (27)	Reduced
17	DMD	male	7	c.597_614delinsCTAGTTTC	p.Phe200X	Yes	Absent
8	DMD	male	15	c.1721G>A	p.Trp574X	Yes	ND
24	DMD	male	22	c.2929C>T	p.Gln977X	Yes	ND
9	DMD	male	23	c.3097_3098del	p.Ser1033LeufsX5	Yes	Absent
16	DMD	male	23	c.3151C>T	p.Arg1051X	No (27)	Absent
23	obligate carrier	female	26	c.3516G>A	p.Trp1172X	Yes	ND
26	DMD	male	26	c.3603+2T>A	p.Lys1201_Arg1202insX25	No (27)	Absent
5	BMD	male	34	c.4845+1G>A		Yes	ND
12	obligate carrier	female	40	c.5697del	p.Lys1899AsnfsX2	No (27)	ND
31	DMD	male	41	c.5771_5772del	p.Glu1924GlyfsX7	No (27)	Absent
10	DMD	male	44	c.6291-1G>T		Yes	Absent
30	DMD	male	45	c.6611dup	p.Arg2205GlufsX18	Yes	Absent
11	obligate carrier	female	51	c.7538dup	p.Lys2514GlufsX34	Yes	ND
18	symptomatic carrier	female	58	c.8641del	p.Leu2881X	Yes	Mosaic
1	DMD	male	67	c.9807+1G>C		No (27)	Absent
34	obligate carrier	female	70	c.10141C>T	p.Arg3381X	No (27)	ND

ND, not done.
IHC, immunohistochemistry.

In three cases splice-site mutations were found: two of these (c.3603 + 2T > A; c.9807 + 1G > C) have been described before in DMD patients [27]. One novel mutation (c.6291-1G > T) in the splice-site of exon 44 was predicted to skip exon 44 thereby shifting the reading frame of the DMD gene. Two mutations were found among 10 patients suspected of having BMD based on clinical symptoms. In a 33-year-old BMD patient from a large BMD family with six patients, a novel splice-site mutation of exon 34 was found (c.4845 + 1G > A) predicting an "in-frame" skip of exon 34 in this family. The second mutation was found in a 10-year-old sporadic BMD patient with reduced dystrophin levels on a Western blot. A splice-site mutation of exon 4 (c.187-2A > G) was identified, which is likely to skip (in-frame) exon 4. The same mutation has been reported before in a BMD patient [27]. No mutation was detected in the remaining eight BMD cases. So, it is possible that these patients are suffering from other types of muscular dystrophy such as LGMD. Most of the BMD-like patients are sporadic except for one family in which recent haplotyping showed that X-linked inheritance is unlikely. A novel heterozygous frameshift mutation was detected in a young symptomatic female (c.8641delC; p.Leu2881ArgfsX13), who appeared to be a DMD carrier, and in whom previous cytogenetic analysis had excluded a translocation in band Xp21. A mutation was identified in four out of five obligate carriers of this study: two frameshift mutations, one of which is novel (c.7538dupA; p.Lys2514GlufsX34), and two nonsense mutations, one of which is novel (c.3516G > A; p.Trp1172X). However, no mutation was identified in any of the five possible DMD carriers. In addition to these mutations we identified 30 different variants. All of these have been reported before [27] and are shown in Table 2.

Discussion

HR-MCA in combination with dsDNA dye LCGreen Plus was used to scan the DMD gene and to genotype frequent variants. LCGreen Plus dye does not inhibit Taq polymerase and can be used at a concentration that will saturate newly synthesized double stranded DNA during PCR. Saturating all the available double stranded sites is a critical characteristic that eliminates the potential for a dye molecule to redistribute during the melting process of the PCR product. Another advantage is that because the dye is added to the PCR before amplification, no further processing or labeling of primers is required.

For HR-MCA one needs to pay careful attention to the design of the primers as mutation detection is easier when there are only one or two melting domains. We found that breaking up exons with three melting domains into multiple fragments allowed the detection of all variants that were tested. This is exemplified by exon 23 in which the two variants (c.2994T > A) and (c.3059C > G) were not observed initially before breaking up the exons. We also manipulated the melting by adding a GC stretch (7–11 bp) to 10 primers in order to avoid three melting domains and to maximize the sensitivity of the technique.

After optimizing the various parameters, all 40 heterozygous and 24 out of 34 of the hemizygous variants that were located anywhere between two primers could be detected. Although the majority of the X-linked hemizygous variants were detected directly, 10 of the hemizygous variants were missed, indicating that post-PCR-mixing from two non-related patients is needed to ensure heteroduplex formation and mutation detection. To avoid the risk that a variant would be missed using this approach because two patients may carry the same variant in the DMD gene, post-PCR mixing between patient sample and a non-affected male control sample would remove this risk. Post-PCR mixing is preferred because pre-PCR mixing requires an accurate quantification of DNA [28], and non amplification of one of the fragments could lead to false negative results.

All amplicons with abnormal melting profiles were sequenced, as there is no distinction between polymorphisms and pathogenic mutations. To avoid part of the sequencing, five most frequent variants throughout the DMD gene were genotyped by HR-MCA. All three possible genotypes within the tested sample set were recognized by a single unlabeled probe. In addition unexpected sequence variants under the probe could be detected. Use of unlabeled probes conveniently eliminates the need for expensive fluorescent labeled probes [29].

There have been numerous methods employed to detect small mutations in the DMD gene, such as DGGE [16], dHPLC [17], SSCP [18], FM-CSCE [19], PTT [21] and sequencing [20]. All of these technologies require post-PCR processing and separation on a gel or another matrix, which makes these techniques laborious and time consuming, as compared to HR-MCA, which is fast and has minimal post-PCR processing requirements. We conclude from our data that HR-MCA is at least as sensitive as DGGE, dHPLC and FM-CSCE. However, a comparative study has

recently shown that HR-MCA has higher sensitivity and specificity than dHPLC [30]. Moreover, all fragments analyzed by dHPLC need to be run under different denaturing conditions to maximize the mutation detection.

HR-MCA has an advantage over the DGGE method. DGGE requires considerable effort to design and optimize, making it more labor-intensive than HR-MCA for routine diagnostic use.

HR-MCA is a mutation scanning technique that requires accurate PCR amplification with normal unlabeled primers, whereas the FM-CSCE method needs the fluorophore labeling of one primer for each pair of primers. However, the major advantage of the FM-CSCE method is that nearly all mutation types can be detected simultaneously [19], whereas HR-MCA is suitable for the detection of only small mutations. For a complete mutation scanning strategy, HR-MCA should be combined with other methods such as MLPA [15].

The HR-MCA method makes mutation detection cost effective as it significantly reduces the amount of sequencing that needs to be performed. Furthermore, HR-MCA is a non-destructive and high throughput method for mutation scanning and genotyping, that can analyze 96 or 384 samples per run, and is thus exquisitely suitable for the screening of large multi-exonic genes, like the DMD gene.

As compared to RNA based methods, such as PTT, our HR-MCA technique is less laborious and less time consuming. PTT requires isolation of RNA, preferably from muscle tissue, which is not always available from affected patients. Although isolation of dystrophin mRNA is also possible from lymphocytes, the yield is very low [16]. Furthermore, only truncating mutations can be detected by PTT, whereas HR-MCA is able to detect all sequence changes, missense mutations, silent mutations, single nucleotide polymorphism (SNP's) and variations of unknown significance.

After sequencing of amplicons with abnormal melting profiles, about 83% of small mutations could be identified in our population suspected of suffering from DMD. It is very likely that a higher percentage of mutations would have been found if DMD had been confirmed in all cases by dystrophin analysis of muscle tissue. In two of the DMD-like cases in which no mutations were found, it seems plausible that other types of muscular dystrophies were involved. The fact

that no mutations were found in the majority of BMD-like patients (8/10), suggests that there is a large clinical overlap between BMD and other types of muscular dystrophy such as LGMD. It is, therefore, recommended that immunobiochemical analysis of muscle tissue be performed in patients suspected of having BMD before screening for small mutations. HR-MCA has been shown to be a quick and sensitive technique for further screening for small mutations in cases where dystrophin is absent or reduced in muscle tissue. An explanation for cases where no small mutation is found may be that the mutation is located either deep in an intron or in a regulatory region. Pathogenic mutations were found in four of the five obligate carrier females (80%). Determination of the carrier status is important for prenatal diagnosis, genetic counseling and prevention of the disease. It is possible that in the only family without a mutation, DMD is caused by a mutation deep in one of the introns activating cryptic exons or by a mutation in the promoter area of the DMD gene.

The majority of mutations that were identified were novel (60%), and were scattered throughout the gene. There were six different nonsense mutations which resulted in a truncated, nonfunctional protein, six different frame shift mutations and five changes that are expected to affect the splicing. All these 17 pathogenic mutations that were detected are unique to each family.

In conclusion, HR-MCA was found to be a highly reliable and quick method for mutation scanning and genotyping, requiring only direct analysis of the PCR reaction with a simple instrument. This technique offers many advantages over other techniques, and is a welcome addition to the screening strategy of laboratories involved in the diagnostic service for Duchenne and Becker muscular dystrophy.

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Supplementary data:

Supplementary Table 1 Sequences of primers, annealing temperatures and fragment sizes for HR-MCA.

Amplicon Number	Primer sequences	Fragment size	Annealing temp.
DMDEX01-F	TGTAAAACGACGGCCAGTGCAGGTCCTGGAATTTGA	405	61Cº
DMDEX01-R	CAGGAAACAGCTATGACCCAAACTAAACGTTATGCCACA		
DMDEX02-F	TGTAAAACGACGGCCAGTCACTAACACATCATAATGG	269	61Cº
DMDEX02-R	CAGGAAACAGCTATGACCGATACACAGGTACATAGTC		
DMDEX03 A-F	TGTAAAACGACGGCCAGTTCATCCGTCATCTTCGGCAGATTAA	176	61Cº
DMDEX03 A-R	CAGGAAACAGCTATGACCCAGGCGGTAGAGTAtgccaaatgaaaatca		
DMDEX03B-F	TCTTCAGTGACCTACAGGATGG	184	64Cº
DMDEX03B-R	CGCCCGCCGtgctgtttcaatcagtacctagtca		
DMDEX04-F	TGTAAAACGACGGCCAGTTTGTCGGTCTCTCTGCTGGTCAGTG	233	60Cº
DMDEX04-R	CAGGAAACAGCTATGACCCCAAAGCCCTCACTCAAAC		
DMDEX05-F	TGTAAAACGACGGCCAGTCAACTAGGCATTTGGTCTC	261	61Cº
DMDEX05-R	CAGGAAACAGCTATGACCTTGTTTCACACGTCAAGGG		
DMDEX06-F	TGTAAAACGACGGCCAGTTGGTTCTTGCTCAAGGAATG	335	61Cº
DMDEX06-R	CAGGAAACAGCTATGACCTGGGGAAAAATATGTCATCAG		
DMDEX07-F	TGTAAAACGACGGCCAGTCTATGGGCATTGGTTGTC	296	60Cº
DMDEX07-R	CAGGAAACAGCTATGACCAAAAGCAGTGGTAGTCCAG		
DMDEX08-F	TGTAAAACGACGGCCAGTTCGTCTTCCTTTAACTTTG	343	61Cº
DMDEX08-R	CAGGAAACAGCTATGACCTCTTGAATAGTAGCTGTCC		
DMDEX09-F	TGTAAAACGACGGCCAGTTCTATCCACTCCCCCAAACC	318	61Cº
DMDEX09-R	CAGGAAACAGCTATGACCAACAAACCAGCTCTTCAC		
DMDEX10-F	CGACGTTGTAAAACGACGGCCAGTGGAACAATCTGCAAAGAC	350	61Cº
DMDEX10-R	CAGGAAACAGCTATGACCAAAGGATGACTTGCCATTATAAC		
DMDEX11-F	TGTAAAACGACGGCCAGTCAAATAAAACTCAAAACCACACC	337	61Cº
DMDEX11-R	CAGGAAACAGCTATGACCCTTCCAAAACTTGTTAGTCTTC		
DMDEX12-F	TGTAAAACGACGGCCAGTCTTTCAAAGAGGTCATAATAGG	305	61Cº
DMDEX12-R	CAGGAAACAGCTATGACCCATCTGTGTTACTGTGTATAGG		
DMDEX13-F	TGTAAAACGACGGCCAGTGCAAATCATTTCAACACAC	387	60Cº
DMDEX13-R	CAGGAAACAGCTATGACCTCTTTAAATCACAGCACTTC		
DMDEX14-15-F	TGTAAAACGACGGCCAGTTGGCAAATTATTCATGCCATT	548	63Cº
DMDEX14-15-R	CAGGAAACAGCTATGACCTGATCCAAGCAAAAATAAACATT		
DMDEX16-F	TGTAAAACGACGGCCAGTATGCAACCCAGGCTTATTC	286	61Cº
DMDEX16-R	CAGGAAACAGCTATGACCCTGTAGCATGATAATTGGTATCAC		
DMDEX17-F	TGTAAAACGACGGCCAGTTTTTCCTTTGCCACTCCAAG	362	61Cº
DMDEX17-R	CAGGAAACAGCTATGACCCACCACCAACAAAACTGCTG		
DMDEX18-F	CGACGTTGTAAAACGACGGCCAGTTGTCAGGCAGGAGTCTCAgat	339	63Cº
DMDEX18-R	CAGGAAACAGCTATGACCCGGAGTTTACAAGCAGCACA		

DMDEX19-F	TGTAAAACGACGGCCAGT gattcacgtgataagctgacaga	286	63Cº
DMDEX19-R	CAGGAAACAGCTATGACCCGCCCGCCGCGCttcagctgataaatatgaacc tatgt		
DMDEX20-F	TGTAAAACGACGGCCAGTTGGCTTTCAGATCATTTCTTTC	393	61Cº
DMDEX20-R	CAGGAAACAGCTATGACCAAATACCTATTGATTATGCTCC		
DMDEX21-F	TGTAAAACGACGGCCAGTGCAAAATGTAATGTATGCAAAG	355	63Cº
DMDEX21-R	CAGGAAACAGCTATGACCATGTTAGTACCTTCTGGATTTC		
DMDEX22-F	TGTAAAACGACGGCCAGTAGGAAAACATGGCAAAGTGTG	370	63Cº
DMDEX22-R	CAGGAAACAGCTATGACCTGCTCAATGGGCAAACTACC		
DMDEX23 A-F	TGTAAAACGACGGCCAGTACTCATCAATTATTAttcatcaattagggt	126	61Cº
DMDEX23 A-R	CAGGAAACAGCTATGACCCATCTCTTTCACAGTGGTGC		
DMDEX23 B-F	TGTAAAACGACGGCCAGTAGCAACAAAGTGGCCTATAC	135	61Cº
DMDEX23 B-F	CAGGAAACAGCTATGACCGCTGGGAGGAGAGCTTC		
DMDEX23 C-F	TGTAAAACGACGGCCAGTTTGAAGAAATTGAGGGACGC	175	61Cº
DMDEX23C-R	CAGGAAACAGCTATGACCCTTTACAGTTTACAGTGTATcgttagg		
DMDEX24-F	TGTAAAACGACGGCCAGTTTGGGCCTGTGTTTAGACATA	327	63Cº
DMDEX24-R	CAGGAAACAGCTATGACCAAATCCACCCCAGCTGTAAAA		
DMDEX25-F	TGTAAAACGACGGCCAGTTGTGGCAGTAATTTTTTTCAG	296	61Cº
DMDEX25-R	CAGGAAACAGCTATGACCAGGAAATCTTAGTTAAGTACG		
DMDEX26-F	TGTAAAACGACGGCCAGTTGAGTGTATCTGATCCCCATGA	438	61Cº
DMDEX26-R	CAGGAAACAGCTATGACCTGTTGCATTTCTTTCTTTTTC		
DMDEX27-F	TGTAAAACGACGGCCAGTTGGGATGTTGTGAGAAAGAA	365	63Cº
DMDEX27-R	CAGGAAACAGCTATGACCTGACCATGTATTGACATATCATTGA		
DMDEX28-F	TGTAAAACGACGGCCAGTGAAGTTTTAATAATGAAATGGCaaaa	311	61Cº
DMDEX28-R	CAGGAAACAGCTATGACCTGACCTCTTTTAATACTGCATAT		
DMDEX29-F	TGTAAAACGACGGCCAGTCCAATGTATTTAGAAAAAAAAGGAG	279	63Cº
DMDEX29-R	CAGGAAACAGCTATGACCGCAAATTAGATTAAAGAGAtttttCAC		
DMDEX30-F	TGTAAAACGACGGCCAGTTACAGAAAAGCTATCAAGAG	297	61Cº
DMDEX30-R	CAGGAAACAGCTATGACCAAAAACAAAAGAATGGAAGC		
DMDEX31-F	TGTAAAACGACGGCCAGTATGGTAGAGGTGGTTGAGGA	296	61Cº
DMDEX31-R	CAGGAAACAGCTATGACCTATAATGCCCAACGAAAACA		
DMDEX32-F	TGTAAAACGACGGCCAGTCAGTTATTGTTTGAAAGGCAAA	322	61Cº
DMDEX32-R	CAGGAAACAGCTATGACCCTTCTTAATGAGGAAAGTCAAGG		
DMDEX33-F	CGACGTTGTAAAACGACGGCCAGTTGGAATAGCAATTAAGGG	393	60Cº
DMDEX33-R	CAGGAAACAGCTATGACCGCTAAGACTCTAATCATAC		
DMDEX34-F	TGTAAAACGACGGCCAGTCAGAAATATAAAAGTTCCaaataagtg	374	61Cº
DMDEX34-R	CAGGAAACAGCTATGACCCATGTTAATACTTCCTTACAAAATC		
DMDEX35-F	TGTAAAACGACGGCCAGTCCGTTTCATAAGCATTAAATC	307	61Cº
DMDEX35-R	CAGGAAACAGCTATGACCAGCTTCTAGCCTTTTCTC		
DMDEX36-F	CGACGTTGTAAAACGACGGCCAGTTGTCTAACCAATAATGCcatg	257	64Cº
DMDEX36-R	CAGGAAACAGCTATGACCCTGGTGTACAATTTGGACA		
DMDEX37-F	CGACGTTGTAAAACGACGGCCAGTCTTTCTCACTCTTCTCGctcac	377	61Cº
DMDEX37-R	CAGGAAACAGCTATGACCTTCGCAAGAGACCATTTAGCAC		

DMDEX38-F	TGTAAAACGACGGCCAGTTTTAGCAACAGGAGGTTGAA	267	64Cº
DMDEX38-R	CAGGAAACAGCTATGACCTTCTTTCCAAATATTTATTTCCACT		
DMDEX39-F	TGTAAAACGACGGCCAGTCTCTGTTAACAATGTACAGCTTTTT	365	64Cº
DMDEX39-R	CAGGAAACAGCTATGACCAAAAACCACAGGCAAGGTAT		
DMDEX40-F	TGTAAAACGACGGCCAGTTACAAAAAGATGAGGGAC	387	61Cº
DMDEX40-R	CAGGAAACAGCTATGACCAATAGAAACAAGAACATCAAC		
DMDEX41-F	TGTAAAACGACGGCCAGTGTTAGCTAACTGCCCTGGGccctgtattg	311	61Cº
DMDEX41-R	CAGGAAACAGCTATGACCTAGAGTAGTAGTTGCaaacacatacgtgg		
DMDEX42-F	TGTAAAACGACGGCCAGTATGGAGGAGGTTTCACTGTT	408	61Cº
DMDEX42-R	CAGGAAACAGCTATGACCCCATGTGAAAGTCAAAATGC		
DMDEX43-F	TGTAAAACGACGGCCAGTTTTCTATAGACAGCTAATTCATTTTT	287	63Cº
DMDEX43-R	CAGGAAACAGCTATGACCACAGTTCCCTGAAAACAAATC		
DMDEX44-F	TGTAAAACGACGGCCAGTGTTACTTGAAACTAAACTCTGCaaatg	444	61Cº
DMDEX44-R	CAGGAAACAGCTATGACCACAACAACAGTCAAAAGTAAtttccatc		
DMDEX45-F	TGTAAAACGACGGCCAGTTTCTTTGCCAGTACAACTGC	357	61Cº
DMDEX45-R	CAGGAAACAGCTATGACCTCTGCTAAAATGTTTTCATTCC		
DMDEX46-F	TGTAAAACGACGGCCAGTCCAGTTTGCATTAACAAATAGtttgag	409	64Cº
DMDEX46-R	CAGGAAACAGCTATGACCAGGGTTAAGAAGAAATAAAgttgtgag		
DMDEX47-F	TGTAAAACGACGGCCAGTTGATAGACTAATCAATAGaagcaaagac	399	61Cº
DMDEX47-R	CAGGAAACAGCTATGACCAACAAAACAAAACAACAATccacatacc		
DMDEX48 A-F	TGTAAAACGACGGCCAGTTTTGGCTTATGCCTTGAGAAT	175	61Cº
DMDEX48 A-R	CAGGAAACAGCTATGACCATAACCACAGCAGCAGATG		
DMDEX48 B-F	TGTAAAACGACGGCCAGTGCTTGAAGACCTTGAAGAGC	185	61Cº
DMDEX48 B-R	CAGGAAACAGCTATGACCAAATGAGAAAATTCAGTGATATTGCC		
DMDEX49-F	TGTAAAACGACGGCCAGTGTGCCCTTATGTACCAGGCAGAAATTG	475	61Cº
DMDEX49-R	CAGGAAACAGCTATGACCGCAATGACTCGTTAATAGCCTTAAGATC		
DMDEX50-F	TGTAAAACGACGGCCAGTCACCAAATGGATTAAGATGTTCATGAAT	307	64Cº
DMDEX50-R	CAGGAAACAGCTATGACCTCTCTCTCACCCAGTCATCACTTCATAG		
DMDEX51-F	TGTAAAACGACGGCCAGTGAAATTGGCTCTTTAGCTTGTGTTTC	424	64Cº
DMDEX51-R	CAGGAAACAGCTATGACCGGAGAGTAAAGTGATTGGTGGAAAATC		
DMDEX52-F	TGTAAAACGACGGCCAGTGTGTTTTGGCTGGTCTCACA	298	63Cº
DMDEX52-R	CAGGAAACAGCTATGACCCATGCATCTTGCTTTGTGTGT		
DMDEX53 A-F	TGTAAAACGACGGCCAGTAAGAATCCTGTTGTTCATCATCCTAGC	252	64Cº
DMDEX53 A-R	CAGGAAACAGCTATGACC CCAGCCATTGTGTTGAATCCTTTAAC		
DMDEX53 B-F	TGTAAAACGACGGCCAGT AGTACAAGAACACCTTCAGAACCG	278	64Cº
DMDEX53 B-R	CAGGAAACAGCTATGACCactttacattaaacatcattaaattacaatctatgg		
DMDEX54-F	CGACGTTGTAAAACGACGGCCAGTGTATTCTGACCTGAGGATTC	378	61Cº
DMDEX54-R	CAGGAAACAGCTATGACCCATGGTCCATCCAGTTTC		
DMDEX55-F	TGTAAAACGACGGCCAGTAATTTAGTTCCTCCATCTTTCTCT	445	61Cº
DMDEX55-R	CAGGAAACAGCTATGACCAAATACATCAGGCTGTATAAAAGC		
DMDEX56-F	TGTAAAACGACGGCCAGTATTCTGCACATATTCTTCTTCCTGC	353	63Cº

DMDEX56-R	CAGGAAACAGCTATGACCGGATGATTTACGTAGACATGTGAG		
DMDEX57-F	TGTAAAACGACGGCCAGTCAATGGAATTGTTAGAATCATCA	320	63Cº
DMDEX57-R	CAGGAAACAGCTATGACCCACTGGATTACTATGTGCTTAACAT		
DMDEX58-F	TGTAAAACGACGGCCAGTTTTTGAGAAGAATGCCACAAGCC	315	63Cº
DMDEX58-R	CAGGAAACAGCTATGACCAAAATATGAGAGCTATCCAGACCC		
DMDEX59-F	TGTAAAACGACGGCCAGTAAAGAATGTGGCCTAAAACC	433	64Cº
DMDEX59-R	CAGGAAACAGCTATGACCTTGTGGGAAGATAACACTGC		
DMDEX60-F	TGTAAAACGACGGCCAGTTAAATATTCTCATCTTCCAATTTGC	267	63Cº
DMDEX60-R	CAGGAAACAGCTATGACCTTACTGTAACAAAGGACAACAATG		
DMDEX61A-F	TGTAAAACGACGGCCAGTCGCCCGCCGCttgctttagtgttctcagtcttgg	169	63Cº
DMDEX61A-R	CAGGAAACAGCTATGACCAAAGTCCCTGTGGGCTTCAT		
DMDEX61B-F	TGTAAAACGACGGCCAGTCGTCGAGGACCGAGTCAG	210	63Cº
DMDEX61B-R	CAGGAAACAGCTATGACCCGCCCGCCGCcaggatgatttatgcttctactgc		
DMDEX62-F	TGTAAAACGACGGCCAGTTAATGTTGTCTTTCCTGTTTGCG	221	63Cº
DMDEX62-R	CAGGAAACAGCTATGACCATACAGGTTAGTCACAATAAATGC		
DMDEX63-F	TGTAAAACGACGGCCAGTTACTCATTGTAAATGCTAAAGTC	229	63Cº
DMDEX63-R	CAGGAAACAGCTATGACCTAGCAAGTAACTTTCACACTGC		
DMDEX64-F	TGTAAAACGACGGCCAGTTTCTGATGGAATAACAAATGCT	322	61Cº
DMDEX64-R	CAGGAAACAGCTATGACCCATTCTAGGCAAACTCTAGGC		
DMDEX65-F	TGTAAAACGACGGCCAGTagtgtggttcacgtttggt	386	64Cº
DMDEX65-R	CAGGAAACAGCTATGACCtgtacgctaagcctcctgtg		
DMDEX66-F	TGTAAAACGACGGCCAGTGTCAGTAATTGTTTTCTGCTTTG	246	61Cº
DMDEX66-R	CAGGAAACAGCTATGACCATAAGAACAGTCTGTCATTTCCC		
DMDEX67 A-F	TGTAAAACGACGGCCAGTTCAGGTTCTGCTGGCATC	172	60Cº
DMDEX67 A-R	CAGGAAACAGCTATGACCTGCAACTTCACCCAACTGTC		
DMDEX67 B-F	TGTAAAACGACGGCCAGTGCCTCCTTCTGCATGATT	187	61Cº
DMDEX67 B-R	CAGGAAACAGCTATGACCAGAAAACGAAGCTCTGTGG		
DMDEX68 A-F	TGTAAAACGACGGCCAGTCGCCCGCCcagcctagctttgcaaccat	249	61Cº
DMDEX68 A-R	CAGGAAACAGCTATGACCACTGGGGTTCCAGTCTCATC		
DMDEX68 B-F	TGTAAAACGACGGCCAGTAGCGGCCCTCTTCCTAGACT	236	61Cº
DMDEX68 B-R	CAGGAAACAGCTATGACCCGCCCGC taacagcaactggcacagga		
DMDEX69-F	TGTAAAACGACGGCCAGTGAACGTGGTAGAAGGTTTATTAAA	267	61Cº
DMDEX69-R	CAGGAAACAGCTATGACCCTAACTCTCACGTCAGGCTG		
DMDEX70-F	TGTAAAACGACGGCCAGTTGGTCATTAGTTTTGAAATCATC	273	63Cº
DMDEX70-R	CAGGAAACAGCTATGACCCATCAAACAAGAGTGTGTTCTG		
DMDEX71-F	TGTAAAACGACGGCCAGTGGCTGAGTTTGCGTGTGTCT	174	61Cº
DMDEX71-R	CAGGAAACAGCTATGACCGAGCGAATGTGTTGGTGGTA		
DMDEX72-F	TGTAAAACGACGGCCAGTAAGCATTCTAGGCCATGTGT	261	61Cº
DMDEX72-R	CAGGAAACAGCTATGACCGGTTAGCTTTCCTTGGTTAGTT		
DMDEX73-F	TGTAAAACGACGGCCAGTACGTCACATAAGTTTTAATGAGC	238	63Cº
DMDEX73-R	CAGGAAACAGCTATGACCATGCTAATTCCTATATCCTGTGC		
DMDEX74-F	TGTAAAACGACGGCCAGTATAAGGGGGGGAAAAAAC	290	63Cº
DMDEX74-R	CAGGAAACAGCTATGACCTGCAAGTGTATGCACTCTG		

DMDEX75-F	TGTAAAACGACGGCCAGTTCTTTTTTACTTTTTTGATGC	380	60Cº
DMDEX75-R	CAGGAAACAGCTATGACCAGTGCTCTCTGAGGTTTAG		
DMDEX76 A-F	TGTAAAACGACGGCCAGTacaatctttgggagggcttc	231	63Cº
DMDEX76 A-R	CAGGAAACAGCTATGACCCTGACTGCTGTCGGACCTCT		
DMDEX76 B-F	TGTAAAACGACGGCCAGTCACAACGGTGTCCTCTCCTT	216	63Cº
DMDEX76 B-R	CAGGAAACAGCTATGACCttcagtggctccctgatacc		
DMDEX77-F	TGTAAAACGACGGCCAGTTAATCATGGCCCTTTAATATCTG	306	63Cº
DMDEX77-R	CAGGAAACAGCTATGACCGATACTGCGTGTTGGCTTCC		
DMDEX78-F	TGTAAAACGACGGCCAGTTTCTGATATCTCTGCCTCTTCC	267	61Cº
DMDEX78-R	CAGGAAACAGCTATGACCCATGAGCTGCAAGTGGAGAGG		
DMDEX79 A-F	TGTAAAACGACGGCCAGTAGAGTGATGCTATCTATCTGCAC	385	61Cº
DMDEX79 A-R	CAGGAAACAGCTATGACCTGCATAGACGTGTAAAACCTGCC		
DMDEX79B 1 – F	TGTAAAACGACGGCCAGTTTGTGAAGGGTAGTGGTATTATACTG	323	60Cº
DMDEX79B 1 – R	CAGGAAACAGCTATGACCTGCCTCAAAGTTTTGTGTGTG		
DMDEX79B 2 – F	TGTAAAACGACGGCCAGTCGCCCGCCGAACGCATTTTGGGTTGTTT	284	60Cº
DMDEX79B 2 – R	CAGGAAACAGCTATGACCTCAAATGAGCAGTGTGTAGTAGTCA		
DMDEX79C1-F	TGTAAAACGACGGCCAGTCTTCCTCTACCACCACACCAA	242	60Cº
DMDEX79 C1-R	CAGGAAACAGCTATGACCAAGCAGGTAAGCCTGGATGA		
DMDEX79 C2-F	TGTAAAACGACGGCCAGTTGTTCATGTCACATCCTAATAGAAA	309	60Cº
DMDEX79 C2-R	CAGGAAACAGCTATGACCCGCCCGCCGTAGCAGCAGGAAGCTGAATG		
DMDEX79D 1 – F	TGTAAAACGACGGCCAGTCGCCCGCCGCGAGTAATCGGTTGGTTGGttga	265	60Cº
DMDEX79D 1 -R	CAGGAAACAGCTATGACC TCCTTCACTTAAAGAGTGGCCTA		
DMDEX79D 2 – F	TGTAAAACGACGGCCAGTGCTGGAGGGCTATGGATTC	280	60Cº
DMDEX79D 2 – R	CAGGAAACAGCTATGACCCGCCCGCCGTCACAAATGTGATGGGGCTA		
DMDEX79E –F	TGTAAAACGACGGCCAGTAATAAACTTTGGGAAAAGGTG	536	64Cº
DMDEX79E –R	CAGGAAACAGCTATGACCGAAGCCGTGTTTGATGTTAAT		
DMDEX79F-F	TGTAAAACGACGGCCAGTGAGAGTGGGCTGACATCAA	532	61Cº
DMDEX79F-R	CAGGAAACAGCTATGACCTCACTCCAGAGCTAATGTGTCT		
DMDEX79G-F	TGTAAAACGACGGCCAGTAGTAAGTTTCATTCTAAAATCAGAGG	531	61Cº
DMDEX79G-R	CAGGAAACAGCTATGACCGTGTTTTCACTGTCTTTCTGGA		

Supplementary Table 2 Exon (fragment) number, PCR buffers and $MgCl_2$ concentrations for sequencing.

Fragment #	**Buffer system**	**$MgCl_2$**
5,22,48,53	10x AT(Applied Biosystems)	1.5mM
4,6,7,8,9,10,12,13, 16,17,18,19,20,21, 25,26,29,30 ,32, 35, 36, 37,38,39,40,41,42,44, 45,46,47,49,50,51,54, 55,58,59,60,61,62,64,66,68,69,72,73, 75,76,77,79A,79G	10x ST (Promega)	1.5mM
1,2,23,24,31,33,34,43, 56,57,71,74	10x ST (Promega)	3 mM
3,11,27,28,63,65,67,70,78,79B,79C,79D,79F	5x STR (LDGA)	1.5 mM
14/15,52	5x STR (LDGA)	3 mM

LDGA: Laboratory of Diagnostic Genome Analysis

Supplementary Table 3 Primer sequences (with M13 tail) that were used for the amplification of DMD amplicons for sequencing.

Amplicon number	Primer sequences
DMDEX01-F4	TGTAAAACGACGGCCAGTGCAGGTCCTGGAATTTGA
DMDEX01-R4	CAGGAAACAGCTATGACCCAAACTAAACGTTATGCCACA
DMDEX02-F5	TGTAAAACGACGGCCAGTCACTAACACATCATAATGG
DMDEX02-R2	CAGGAAACAGCTATGACCGATACACAGGTACATAGTC
DMDEX03-F5	TGTAAAACGACGGCCAGTTCATCCGTCATCTTCGGCAGATTAA
DMDEX03-R4	CAGGAAACAGCTATGACCCAGGCGGTAGAGTATGCCAAATGAAAATCA
DMDEX04-F3	TGTAAAACGACGGCCAGTTTGTCGGTCTCTCTGCTGGTCAGTG
DMDEX04-R2	CAGGAAACAGCTATGACCCCAAAGCCCTCACTCAAAC
DMDEX05-F3	TGTAAAACGACGGCCAGTCAACTAGGCATTTGGTCTC
DMDEX05-R3	CAGGAAACAGCTATGACCTTGTTTCACACGTCAAGGG
DMDEX06-F6	TGTAAAACGACGGCCAGTTGGTTCTTGCTCAAGGAATG
DMDEX06-R6	CAGGAAACAGCTATGACCTGGGGAAAAATATGTCATCAG
DMDEX07-F3	TGTAAAACGACGGCCAGTCTATGGGCATTGGTTGTC
DMDEX07-R3	CAGGAAACAGCTATGACCAAAAGCAGTGGTAGTCCAG
DMDEX08-F5	TGTAAAACGACGGCCAGTTCGTCTTCCTTTAACTTTG
DMDEX08-R5	CAGGAAACAGCTATGACCTCTTGAATAGTAGCTGTCC
DMDEX09-F4	TGTAAAACGACGGCCAGTTCTATCCACTCCCCCAAACC
DMDEX09-R4	CAGGAAACAGCTATGACCAACAAACCAGCTCTTCAC
DMDEX10-F1	CGACGTTGTAAAACGACGGCCAGTGGAACAATCTGCAAAGAC
DMDEX10-R1	CAGGAAACAGCTATGACCAAAGGATGACTTGCCATTATAAC
DMDEX11-F5	TGTAAAACGACGGCCAGTCAAATAAAACTCAAAACCACACC
DMDEX11-R3	CAGGAAACAGCTATGACCCTTCCAAAACTTGTTAGTCTTC
DMDEX12-F2	TGTAAAACGACGGCCAGTCTTTCAAAGAGGTCATAATAGG

DMDEX12-R2	CAGGAAACAGCTATGACCCATCTGTGTTACTGTGTATAGG
DMDEX13-F3	TGTAAAACGACGGCCAGTGCAAATCATTTCAACACAC
DMDEX13-R3	CAGGAAACAGCTATGACCTCTTTAAATCACAGCACTTC
DMDEX14-F2	TGTAAAACGACGGCCAGTTGGCAAATTATTCATGCCATT
DMDEX14-R2	CAGGAAACAGCTATGACCTGATCCAAGCAAAAATAAACATT
DMDEX16-F3	TGTAAAACGACGGCCAGTATGCAACCCAGGCTTATTC
DMDEX16-R3	CAGGAAACAGCTATGACCCTGTAGCATGATAATTGGTATCAC
DMDEX17-F6	TGTAAAACGACGGCCAGTTTTTCCTTTGCCACTCCAAG
DMDEX17-R4	CAGGAAACAGCTATGACCCACCACCAACAAAACTGCTG
DMDEX18-F1	CGACGTTGTAAAACGACGGCCAGTTGTCAGGCAGGAGTCTCAGAT
DMDEX18-R1	CAGGAAACAGCTATGACCCGGAGTTTACAAGCAGCACA
DMDEX19-F4	TGTAAAACGACGGCCAGTGATGGCAAAAGTGTTGAGAAAAAGTC
DMDEX19-R4	CAGGAAACAGCTATGACCTTCTACCACATCCCATTTTCTTCCA
DMDEX20-F2	TGTAAAACGACGGCCAGTTGGCTTTCAGATCATTTCTTTC
DMDEX20-R2	CAGGAAACAGCTATGACCAAATACCTATTGATTATGCTCC
DMDEX21-F3	TGTAAAACGACGGCCAGTGCAAAATGTAATGTATGCAAAG
DMDEX21-R3	CAGGAAACAGCTATGACCATGTTAGTACCTTCTGGATTTC
DMDEX22-F2	TGTAAAACGACGGCCAGTAGGAAAACATGGCAAAGTGTG
DMDEX22-R2	CAGGAAACAGCTATGACCTGCTCAATGGGCAAACTACC
DMDEX23-F3	TGTAAAACGACGGCCAGTTCATCTACTTTGTTTACATGTTTGAA
DMDEX23-R3	CAGGAAACAGCTATGACCACAGTGTATCGTTAGGGAAAAA
DMDEX24-F4	TGTAAAACGACGGCCAGTTTGGGCCTGTGTTTAGACATA
DMDEX24-R3	CAGGAAACAGCTATGACCAAATCCACCCCAGCTGTAAAA
DMDEX25-F2	TGTAAAACGACGGCCAGTTGTGGCAGTAATTTTTTTCAG
DMDEX25-R2	CAGGAAACAGCTATGACCAGGAAATCTTAGTTAAGTACG
DMDEX26-F3	TGTAAAACGACGGCCAGTTGAGTGTATCTGATCCCCATGA
DMDEX26-R1	CAGGAAACAGCTATGACCTGTTGCATTTCTTTCTTTTTC
DMDEX27-F2	TGTAAAACGACGGCCAGTTGGGATGTTGTGAGAAAGAA
DMDEX27-R4	CAGGAAACAGCTATGACCTGACCATGTATTGACATATCATTGA
DMDEX28-F2	TGTAAAACGACGGCCAGTGAAGTTTTAATAATGAAATGGCAAAA
DMDEX28-R3	CAGGAAACAGCTATGACCTGACCTCTTTTAATACTGCATAT
DMDEX29-F4	TGTAAAACGACGGCCAGTCCAATGTATTTAGAAAAAAAAGGAG
DMDEX29-R5	CAGGAAACAGCTATGACCGCAAATTAGATTAAAGAGATTTTTCAC
DMDEX30-F4	TGTAAAACGACGGCCAGTTACAGAAAAGCTATCAAGAG
DMDEX30-R3	CAGGAAACAGCTATGACCAAAAACAAAAGAATGGAAGC
DMDEX31-F2	TGTAAAACGACGGCCAGTATGGTAGAGGTGGTTGAGGA
DMDEX31-R2	CAGGAAACAGCTATGACCTATAATGCCCAACGAAAACA
DMDEX32-F2	TGTAAAACGACGGCCAGTCAGTTATTGTTTGAAAGGCAAA
DMDEX32-R2	CAGGAAACAGCTATGACCCTTCTTAATGAGGAAAGTCAAGG
DMDEX33-F1	CGACGTTGTAAAACGACGGCCAGTTGGAATAGCAATTAAGGG
DMDEX33-R1	CAGGAAACAGCTATGACCGCTAAGACTCTAATCATAC
DMDEX34-F3	TGTAAAACGACGGCCAGTCAGAAATATAAAAGTTCCAAATAAGTG
DMDEX34-R3	CAGGAAACAGCTATGACCCATGTTAATACTTCCTTACAAAATC

DMDEX35-F2	TGTAAAACGACGGCCAGTCCGTTTCATAAGCATTAAATC
DMDEX35-R3	CAGGAAACAGCTATGACCAGCTTCTAGCCTTTTCTC
DMDEX36-F1	CGACGTTGTAAAACGACGGCCAGTTGTCTAACCAATAATGCCATG
DMDEX36-R1	CAGGAAACAGCTATGACCCTGGTGTACAATTTGGACA
DMDEX37-F1	CGACGTTGTAAAACGACGGCCAGTCTTTCTCACTCTTCTCGCTCAC
DMDEX37-R1	CAGGAAACAGCTATGACCTTCGCAAGAGACCATTTAGCAC
DMDEX38-F3	TGTAAAACGACGGCCAGTTTTAGCAACAGGAGGTTGAA
DMDEX38-R3	CAGGAAACAGCTATGACCTTCTTTCCAAATATTTATTTCCACT
DMDEX39-F3	TGTAAAACGACGGCCAGTCTCTGTTAACAATGTACAGCTTTTT
DMDEX39-R3	CAGGAAACAGCTATGACCAAAAACCACAGGCAAGGTAT
DMDEX40-F2	TGTAAAACGACGGCCAGTTACAAAAAGATGAGGGAC
DMDEX40-R2	CAGGAAACAGCTATGACCAATAGAAACAAGAACATCAAC
DMDEX41-F2	TGTAAAACGACGGCCAGTGTTAGCTAACTGCCCTGGGCCCTGTATTG
DMDEX41-R2	CAGGAAACAGCTATGACCTAGAGTAGTAGTTGCAAACACATACGTGG
DMDEX42-F3	TGTAAAACGACGGCCAGTATGGAGGAGGTTTCACTGTT
DMDEX42-R3	CAGGAAACAGCTATGACCCCATGTGAAAGTCAAAATGC
DMDEX43-F6	TGTAAAACGACGGCCAGTTTTCTATAGACAGCTAATTCATTTTT
DMDEX43-R6	CAGGAAACAGCTATGACCACAGTTCCCTGAAAACAAATC
DMDEX44-F5	TGTAAAACGACGGCCAGTGTTACTTGAAACTAAACTCTGCAAATG
DMDEX44-R2	CAGGAAACAGCTATGACCACAACAACAGTCAAAAGTAATTTCCATC
DMDEX45-F5	TGTAAAACGACGGCCAGTTTCTTTGCCAGTACAACTGC
DMDEX45-R4	CAGGAAACAGCTATGACCTCTGCTAAAATGTTTTCATTCC
DMDEX46-F4	TGTAAAACGACGGCCAGTCCAGTTTGCATTAACAAATAGTTTGAG
DMDEX46-R4	CAGGAAACAGCTATGACCAGGGTTAAGAAGAAATAAAGTTGTGAG
DMDEX47-F4	TGTAAAACGACGGCCAGTTGATAGACTAATCAATAGAAGCAAAGAC
DMDEX47-R4	CAGGAAACAGCTATGACCAACAAAACAAAACAACAATCCACATACC
DMDEX48-F4	TGTAAAACGACGGCCAGTTTGAATACATTGGTTAAATCCCAACATG
DMDEX48-R4	CAGGAAACAGCTATGACCCCTGAATAAAGTCTTCCTTACCACAC
DMDEX49-F4	TGTAAAACGACGGCCAGTGTGCCCTTATGTACCAGGCAGAAATTG
DMDEX49-R4	CAGGAAACAGCTATGACCGCAATGACTCGTTAATAGCCTTAAGATC
DMDEX50-F3	TGTAAAACGACGGCCAGTCACCAAATGGATTAAGATGTTCATGAAT
DMDEX50-R2	CAGGAAACAGCTATGACCTCTCTCTCACCCAGTCATCACTTCATAG
DMDEX51-F3	TGTAAAACGACGGCCAGTGAAATTGGCTCTTTAGCTTGTGTTTC
DMDEX51-R3	CAGGAAACAGCTATGACCGGAGAGTAAAGTGATTGGTGGAAAATC
DMDEX52-F4	TGTAAAACGACGGCCAGTGTGTTTTGGCTGGTCTCACA
DMDEX52-R4	CAGGAAACAGCTATGACCCATGCATCTTGCTTTGTGTGT
DMDEX53-F3	TGTAAAACGACGGCCAGTTCCTCCAGACTAGCATTTAC
DMDEX53-R3	CAGGAAACAGCTATGACCTTAGCCTGGGTGACAGTG
DMDEX54-F2	CGACGTTGTAAAACGACGGCCAGTGTATTCTGACCTGAGGATTC
DMDEX54-R2	CAGGAAACAGCTATGACCCATGGTCCATCCAGTTTC
DMDEX55-F3	TGTAAAACGACGGCCAGTAATTTAGTTCCTCCATCTTTCTCT
DMDEX55-R7	CAGGAAACAGCTATGACCAAATACATCAGGCTGTATAAAAGC
DMDEX56-F2	TGTAAAACGACGGCCAGTATTCTGCACATATTCTTCTTCCTGC

DMDEX56-R2	CAGGAAACAGCTATGACCGGATGATTTACGTAGACATGTGAG
DMDEX57-F2	TGTAAAACGACGGCCAGTCAATGGAATTGTTAGAATCATCA
DMDEX57-R2	CAGGAAACAGCTATGACCCACTGGATTACTATGTGCTTAACAT
DMDEX58-F4	TGTAAAACGACGGCCAGTTTTTGAGAAGAATGCCACAAGCC
DMDEX58-R4	CAGGAAACAGCTATGACCAAAATATGAGAGCTATCCAGACCC
DMDEX59-F6	TGTAAAACGACGGCCAGTAAAGAATGTGGCCTAAAACC
DMDEX59-R6	CAGGAAACAGCTATGACCTTGTGGGAAGATAACACTGC
DMDEX60-F3	TGTAAAACGACGGCCAGTTAAATATTCTCATCTTCCAATTTGC
DMDEX60-R3	CAGGAAACAGCTATGACCTTACTGTAACAAAGGACAACAATG
DMDEX61-F3	TGTAAAACGACGGCCAGTCATTGTTTTAATTGTTCCTCATT
DMDEX61-R3	CAGGAAACAGCTATGACCTTCAACTCTTAATTCTTTTGTTTTT
DMDEX62-F4	TGTAAAACGACGGCCAGTTAATGTTGTCTTTCCTGTTTGCG
DMDEX62-R4	CAGGAAACAGCTATGACCATACAGGTTAGTCACAATAAATGC
DMDEX63-F3	TGTAAAACGACGGCCAGTTACTCATTGTAAATGCTAAAGTC
DMDEX63-R3	CAGGAAACAGCTATGACCTAGCAAGTAACTTTCACACTGC
DMDEX64-F2	TGTAAAACGACGGCCAGTTTCTGATGGAATAACAAATGCT
DMDEX64-R2	CAGGAAACAGCTATGACCCATTCTAGGCAAACTCTAGGC
DMDEX65-F4	TGTAAAACGACGGCCAGTGGTTTTACTCTTTGAGTCATTTGT
DMDEX65-R4	CAGGAAACAGCTATGACCTACGCTAAGCCTCCTGTGAC
DMDEX66-F5	TGTAAAACGACGGCCAGTGTCAGTAATTGTTTTCTGCTTTG
DMDEX66-R3	CAGGAAACAGCTATGACCATAAGAACAGTCTGTCATTTCCC
DMDEX67-F3	TGTAAAACGACGGCCAGTGAAGTAACCCCACTCTGTGGAA
DMDEX67-R2	CAGGAAACAGCTATGACCAAACGAAGCTCTGTGGGTTT
DMDEX68-F1	TGTAAAACGACGGCCAGTTAATCGAACTGATATACACCTCC
DMDEX68-R1	CAGGAAACAGCTATGACCACTAACAGCAACTGGCACAGG
DMDEX69-F3	TGTAAAACGACGGCCAGTGAACGTGGTAGAAGGTTTATTAAA
DMDEX69-R3	CAGGAAACAGCTATGACCCTAACTCTCACGTCAGGCTG
DMDEX70-F3	TGTAAAACGACGGCCAGTTGGTCATTAGTTTTGAAATCATC
DMDEX70-R3	CAGGAAACAGCTATGACCCATCAAACAAGAGTGTGTTCTG
DMDEX71-F5	TGTAAAACGACGGCCAGTGGCTGAGTTTGCGTGTGTCT
DMDEX71-R3	CAGGAAACAGCTATGACCGAGCGAATGTGTTGGTGGTA
DMDEX72-F3	TGTAAAACGACGGCCAGTAAGCATTCTAGGCCATGTGT
DMDEX72-R3	CAGGAAACAGCTATGACCGGTTAGCTTTCCTTGGTTAGTT
DMDEX73-F2	TGTAAAACGACGGCCAGTACGTCACATAAGTTTTAATGAGC
DMDEX73-R2	CAGGAAACAGCTATGACCATGCTAATTCCTATATCCTGTGC
DMDEX74-F1	TGTAAAACGACGGCCAGTATAAGGGGGGGAAAAAAC
DMDEX74-R1	CAGGAAACAGCTATGACCTGCAAGTGTATGCACTCTG
DMDEX75-F1	TGTAAAACGACGGCCAGTTCTTTTTTACTTTTTTGATGC
DMDEX75-R1	CAGGAAACAGCTATGACCAGTGCTCTCTGAGGTTTAG
DMDEX76-F4	TGTAAAACGACGGCCAGTGGGTCAAAATTTATGAGTCCTG
DMDEX76-R3	CAGGAAACAGCTATGACCTTCATGTCCCTGTAATACGACT
DMDEX77-F1	TGTAAAACGACGGCCAGTTAATCATGGCCCTTTAATATCTG
DMDEX77-R1	CAGGAAACAGCTATGACCGATACTGCGTGTTGGCTTCC

DMDEX78-F2	TGTAAAACGACGGCCAGTTTCTGATATCTCTGCCTCTTCC
DMDEX78-R3	CAGGAAACAGCTATGACCCATGAGCTGCAAGTGGAGAGG
DMDEX79-F2	TGTAAAACGACGGCCAGTAGAGTGATGCTATCTATCTGCAC
DMDEX79-R2	CAGGAAACAGCTATGACCTGCATAGACGTGTAAAACCTGCC
DMDEX79-F3	TGTAAAACGACGGCCAGTATTTTTGTGAAGGGTAGTGGT
DMDEX79-R3	CAGGAAACAGCTATGACCGAAAAAGTCAGTCTATAGAAATTCG
DMDEX79-F4	TGTAAAACGACGGCCAGTCCACCACACCAAATGACTAC
DMDEX79-R4	CAGGAAACAGCTATGACCATCTAAATCGTGGCATTGCT
DMDEX79-F5	TGTAAAACGACGGCCAGTAGTAATCGGTTGGTTGGTTG
DMDEX79-R5	CAGGAAACAGCTATGACCAACACAGTTCATGGGCTTCT
DMDEX79-F6	TGTAAAACGACGGCCAGTAATAAACTTTGGGAAAAGGTG
DMDEX79-R6	CAGGAAACAGCTATGACCGAAGCCGTGTTTGATGTTAAT
DMDEX79-F7	TGTAAAACGACGGCCAGTGAGAGTGGGCTGACATCAA
DMDEX79-R7	CAGGAAACAGCTATGACCTCACTCCAGAGCTAATGTGTCT
DMDEX79-F8	TGTAAAACGACGGCCAGTAGTAAGTTTCATTCTAAAATCAGAGG
DMDEX79-R8	CAGGAAACAGCTATGACCGTGTTTTCACTGTCTTTCTGGA