Evaluation of a Single-Tube, Long-Read, Two-Mode PCR Technology that Reports the Categorical Range of *DMPK* CTG Expansions and can Resolve up to 1900 Repeats in Myotonic Dystrophy Type 1

Bradley Hall[1], Jessica Cooley[2], Eric Bend[2], Jacob Wisotsky[1], and Jennifer A. Lee[2]
Asuragen, Inc., Austin, TX; [2]Greenwood Genetic Center, Greenwood, SC

Summary

- Triplet repeat CTG expansions in the *DMPK* gene cause Myotonic Dystrophy Type 1.
- Current laboratory workflows require PCR and Southern blot analysis to adjudicate DM1 repeat status.
- Asuragen developed a single-tube, long-read, two-mode PCR/CE assay based on AmplideX® technology that resolves ≤200 CTG repeats and flags larger expanded alleles by both stutter pattern and pile-up peak.
- For many larger expansions, an agarose gel electrophoresis method can categorically size alleles.
- This technology unifies gene-specific and repeat-primed designs in a streamlined procedure and can genotype normal and expanded alleles.

Introduction

Myotonic Dystrophy is the most common adult-onset muscular dystrophy, and the most severe form is type 1 (DM1). The autosomal dominant disease is caused by a pathogenic CTG expansion in the 3' UTR of the myotonic dystrophy protein kinase gene (*DMPK*). In the absence of family history, early DM1 symptoms are typically mild, overlapping with other disorders (e.g. baldness, myalgia, cataracts) which leads to under- or misdiagnosis of patients[1]. With the introduction of molecular testing, disease prevalence could be as high as ~1:2000[2].

In clinical labs, DM1 molecular testing requires both PCR and Southern blot (SB) analysis because most laboratory-developed PCR tests (LDTs) cannot consistently amplify >100 repeats, which is the threshold for the classical disease category. Not only does SB take approximately one week, but due to expansion allele dropout by sizing PCR, expansion detection must often be performed by an independent PCR or other assay. Here, we describe a novel PCR amplification technology that surmounts this hurdle and reliably resolves normal and expanded alleles using Capillary Electrophoresis (CE) for ≤200 repeats and flags >200. An optional Agarose Gel Electrophoresis (AGE) method was developed for size estimation and categorical genotyping for many larger expansions.

Materials and Methods

Presumed normal specimens (27) were selected (Asuragen, Site 1). Residual clinical specimens (67) were selected with genotypes independently determined using PCR and/or SB at Greenwood Genetic Center (GGC, Site 2). Positive control reference cell lines from Coriell Cell Repository (CCR) (n=7 as described in Kalman et al.[3]) and a calibrator were used at both sites. Specimens covered each of the clinical categories defined by the CTG repeat number (Figure 1). Genomic DNA was amplified using pilot Asuragen AmplideX PCR/CE *DMPK* Kit (RUO)* reagents that were identical to final product formulations. FAM-labeled amplicons were resolved by CE (Thermo Fisher), peaks were called using GeneMapper. Called peaks (bp) were converted to repeat size and category by the *DMPK* Macro using a calibrator at each site. Expansions larger than 50 repeats were resolved by AGE.

*Research Use Only. Not for use in diagnostic procedures.
Conflict of Interest Disclosure: All authors have the financial relationship to disclose: Employment by Asuragen or Greenwood Genetics Center
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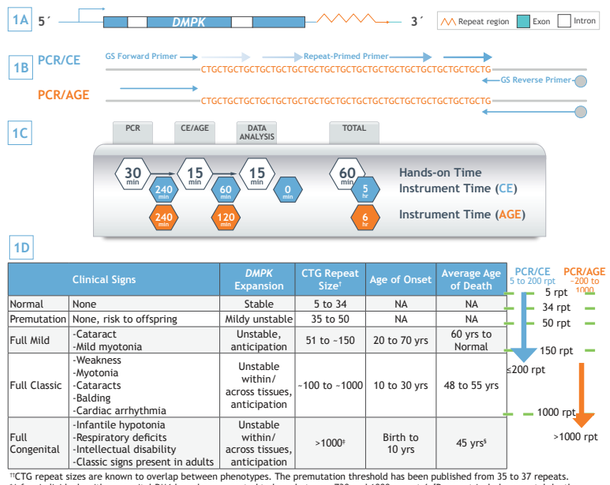
Results



1D

Clinical Signs		*DMPK* Expansion	CTG Repeat Size[1]	Age of Onset	Average Age of Death
Normal	None	Stable	5 to 34	NA	NA
Premutation	None, risk to offspring	Mildy unstable	35 to 50	NA	NA
Full Mild	-Cataract -Mild myotonia	Unstable, anticipation	51 to ~150	20 to 70 yrs	60 yrs to Normal
Full Classic	-Weakness -Myotonia -Cataracts -Balding -Cardiac arrhythmia	Unstable within/ across tissues, anticipation	~100 to ~1000	10 to 30 yrs	48 to 55 yrs
Full Congenital	-Infantile hypotonia -Respiratory deficits -Intellectual disability -Classic signs present in adults	Unstable within/ across tissues, anticipation	>1000[2]	Birth to 10 yrs	45 yrs[3]

PCR/CE 5 to 200 rpt; PCR/AGE ~200 to 1000 rpt
5 rpt
34 rpt
50 rpt
150 rpt
≤200 rpt
1000 rpt
>1000 rpt

[1]CTG repeat sizes are known to overlap between phenotypes. The premutation threshold has been published from 35 to 37 repeats.
[2]A few individuals with congenital DM1 have been reported to have between 730 and 1000 repeats[4]. [3]Does not include neonatal deaths.

Figure 1. *DMPK* Gene Map and Correlation of Phenotype and CTG Repeat Length in DM1 with Assay Sizing Ranges. A) DM1 is caused by an expansion of the trinucleotide repeat CTG in the 3' UTR of *DMPK* located at Chr 19q13.3. **B)** The assay consists of two gene-specific (GS) primers (FAM-labeled reverse primer), and one triplet repeat primer (TP) that hybridizes and primes anywhere in the repeat region creating a stutter repeat pattern (RP). The assay produces two complementary data outputs in a single-tube: GS amplicons provide sizing of normal and expanded alleles (CE and AGE), whereas short and long alleles are either enumerated or flagged by RP and pile-up (CE only). **C)** The streamlined workflow can be performed within a 7 hr work-shift for either PCR/CE (4-24 samples with per injection run times) or PCR/AGE (11 samples and 1 ladder lane) on Lonza Reliant 1% agarose gels. Total hands on time is ~1 hr. **D)** Correlation of phenotype to genotype adapted from Bird et al.[5] & ACMG categorical guidelines[6].

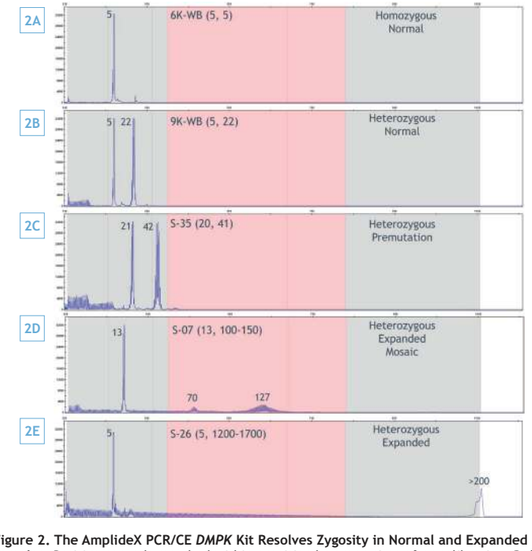


Figure 2. The AmplideX PCR/CE *DMPK* Kit Resolves Zygosity in Normal and Expanded Samples. Positive controls matched within precision between sites after calibration. Reference genotypes are provided at the top of each trace. **A-D)** Normal and expanded specimens were sized from GS peaks. Expanded repeat stutter pattern differentiates zygosity. **E)** Specimens containing >200 CTG repeats also manifest an aggregated "pile-up" peak at >950 bp.

3A

Target Precision	
CTG Repeat Range	Precision
<50	± 1 CTG
50 - 120	± 2 CTG
121 - 200	± 5%

3B

AmplideX *DMPK* PCR/CE Kit	Orthogonal Method (Site 2 LDT) >50	Orthogonal Method (Site 2 LDT) ≤50 or null
>50	64[a]	0[b]
≤50 or null	0[c]	124[d]

Percent Positive Agreement	64/(64+0) = 100%
Percent Negative Agreement	124/(0+124) = 100%
Overall Percent Agreement	(64+124)/(64+0+0+124) = 100%

[a]valid allele calls >50 in both comparator and test data sets that were within the predetermined tolerances (true positive)
[b]allele calls <50 that appeared in the test data but not the comparator data set or were outside tolerance (false positive)
[c]allele calls <50 that appeared in the comparator data set but not the test data or were outside tolerance (false negative)
[d]valid allele calls <50 in both comparator and test data sets that were within the predetermined tolerances (true negative)

3C

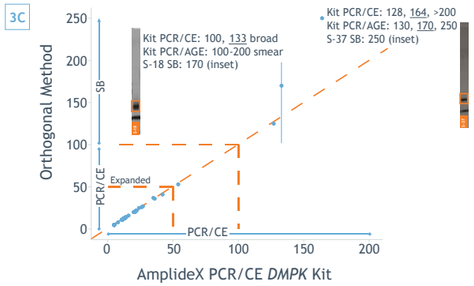


Figure 3. The AmplideX PCR/CE *DMPK* Kit Identified Sample Genotypes (Allele Sizes and Categorical Genotypes) with >98% Concordance Compared to an Orthogonal Method for all 94 Specimens. A) Precision between sites for positive controls were within guidelines described in the Kit Protocol Guide. **B)** Accuracy of differentiating expanded specimens (>50 CTGs) using each assay compared in a 2x2 contingency table shows 100% agreement. Each allele (188) treated separately. **C)** The Kit was >98% concordant within ±10% sizing alleles ≤200 CTGs to reference results for the 2 major alleles of all 94 specimens (126/128 alleles). 100% of the alleles flagged >200 CTGs (60) were expanded in the reference. Four additional mosaic alleles were identified by the Kit and not in the reference. The dotted line represents a slope of 1 (identical agreement).

Sample Name	Site 2 SB Reference	AmplideX PCR/CE	AmplideX PCR/AGE (>200 allele)
S-01	11, 300-500	11, >200	Smear, High Mosaic 300-500
S-02	5, 1200-1350	5, >200	~1100
S-03	5, 1100-1500	5, >200	Smear, High Mosaic >500
S-04	12, 200-300	12, >200	~260-350
S-05	5, 800-900	5, >200	~650-700
S-06	26, 1050	26, >200	~1000
S-07	13, 100-150	13, 71, 129	70, 130
S-08	12, 1900-2220	12, >200	700, 1900
S-09	22, 300	22, >200	Mosaic, 261
S-10	5, 50-250	5, 141, >200	150, Smear 200-500

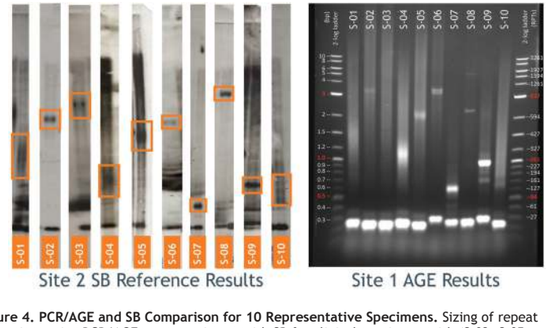


Figure 4. PCR/AGE and SB Comparison for 10 Representative Specimens. Sizing of repeat expansions using PCR/AGE were consistent with SB for clinical specimens with (S-02, S-05, S-06, S-07, S-08, S-09). Specimens with known mosaicism by SB appeared as a smear on the PCR/AGE assay (S-01, S-03, S-04, S-10) and in some cases could not easily be interpreted by AGE (S-03, S-10).

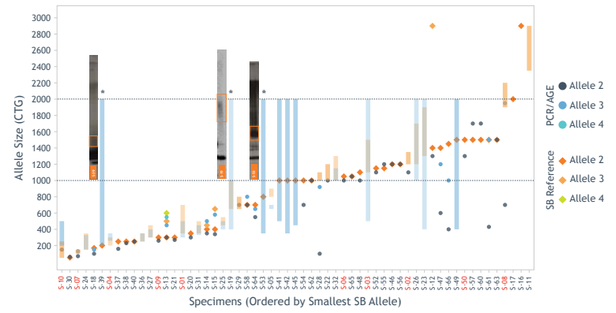


Figure 5. PCR/AGE Categorical Agreement with Reference SB Method for 59 specimens with >50 CTGs. Mosaic smeared bands observed by SB were more pronounced in PCR/AGE. Specimens without a blue (PCR/AGE) data point are hidden by the overlapping SB data point. Red text indicates samples in Figure 4. Category (<1000 CTGs): 23 of 27 specimens (85%) sized by PCR/AGE were within 10% of the reference, 1 was >10% (S2-05), and 3 (11%) were unresolved smeared AGE bands wherein the SB could also be interpreted as a smear (* SB inset). (1000-2000 CTGs): 20 of 29 specimens (69%) sized by PCR/AGE were within 10% of the reference and 2 more were within 20%. Unresolved smeared AGE bands were observed in 7 of 29 specimens (24%), and 3 were also smears by SB. (>2000 CTGs): No reference alleles could be identified by PCR/AGE (5/5), though 2 specimens (S2-12, S2-08) with mosaic alleles in the 1000-2000 category were identified. PCR failed for 5/67 specimens. Only PCR/CE was repeated for these specimens and 100% were successfully flagged with >200 CTGs.

Conclusions

- We report the first single-tube, long-read PCR technology that can resolve *DMPK* zygosity and genotype alleles across the categorical range of repeat expansions.
- The PCR/CE assay showed 100% agreement in identifying expansions with >50 CTGs, differentiating normal, mosaic and expanded samples, and flagging larger expansions across all 67 samples. The assay also demonstrated >98% concordance for sizing alleles up to 200 repeats within 10% of reference.
- The PCR/AGE assay demonstrated good categorical agreement to SB analysis up to 1000 repeats (85% were sized within 10% of reference), however the smeared bands sometimes appeared more diffuse than on SB.
- The largest allele detected and sized by PCR/AGE in this study was 1900 repeats.
- This PCR-based workflow could potentially reduce the labor and turnaround time from sample to answer compared to SB analysis. For samples with >1900 repeats and for any sample with an unresolved AGE smear, SB may be required if sizing is necessary.

References

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