Defining the performance parameters of a rapid screening tool for myotonic dystrophy type 1 based on triplet-primed PCR and melt curve analysis

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Abstract

Background: DMPK CTG-repeat expansions that cause myotonic dystrophy type 1 (DM1) can be detected more rapidly, cost-effectively, and simply by combining triplet-primed PCR (TP-PCR) with melting curve analysis (MCA). We undertook a detailed technical validation study to define the optimal operational parameters for performing bidirectional TP-PCR MCA assays.

Methods: We determined the assays’ analytic specificity and sensitivity, assessed the effect of reaction volumes, DNA diluents, and common contaminants on melt peak temperature, determined the assays’ sensitivity in detecting low-level mosaicism for repeat expansion, and evaluated their performance on two real-time PCR platforms.

Results: Both assays were highly specific and sensitive, and performed optimally under a broad range of parameters. Bidirectional TP-PCR MCA analysis also reduces the risk of generating false-negative results associated with the rare CCG-interruptions that may be present at either end of expanded alleles.

Conclusion: The DMPK TP-PCR MCA is a highly specific, sensitive, and significantly cost-saving screening tool for DM1.

1. Introduction

Myotonic dystrophy type 1 (DM1) is the most common adult-onset muscular dystrophy, with an overall worldwide prevalence of approximately 1 in 20,000 or much higher at 1 in 3000 in Finnish population and 1 in 500 among founder population in Quebec [1–3]. It is autosomal dominantly inherited and is a progressive disorder exhibiting myotonia, muscle weakness, cardiac arrhythmia, respiratory failure, and cataracts, among others [3]. DM1 is caused by presence of an expanded CTG-repeat (≥50 repeats) in the 3′ untranslated region of the dystrophia myotonica protein kinase (DMPK) gene located on chromosome 19q13.3 [4–6]. DM1-unaffected individuals carry 5–34 repeats, while carriers of a premutation allele (35–49 repeats) are asymptomatic but risk conceiving DM1-affected children [7,8]. Affected individuals with 50–150 repeats have mild phenotypes, whereas those with 100–1000 and >1000 repeats present with classic DM1 phenotypes and congenital DM1, respectively [1].

The wide clinical phenotypes exhibited by DM1 and their similarities with other genetically distinct disorders [9,10] necessitate molecular confirmation of the disease. The diagnostic strategy for DM1 has in the past been the use of PCR to detect small alleles and Southern blot analysis to estimate the size of the larger ones [11–13]. However, a more sensitive yet less labor-intensive triplet-primed PCR (TP-PCR) method developed by Warner et al. [14] has reliably been utilized to detect the presence of the CTG expansion. Most TP-PCR products are evaluated for their sizes using capillary electrophoresis, which becomes expensive when used to analyze large numbers of samples. We recently proposed a more economical one-step rapid screen for DM1 using TP-PCR followed by melting curve analysis (MCA), where the higher melt peak temperatures (TmS) of DM1 samples clearly distinguished them from non-DM1 samples, which displayed comparatively lower TmS [15].

Nonetheless, in order for TP-PCR MCA to be used confidently as a screening tool, it is necessary to identify the optimal parameters and tolerance ranges of the assay as well as external and internal factors that could potentially lead to false-positive or false-negative genotype classification of samples. In this study, we investigated the assays’ specificity for the DMPK CTG-repeat locus, examined the effect of input DNA, reaction volumes, DNA diluents, and common DNA contaminants on the Tm, determined the assays’ sensitivity in detecting low-level mosaicism for...
expanded alleles, and evaluated their performance on two real-time PCR platforms.

2. Methods

2.1. DNA samples

Fifteen lymphoblastoid cell lines and three genomic DNA samples obtained from Coriell Cell Repository (Coriell Institute for Medical Research, Camden, NJ, USA) (Table 1) were used in this study. Samples carrying other trinucleotide repeat expansions were used to evaluate locus specificity of the assay. GM16206 and GM05164 were used to test the effect of input DNA, PCR reaction volume, DNA diluents (HPLC grade water or 10 mM Tris, 1 mM EDTA, pH 8.0 (1× TE) buffer), and impurities (glycogen and salt) on Tm.

To generate model mixtures mimicking mosaicism for the full penetrance allele, GM07426 (12/13 CTGs) and GM03989 (13/>180 CTGs) DNAs were mixed in different proportions to achieve 1–50% of full penetrance allele per 100 ng DNA. To generate model mixtures containing 1–50% of the premutation allele, AC1080 (12/48 CTGs) was mixed with GM07426 DNA. Model mixtures containing different percentages of non-disease-associated allele, premutation, and full penetrance allele, AC1080 (12/48 CTGs) was mixed with GM07426 DNA. Model mixtures containing different percentages of non-disease-associated allele, premutation, and full penetrance repeat-containing plasmids were prepared by combining pDMPK(CTG)12, pDMPK(CTG)48, and pDMPK(CTG)77 [15], with total plasmid DNA maintained at 0.5 pg. Plasmids pDMPK(CTG)35 and pDMPK(CTG)48 [15] were used to indicate threshold temperatures that separate melt peaks of DM1 and non-DM1 samples. Plasmid pDMPK(CTG)48 was also employed to assess well-to-well Tm uniformity in two real-time PCR platforms.

In addition to the Coriell samples, a DM1 sample from the KK Women’s and Children’s Hospital carrying an expansion of approximately 400–480 CTGs and a DM1 sample from the 2012 European Molecular Genetics Quality Network (EMQN) external quality assessment scheme carrying approximately 400–500 CTGs were used to characterize the effect of 5′ and 3′ CCG-interruptions, respectively. Purified samples (~10–20 ng) were bidirectionally sequenced, using 3.2 pmol of a modified P2 primer (12 nucleotides shorter from the 3′ end) and the modified 3′ R primer. The EMQN sample produced multiple amplification fragments suggestive of somatic heterogeneity of the expanded allele (data not shown). The strongest expanded allele band was isolated and sequenced. Sequencing was performed in the presence of 2× Q-solution (Qiagen), with annealing temperature at 60°C. Nucleotide sequences were correlated to the corresponding TP-PCR capillary electrophoretic peak patterns.

This study was approved by the National University of Singapore Institutional Review Board (Ref: 07-123E and 13-309E) and the SingHealth Centralised Institutional Review Board (Ref: 2013/073/A).

2.2. TP-PCR and MCA

The 5′ and 3′ TP-PCR MCA assays were performed separately using genomic and/or plasmid DNAs on LightCycler 480 Real-Time PCR System (LC480, Roche Diagnostics, Mannheim, Germany) as previously described [15]. Unless otherwise stated, all TP-PCR MCA assays were performed in 15 µL reaction volume with 100-ng genomic DNA or 0.5-pg plasmid DNA. To investigate the effect of reaction volume on Tm, 900 ng of genomic DNA was added to 135 µL of PCR master mix to obtain a DNA concentration of 6.67 ng/µL and 5, 10, 15, 20, 25, and 50 µL aliquots of the master mix were subjected to TP-PCR MCA. To examine the effect of common DNA diluents on Tm, template DNA was diluted in HPLC-purified water or in 1× TE buffer. Varying concentrations of up to 1.33 µg/µL of glycogen (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) and up to 100 mM sodium acetate, pH 5.2 (Sigma-Aldrich, St. Louis, MO, USA) were added to TP-PCR MCA reactions to examine their effect on Tm. For the Tm, uniformity study, 0.5 pg of pDMPK(CTG)48 DNA was assayed in both LC480 and Rotor-Gene Q (RGQ, Qiagen, Hilden, Germany) instruments. MCA on the RGQ utilized the HRM module which involved a 1-min denaturation step at 95°C, followed by temperature ramping from 65 to 95°C, increasing by 0.5°C every 5 s.

2.3. Primer extension and capillary electrophoresis

TP-PCR product labeling using fluorescently tagged primers and subsequent capillary electrophoresis were performed as previously described [15].

2.4. Repeat-flanking PCR and sequencing

Repeat-flanking PCR was performed on 100 ng DNA of a DM1 clinical sample from KK Women’s and Children’s Hospital and a DM1 sample from the 2012 EMQN proficiency testing exercise, using 0.6 µmol/L forward primer P2 [14] and 0.6 µmol/L of a modified reverse primer 3′ R [15] (7 nucleotides shorter from the 3′ end). Thermocycling was performed in a GeneAmp PCR System 9700 (Applied Biosystems-Thermo Fisher Scientific), which involved a 15-min enzyme activation at 95°C, 30 rounds of 98°C for 1 min, 60°C for 1 min, and 72°C for 6 min with 6-s increment per cycle, and a 10-min final extension at 72°C. PCR products were resolved on a 1% agarose gel in 1× TBE buffer, a band corresponding to the expanded allele was excised and purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Pte Ltd, Singapore), and the purified DNA was sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Purified samples (~10–20 ng) were bidirectionally sequenced, using 3.2 pmol of a modified P2 primer (12 nucleotides shorter from the 3′ end) and the modified 3′ R primer. The EMQN sample produced multiple amplification fragments suggestive of somatic heterogeneity of the expanded allele (data not shown). The strongest expanded allele band was isolated and sequenced. Sequencing was performed in the presence of 2× Q-solution (Qiagen), with annealing temperature at 60°C. Nucleotide sequences were correlated to the corresponding TP-PCR capillary electrophoretic peak patterns.

3. Results

3.1. Locus specificity of the DMPK TP-PCR MCA assay

To demonstrate that the TP-PCR primers anneal specifically to the DMPK CTG-repeat without cross-annealing to other trinucleotide repeat loci, 5′ and 3′ TP-PCR MCA assays were performed on DNA samples carrying expanded alleles of the Friedreich ataxia (FRDA) FXN GAA-repeat, fragile X syndrome (FXS) FMR1 CGG-repeat, and Huntington disease (HD) HTT CAG-repeat (Table 1). Figure 1 illustrates the TP-PCR primer annealing sites at the DMPK CTG repeat locus, the expected
Table 1. Cell line samples from the CCR that were used in this study.

| No. | Sample ID | Genotype information | Tm (°C) |
|-----|-----------|----------------------|---------|
|     |           | DMKP (CTG)n: N~2000 | 87.58/87.02 |
|     |           | DMKP (CTG)n: 21~340 | 87.30/86.74 |
|     |           | DMKP (CTG)n: 21/377 (±53) | 87.30/86.74 |
|     |           | DMKP (CTG)n: 12/5670 (±0.9) | 85.91/85.91 |
|     |           | β-thalassemia compound heterozygote | 81.49/81.49 |
|     |           | FXN (GAA)n: ~460/~670 | 81.48/81.48 |
|     |           | FXN (GAA)n: not determined | 81.48/81.48 |
|     |           | DMKP (CTG)n: 29/85 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 29/91 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 20/183~193 | 81.48/81.48 |
|     |           | DMKP (CTG)n: >200 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 501~550 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 11/14 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 11/12 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 5/11 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 5/13 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 34/200 | 81.20/81.20 |
|     |           | DMKP (CTG)n: 17/175 | 81.48/81.48 |
|     |           | DMKP (CTG)n: 22/101 | 81.20/81.48 |
|     |           | DMKP (CTG)n: 15/29 | 81.20/81.48 |
|     |           | DMKP (CTG)n: 18/31 | 81.48/81.48 |

CCR: Coriell Cell Repositories; N: normal; FXN: frataxin gene; DMKP: dystrophia myotonica-protein kinase gene; FMR1: fragile X mental retardation 1 gene; HTT: huntingtin gene.

3.2. Analytic sensitivity of the DMKP TP-PCR MCA assay

We performed both 5’ and 3’ TP-PCR MCA assays on 1, 5, 10, 20, 50, 100, 200, 500 ng, and 1 μg DNA from GM16206 (12/14 CTGs) and GM05164 (21/>180 CTGs). DNA as low as 5 ng and up to 500 ng generated defined melt peaks that did not differ significantly in Tm, while 1-ng DNA produced a weak melt profile and 1 μg produced a right-shifted melt peak with higher Tm (Figure 2(b)). Nonetheless, both samples were segregated into two clusters, with two markedly different Tm ranges consistent with their DMKP expansion status. These results imply that a wide range of input DNA can be used, minimizing the need to dilute all samples to a narrow working concentration.

3.3. Effect of reaction volume variation on Tm

To examine the effect of assay reaction volumes, GM16206 and GM05164 DNAs were assayed in 5, 10, 15, 20, 25, and 50 μL reaction volumes with DNA concentration held constant at 6.67 ng/μL. The lowest reaction volume (5 μL) produced Tm,s ranging from 80.93 to 81.48°C (Figure 2(a)). The absence of both CTG-repeat expansion and cross-amplification of other trinucleotide repeat loci in these samples by the DMKP TP-PCR primers was confirmed by capillary electrophoresis of the TP-PCR products, which showed the presence of only non-disease associated DMKP alleles (Table 1). These observations confirm the specificity of both 5’ and 3’ TP-PCR assays in amplifying only the CTG-repeat of the DMKP locus.

3.4. Effect of common reagents on Tm

To determine if DNA diluent could significantly affect Tm, GM16206 and GM05164 DNAs were diluted in either HPLC H2O or 1× TE buffer. No obvious Tm differences were observed between samples diluted in the two diluents (Figure 2(d)). As for the effect on Tm of common reagents used in DNA precipitation, GM16206 was tested in the presence of 0.33, 0.67, 1, and 1.33 μg/μL glycogen and 1, 10, 50, and 100 mM sodium acetate. Glycogen had negligible effect on the Tm of both 5’ and 3’ TP-PCR amplicons (Figure 3(a)). In marked contrast,
Figure 1. Schematic of DMPK 5’ TP-PCR showing positions of the primers (a), capillary electropherogram of samples from DM1-unaffected and affected individuals (b), and the basis of MCA of shorter (c) and longer (d) TP-PCR products.
Figure 2. Melt peaks of 5’ and 3’ TP-PCR products of DM1 and non-DM1 Coriell samples (including Huntington disease (HD), Friedreich ataxia (FRDA), and fragile X syndrome (FXS) samples) (a), using different amounts of input DNA (b), assayed in different reaction volumes (c), and using DNA template diluted in different diluents (d). Grey peaks which form grey-shaded vertical bars represent the melt profiles of control plasmids pDMPK(CTG)35 and pDMPK(CTG)48, which established threshold temperatures to separate non-DM1 from DM1 samples. -dF/dT, negative first derivative of fluorescence versus temperature.
Figure 3. Melt peaks of 5' and 3' TP-PCR products of GM16206 (12 and 14 CTGs) when different concentrations of glycogen (a) and sodium acetate (b) were added into the reactions. The grey-shaded vertical bars, established by control plasmids pDMPK((CTG)35) and pDMPK((CTG)48), separate non-DM1 from DM1 samples. C, Well-to-well T<sub>m</sub> uniformity of the assay performed in Roche LC480 and Qiagen RGQ platforms (T<sub>m</sub> shown as mean±SD). -dF/dT, negative first derivative of fluorescence versus temperature.
increasingly right-shifted melt peaks were observed in the presence of increasing concentrations of sodium acetate between 10 and 50 mM (Figure 3(b)), and the reaction failed completely in the presence of 100 mM of sodium acetate. Thus, the presence of excess sodium acetate in a reaction could lead to misclassification of a non-DM1 sample as DM1-affected.

3.5. Instrument well-to-well \( T_m \) uniformity

To examine well-to-well \( T_m \) uniformity of different real-time PCR platforms, we performed identical 5’ TP-PCR MCA reactions in all 96 positions of the LC480 block. Similarly, we performed 24 identical 5’ TP-PCR MCA reactions in the RGQ instrument, equally distributing six 4-tube strips within the 72-slot rotor. Plasmid pDMPK(CTG)\(_{18}\) was used as template. The LC480 MCA generated three different \( T_m \)s ranging from 84.52 to 85.07°C, while the RGQ HRM produced two \( T_m \)s of 84.92 and 85.08°C (Figure 3(c)). The coefficient of variation of the average \( T_m \) of the LC480 and RGQ replicates were, however, very similar at 0.11% and 0.09%, respectively. It should be noted that while the amount of plasmid DNA used in this experiment (0.5 pg) was approximately five orders of magnitude less than that of genomic DNA (100 ng), the number of copies of repeat-containing template present in 0.5 pg of plasmid is approximately one order of magnitude more than the copies of repeat present in 100 ng of genomic DNA.

3.6. Influence of CCG-interruptions on TP-PCR MCA performance

To assess the effect of interruptions within the CTG-repeat on 5’ and 3’ TP-PCR melt peak profiles, we performed a comparative analysis of a typical DM1 sample carrying an uninterrupted ~2000 CTG-repeat allele (GM03989), a DM1 sample carrying a ~520-CTG-repeat with tandem CCG-interruptions toward the 3’ end, and a DM1 sample carrying a ~400–480-CTG-repeat with interspersed CCG-interruptions toward the 5’ end. We performed 5’ and 3’ TP-PCR MCAs on each sample and also took aliquots of the products to perform extension labeling as described [15]. For the samples with interrupted electrophoretic peaks, we also performed repeat-flanking PCR on the genomic DNA, gel-purified the expanded allele band, and sequenced the expanded alleles bidirectionally using modified P2 and modified 3’ R primers.

For the sample without interruptions, both 5’ and 3’ TP-PCR MCA produced a strong melt peak in the expanded range with \( T_m \)s of 87.58 and 87.02°C, respectively (Figure 4(a)). The corresponding capillary electrophoresis results show continuous peaks that could be counted up to 180 repeats.

For the sample with 3’ interruptions, the 5’ TP-PCR MCA produced a strong melt peak with a \( T_m \) in the disease-associated range of 87.02°C, while the 3’ TP-PCR MCA produced only a weak hump at a \( T_m \) of 87.30°C (Figure 4(b)). Whereas the corresponding capillary electrophoresis results of both 5’ and 3’ TP-PCR products reveal an 11-repeat allele, only the 5’ TP-PCR CE results showed continuous peaks extending up to 180 repeats, consistent with a large expansion. In contrast, the 3’ TP-PCR results revealed an aberrant peak pattern with an apparent gap in fluorescent peaks followed by five peaks of sharply decreasing peak height, after which the peaks were inconspicuous unless magnified (see inset). Sequencing of the most prominent expanded allele fragment obtained by repeat-flanking PCR revealed a single CCG-interruption at the 8th triplet repeat from the 3’ end and 50 tandem CCG-interruptions from the 18th–67th trinucleotides. The interruption at the 8th repeat would have resulted in failure of the 3’ TP primer to anneal efficiently to produce fluorescent peaks representing repeats 8–12. The next efficient annealing position would have been where the primer anneals to repeats 9–13 to produce the fluorescent peak representing 13 repeats. This would be followed by four additional fluorescent peaks, before the tandem CCG-interruptions prevent any further annealing of the 3’ TP primer. Despite the (CCG)\(_{50}\) interruption, inconspicuous but detectable continuous peaks to at least 70 CTG-repeats could be observed. As the TP-PCR was performed on total genomic DNA, the capillary electrophoresis results suggest that one or more of the less prominent expanded allele species of this sample may contain uninterrupted CTG-repeats.

For the sample with 5’ interruptions, both assays produced strong melt peaks with \( T_m \)s in the disease-associated range of 87.30 and 86.73°C, respectively (Figure 4(c)). Interestingly, only the 3’ TP-PCR CE results showed continuous peaks extending up to 180 repeats, consistent with a large expansion. In contrast, although the 5’ TP-PCR capillary electrophoresis also showed the same allele of 13 repeats as the 3’ assay, the fluorescent peak pattern of the other allele showed distinct peaks up to 21 repeats, and very inconspicuous fluorescent peaks thereafter, with somewhat more prominent peaks representing 36, 58, and 64 and greater repeats observed. This aberrant peak pattern is highly likely due to the interspersed CCG-interruptions towards the 5’ end of the repeat as revealed by sequencing. However, unlike the affected sample with the 3’-interrupted expansion where the 3’ TP-PCR failed to generate a melt peak, the 5’ TP-PCR of this sample could generate a strong melt peak in the disease-associated range to detect the expansion. These observations suggest that large tandem interruptions at one end of an expanded allele can result in failure to detect the expansion, depending on the TP-PCR assay used, whereas a more interspersed interruption appears to be less disruptive.

It should be noted that although Sanger sequencing successfully identified interruptions from the ends of expanded alleles, it cannot cover the entire repeat tract, which may contain additional internal interruptions. Likewise, despite the uninterrupted capillary electrophoretic peaks of ~180 repeats from both ends of the repeat stretch in our control sample (GM03989), the possibility of interruptions in the middle of the tract beyond the 180 repeats from either end cannot be ruled out. Also, in samples with heterogeneous expansion sizes, the sequence
may not necessarily represent the other expanded allele species.

3.7. Low-level mosaicism detection sensitivity

The sensitivity of the TP-PCR MCA assay in detecting low-level mosaicism for full penetrance alleles was evaluated by performing the assay on artificial DNA mixtures that comprised 1%, 2%, 5%, 10%, 25%, and 50% of a full penetrance allele (>180 CTGs) in a non-disease-associated, i.e. normal allele (12 and 13 CTGs), background. Both 5′ and 3′ TP-PCR assays efficiently detected the full penetrance allele when present at a concentration as low as 1% in normal/full penetrance mixtures (Figure 5). The corresponding capillary electrophoresis results confirmed the presence of the full penetrance allele in the mixtures. Similarly, both assays detected the premutation allele (48 CTGs) when present as low as 10% in normal/premutation mixtures (Supplemental Figure S1).

Mosaicism for both premutation and full penetrance alleles in a normal/premutation/full penetrance mixture could be detected when present at a minimum concentration of 20% (Figure 6). These observations suggest that TP-PCR MCA is very sensitive in detecting low-level mosaicism for expanded DMPK alleles in an otherwise normal allele background.

4. Discussion

We previously described the utility of MCA as a sensitive and rapid tool for analysis of DMPK CTG-repeat TP-PCR amplicons of up to 180 repeats [15]. The TP-PCR method works by combining a locus-specific flanking primer with a triplet-primed (TP) primer and a tail primer (Figure 1(a)). The random annealing of the TP primer within the CTG repeat stretch leads to the generation of a heterogeneous mixture of PCR products whose sizes differ by three base pairs. Alleles with shorter repeat tracts produce a series of limited products whereas
those with longer repeat tracts generate additional products of larger size (Figure 1(b)). MCA analysis of these heterogeneous products is performed in the presence of the non-saturating SYBR Green I fluorescent dye, which fluoresces when bound to double-stranded DNA. At lower temperatures, the shorter PCR fragments are dissociated, and the dislocated

| NL : FP | 5' TP-PCR | Electropherogram | 3' TP-PCR | Electropherogram |
|---------|-----------|------------------|-----------|------------------|
| 100% : 0% | Melt peak | RFU (x10³) | Melt peak | RFU (x10³) |
| 1 | 81.76°C | 3 | 81.76°C | 3 |
| 50 | 87.30°C | 13 | 87.30°C | 13 |
| 200 | | | | |
| 99% : 1% | Melt peak | RFU (x10³) | Melt peak | RFU (x10³) |
| 1 | 81.76°C | 3 | 81.76°C | 3 |
| 50 | 87.30°C | 13 | 87.30°C | 13 |
| 200 | | | | |
| 98% : 2% | Melt peak | RFU (x10³) | Melt peak | RFU (x10³) |
| 1 | 81.76°C | 3 | 81.76°C | 3 |
| 50 | 87.30°C | 13 | 87.30°C | 13 |
| 200 | | | | |

Figure 5. Melt peaks and the corresponding capillary electropherograms of 5' and 3' TP-PCR products of non-disease associated, i.e. normal/full penetrance mixtures. Domains highlighted in grey and pink represent premutation and full penetrance zones, respectively. Insets show higher resolution of longer TP-PCR products. The presence of full penetrance allele in the mixture could be detected at as low as 1% in the predominantly normal allele background. NL, normal; FP, full penetrance. Full color available online.
dye molecules relocate themselves to the remaining longer double-stranded DNA fragments. As a result, there is no net drop in fluorescence. When the temperature has risen to the point at which the longest PCR fragments are dissociated and all dislocated dye molecules are unbound, the fluorescence level will drop. The temperature at which fluorescence drops, which is also the melt peak temperature, is thus proportional to the longest PCR fragments present in the sample. Thus, non-DM1 samples will produce melt peaks with lower $T_m$ (Figure 1(c)) when compared with DM1 samples (Figure 1(d)).

It should be noted that the actual size of the expanded allele in the DM1 cell lines used in this study may not be exactly as reported by Coriell, due to the unstable nature of the large expansions after repeated passaging of the cells. However, the melt peak temperature for samples with large expansions exceeding 180 repeats will be the same, regardless of how much larger the expanded repeat actually is, since the TP-PCR assays will amplify and only display amplicons of up to ~180 repeats from either end of the repeat.

In this study, we have defined the range of conditions for consistent and optimal performance of the assays. Both assays specifically detect only the DMPK CTG-repeat, with no cross-amplification of other trinucleotide repeat loci. The wide working range of input DNA of both assays provides reassurance that sample DNA concentrations are not required to fall within a narrow range in order to obtain accurate results. The minimal reaction volume of 15 µL also reduces reagent costs, which can be substantial especially when large numbers of samples are analyzed. In addition, the fact that different DNA diluents can be used without noticeable $T_m$ differences confers additional flexibility in template DNA preparation and dilution. However, we observed that sodium acetate, a salt commonly used in DNA precipitation, could shift melt peak $T_m$s sufficiently to lead to a false-positive result when present in sufficiently high amounts. It is therefore advisable to prepare all test samples using a common DNA extraction procedure in order to minimize contaminant-induced $T_m$ variation.

The observation of well-to-well $T_m$ variation in two different real-time PCR instruments may simply reflect the temperature distribution characteristics of block- and rotary drum-based thermal cyclers. Nonetheless, this minor interwell variation should not represent a major constraint of the TP-PCR MCA assay, as long as only samples run on the same instrument are compared and controls are included in all runs, as minor inter-run $T_m$ variations may also occur. As MCA can be programmed to execute automatically after thermocycling without any

![Figure 6. Melt peaks of 5’ and 3’ TP-PCR products of non-disease associated, i.e. normal/premutation/full penetrance mixtures. Domains highlighted in grey and pink represent premutation and full penetrance zones, respectively. The premutation and full penetrance alleles in the mixture could be detected at as low as 20% in the predominantly normal allele background. NL, normal; PM, premutation; FP, full penetrance. Full color available online.](image_url)
Another factor which could adversely affect TP-PCR MCA performance is the presence of CCG-interruptions within the DMPK CTG-repeat. As with other groups who have reported repeat interruptions at the 3′ end [19–23], despite their rarity, we recommend performing bidirectional TP-PCR MCA on all samples to minimize a potential false-negative result that can occur when only unidirectional TP-PCR is performed. Any discordance between the S′ and 3′ TP-PCR MCA results should be further investigated, e.g. using capillary electrophoresis analysis, to resolve the discrepancy.

The sensitivity of the TP-PCR MCA assay in detecting the full penetrance and premutation allele in model mixture experiments down to 1% and 10%, respectively, suggests that this assay is capable of detecting low-level mosaicism for expanded DMPK alleles in DM1-affected individuals, thus performing as well as, if not superior to, Southern blot analysis of restriction-digested genomic DNA [2]. MCA is also superior to the conventional repeat-flanking PCR, which could lead to false-negative results when the expanded allele fails to amplify, by producing a melt peak that is to the right of the threshold temperature regardless of the expansion size. Nonetheless, it should be noted that MCA as a screening tool provides qualitative result as to whether an expansion is present and is not capable of establishing genotype–phenotype correlation in a patient.

The use of MCA to analyze TP-PCR products is also more cost-effective than capillary electrophoresis. We calculated the reagent cost for unidirectional TP-PCR to be ~USD1.26 per sample (for subsequent MCA analysis) and ~USD1.29 per sample (for subsequent capillary electrophoresis). The cost of reagents and consumables was ~USD0.15 for post-PCR MCA analysis on the LC480, and ~USD2.59 for post-PCR capillary electrophoresis analysis on the ABI 3130xl Genetic Analyzer. Using a hypothetical situation which assumes an inflated 1% population prevalence of DM1-affected individuals, screening 1000 samples would cost ~USD1410 for nonfluorescent TP-PCR MCA, and another ~USD40 for the short-cycled primer-labeled extension and capillary electrophoresis on 10 screen-positive samples, for a total reagent cost of ~USD1450. In contrast, if the fluorescent TP-PCR capillary electrophoresis was used instead, it would cost ~USD3880 or ~USD2.43 more per sample tested. As the calculation was only based on unidirectional TP-PCR, the difference between MCA- and capillary electrophoresis-based test costs would double to almost ~USD5 per sample if bidirectional TP-PCR assay were to be performed.

In conclusion, we have shown that the DMPK TP-PCR MCA assay is robust and reliable, performs optimally under a broad range of assay conditions, and is a more economical alternative than capillary electrophoresis-based analysis.

Key issues

- Expansion of a CTG trinucleotide repeat within the dystrophia myotonica protein kinase (DMPK) gene to ≥50 causes myotonic dystrophy type 1 (DM1).
- The wide clinical phenotypes exhibited by DM1 and their similarities with other genetically distinct disorders necessitate molecular confirmation of the disease, which has in the past involved the use of repeat-flanking PCR and Southern blot analysis.
- The use of TP-PCR followed by capillary electrophoresis to detect presence of the expansion has become increasingly common.
- Detection of CTG-repeat expansion could potentially be performed more rapidly, cost-effectively, and simply by combining TP-PCR with melting curve analysis (MCA), which discriminates between DM1 and non-DM1 samples based on the melt peak temperatures (Tm)s of their respective amplification products.
- For DMPK TP-PCR MCA to be used confidently as a screening tool, it is necessary to identify the optimal parameters and tolerance ranges of the assay as well as external and internal factors that could potentially lead to false-positive or false-negative genotype classification of samples.
- We investigated the assays’ specificity for the DMPK CTG-repeat locus, examined the effect of input DNA, reaction volumes, DNA diluents, and common DNA contaminants on Tm, determined the assays’ sensitivity in detecting low-level mosaicism for expanded alleles, and evaluated their performance on two real-time PCR platforms.
- Both assays detected only the DMPK CTG-repeat but not the FMR1 CGG-repeat, HTT CAG-repeat, or FXN GAA-repeat, and displayed analytic sensitivity down to 5 ng of genomic template. Different DNA diluents and glyco- gen amounts had minimal effect, while salt strongly affected Tm. Both assays could detect as low as 1% mosaicism for the expanded allele in an otherwise normal allele background. Tight well-to-well Tm uniformity was observed on both the Roche LC480 and Qiagen RGQ HRM instruments.
- The detection of CTG-repeat expansion by TP-PCR MCA could be adversely affected by the rare CCG-interruptions that may be present at either end of an expanded allele, depending on the structure of the interruption. In order to minimize potential false-negative screen results associated with the interruption, we recommend performing bidirec- tional TP-PCR MCA on all samples. Any discordance between the S′ and 3′ TP-PCR MCA results should be further investigated, for example using capillary electrophoresis analysis, to resolve the discrepancy.

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Declaration of interest

SS Chong is the inventor of the TP-PCR MCA method described in the paper. CG Lee is the co-inventor of the TP-PCR MCA method described in the paper. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
This article summarizes more recent and updated information related to DM1, such as its prevalence, molecular genetic diagnosis, and phenotype classification.

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