Clinical correlations of a streamlined molecular assay based on AmplideX® PCR/CE technology that determines repeat size for both normal and expanded alleles in *DMPK* for myotonic dystrophy type 1

Bradley Hall[1], Pamela J Snyder[2], Jon Kemppainen[1], Thomas W Prior[2], Pranesh R Rao[1], and Gary J Latham[1]
[1]Research and Development, Asuragen, Inc., Austin, TX, [2]Department of Pathology, Ohio State University, Columbus, OH

Summary

- Triplet repeat CTG expansions in the *DMPK* gene are causative for Myotonic Dystrophy Type 1 (DM1).
- Current laboratory workflows require both PCR and Southern blot (SB) analysis to adjudicate DM1 repeat status.
- We developed a single-tube assay based on AmplideX® PCR technology and capillary electrophoresis (CE) that resolves ≥200 CTG repeats and flags larger expanded alleles without the need for SB analysis.
- The prototype *DMPK* PCR/CE assay unifies gene-specific and repeat-primer designs in a streamlined procedure to generate sensitive and specific results across all categories of expanded alleles.

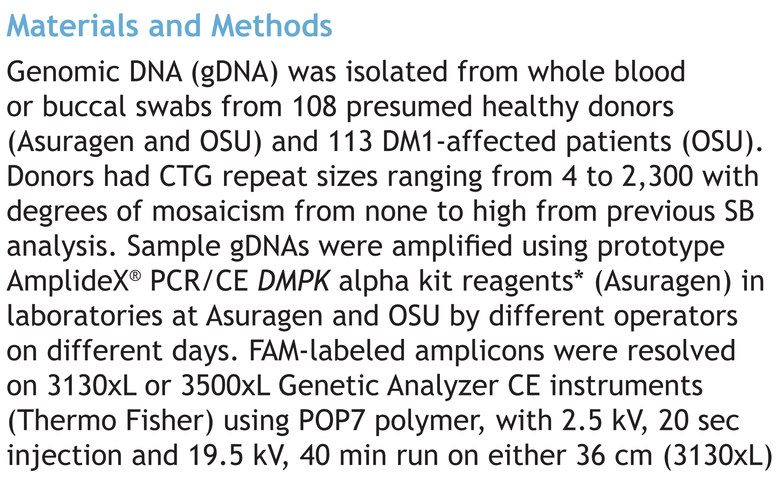
Introduction

Myotonic dystrophy type 1 (DM1) is an autosomal dominant disease characterized by >50 CTG repeats in the 3' UTR of *DMPK*[1]. In the absence of family history, early DM1 symptoms are typically mild, overlapping with other disorders (e.g. baldness, myalgia, cataracts) and leading to under- or misdiagnosis of patients[1]. The introduction of molecular testing suggests a disease prevalence as high as ~1:2000[2].

In clinical labs, DM1 testing requires a combination of multiple PCR reactions and Southern blot (SB) analysis because PCR cannot reliably amplify over 100 repeats. Not only does the process take up to 8 days, but allele drop-outs are often indistinguishable from homozygous samples[3]. The method and results described here enlist Asuragen's AmplideX® PCR technology to enable accurate CE-based sizing of *DMPK* alleles with resolution up to 200 repeats from a single-tube assay. Expanded alleles of up to 2300 repeats can be sized using agarose gel electrophoresis (AGE).

Materials and Methods

Genomic DNA (gDNA) was isolated from whole blood or buccal swabs from 108 presumed healthy donors (Asuragen and OSU) and 113 DM1-affected patients (OSU). Donors had CTG repeat sizes ranging from 4 to 2,300 with degrees of mosaicism from none to high from previous SB analysis. Sample gDNAs were amplified using prototype AmplideX® PCR/CE *DMPK* alpha kit reagents* (Asuragen) in laboratories at Asuragen and OSU by different operators on different days. FAM-labeled amplicons were resolved on 3130xL or 3500xL Genetic Analyzer CE instruments (Thermo Fisher) using POP7 polymer, with 2.5 kV, 20 sec injection and 19.5 kV, 40 min run on either 36 cm (3130xL) or 50 cm (3500xL) capillaries. Genotyping was achieved following analysis using a ROX 1000 size ladder (Asuragen) and a four-point calibrator. Select samples were also sized by AGE using Reliant Mini 12-well precast 1% SeaKem Gold agarose gels (Lonza), Quick-Load 2-Log DNA ladder (NEB) and Bionic Buffer (Sigma).



Clinical Signs			CTG Repeat Size[†]	Age of Onset	Average Age of Death
Normal	None		5 - 37	NA	NA
Premutation	None, risk to offspring		37 - 49	NA	NA
Full Mild	-Cataract -Mild myotonia		50 - ~150	20 - 70 yrs	60 yrs - Normal
Full Classic	-Weakness -Myotonia -Cataracts	-Balding -Cardiac arrhythmia -Others	~100 - ~1000	10 - 30 yrs	48 - 55 yrs
Full Congenital	-Infantile hypotonia -Respiratory deficits	-Intellectual disability -Classic signs present in adults	>1000[‡]	Birth - 10 yrs	45 yrs[§]

[†]CTG repeat sizes are known to overlap between phenotypes. The disease threshold has been published from 34 to 37 repeats.
[‡]A few individuals with congenital DM1 have been reported to have between 730 and 1000 repeats[7]. [§]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 the expansion of the trinucleotide repeat CTG in the 3' UTR of *DMPK* located at Ch 19q13.3. **B)** The assay consists of two gene-specific (GS) primers (FAM-labeled reverse primer), and one repeat primer (RP) that can hybridize and prime anywhere in the repeat region. 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 stutter patterns (CE only). **C)** Table correlating phenotype to genotype adapted from Bird et al.[6] with associated assay sizing.

*Disclaimer: The product is still under development. Its future availability and performance cannot be ensured.
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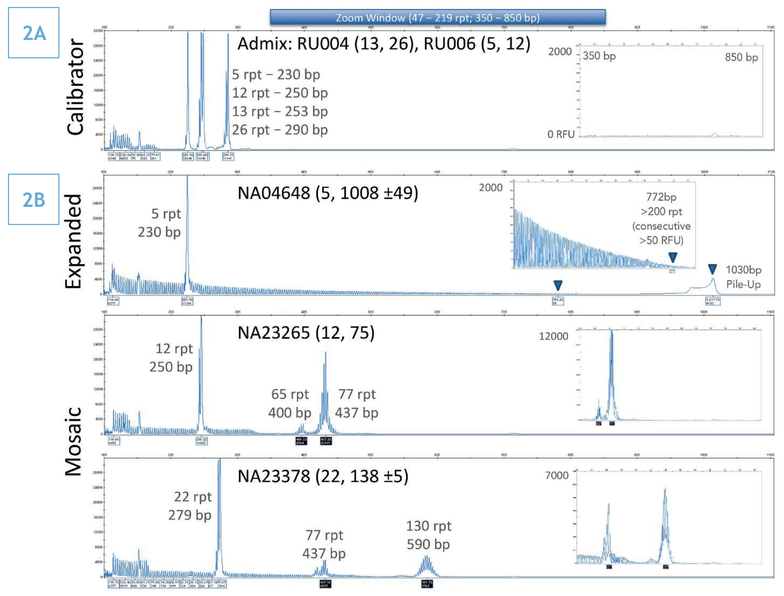
Results



RU004/6 (GS)		Coriell GS (Allele 1)		Coriell GS (Allele Both)	
Repeats	Base Pairs	Repeats	Base Pairs	Repeats	Base Pairs
5	229.92	5	229.92	5	229.92
12	250.28	12	250.22	12	250.22
13	253.16	22	279.09	22	279.09
26	290.39			65	400.33
				77	437.25
				77	437.10
				130	589.72
Slope	2.8769	Slope	2.8920	Slope	2.8754
Intercept	215.66	Intercept	215.48	Intercept	215.42
R^2	1.0000	R^2	1.0000	R^2	1.0000

RU004/6 (RP)		NA23378 (RP)	
Repeats	Base Pairs	Repeats	Base Pairs
4	118.73	4	118.73
10	136.56	10	136.46
15	150.88	15	150.88
20	165.35	20	165.35
25	179.81	25	179.80
		30	194.38
		35	208.97
		40	223.51
		45	238.12
		50	252.67
		55	267.10
Slope	2.9036	Slope	2.9080
Intercept	107.29	Intercept	107.20
R^2	1.0000	R^2	1.0000

Figure 2. Single-Tube, Three-Primer *DMPK* PCR/CE Assay Data. A) Normal samples were sized from GS peaks. Heterozygous samples were differentiated from homozygous with the repeat profile. When only one peak is present, the lack of expanded repeat pattern indicates homozygosity **(Figure 4B)**. Reference genotypes are provided at the top of each trace. **B)** Expanded samples display one normal allele with a larger second GS peak and/or expanded repeat pattern. Samples containing more than 230 CTG repeats also display an aggregated peak at ~1030 bp ("Pile-up"). **C and D)** To determine repeat numbers from fragment mobility, either an admixture of normal (RU004 and RU006) or Coriell samples with known genotypes such as NA04648, NA23265 and NA23378 could be combined to create a calibrator. Either GS **C)** or RP peak mobility **D)** were used for calibration; both provided similar values for the slope (average 2.88) and "0 repeat" y-intercept (average 215 for GS or 107 for RP) used in the linear fit.

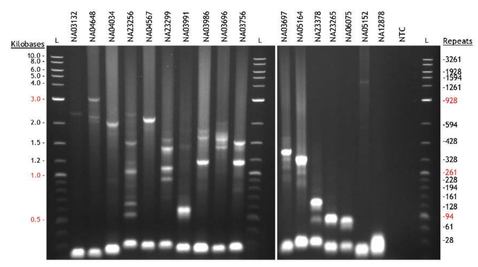


Figure 3. Single-Tube, Two-Primer *DMPK* PCR/AGE Assay Data. Amplification of 17 Coriell samples with specific banding patterns up to 1600 repeats (NA05152) and mosaicism that agrees with results using independent methods such as SB.

Coriell #	Kalman et al.[3]	Asuragen PCR/AGE	PCR/AGE (Allele 2)
NA03132	5, 2078 ± 217	5, >200	~700
NA04648	5, 1008 ± 49	5, >200	~670, ~930
NA04034	700	12, >200	~600
NA23256	25, 75	25, 102, 137, >200	~300, ~420 mosaic
NA04567	21, 637 ± 33	21, >200	~650
NA23299	475 mosaic	22, >200	~300,~400 mosaic
NA03991	NA03990: 78	14, 108-114, >200	110, ~400,~550
NA03986	550 mosaic	12, >200	~380, ~450 mosaic
NA03696	12, 697 ± 13	12, >200	~450 mosaic
NA03756	450 mosaic	13, >200	~320,~430 mosaic
NA03697	12, 412 ± 33	12, >200	~380
NA05164	21, 377 ± 53	21, >200	~350
NA23378	22, 138 ± 5	22, 77, 170	~80, ~140
NA23265	12, 75	12, 65, 77	~80
NA06075	12, 56, 70 ± 0.9	12, 56, 71	~70, ~80
NA05152	5, 1621 ± 30	5, >200	5, ~1600
NA12878		5, 13	<28
NTC			

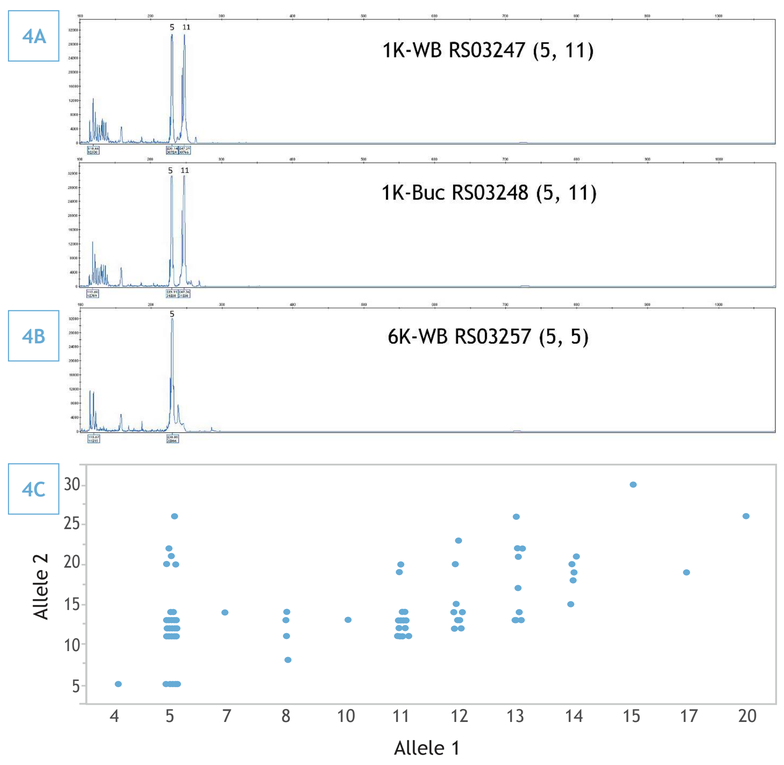
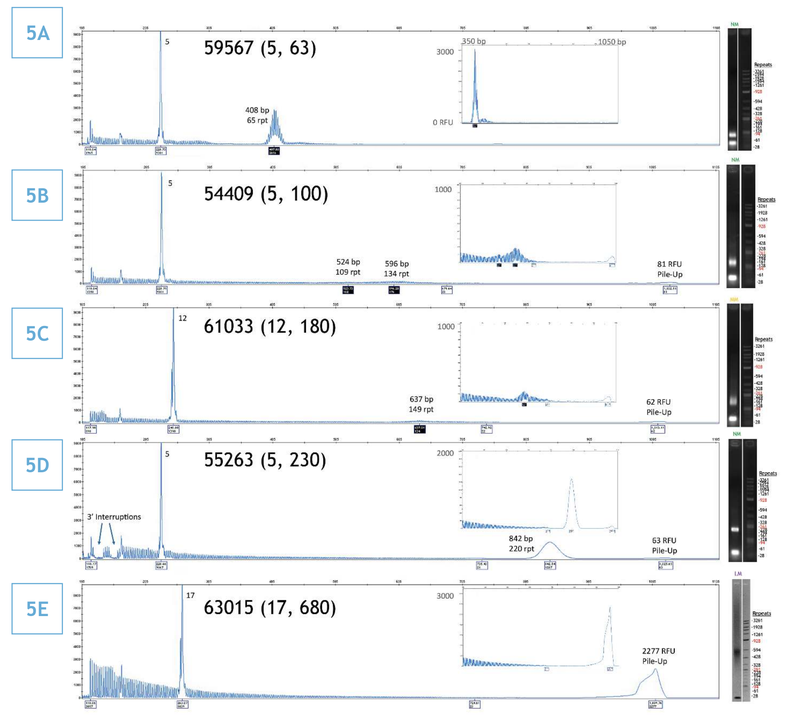


Figure 4. Consistent and Reliable *DMPK* PCR/CE Results Across 108 Presumed Normal Donor Samples. A) The assay was run in duplicate for 24 whole blood (WB) and 10 matched WB and buccal (Buc) samples with 100% concordance and similar traces patterns across sample type. The remaining 74 WB samples were tested once with the assay. **B)** A homozygous (5, 5) is clearly differentiated from a heterozygous (5, 11) and expanded (5, >200; **Figure 5D**). **C)** All the genotypes are represented in a jitter plot. One sample was detected with a genotype (4, 5) whereas the most common genotype was a (5, 13). 24% of samples were homozygous (26 of 108) similar to literature reports[5].



#	Sample ID	Asuragen PCR/AGE Allele 2
1	56738	~1500 ,~1950
2	49148	~1500
3	58716	~1000, ~1500
4	57184	~650-1350
5	55965	>1000
6	59993	>1000
7	51594	~930
8	59374	~960
9	60265	~750-950
10	53175	~700-1200
11	63576	~950

Figure 5. Concordant *DMPK* PCR Assay Results Across 113 DM1 Clinical Samples. A) Representative sample 59567 with well-defined GS peaks by PCR/CE, no mosaicism and no pile-up peaks. Genotypes labeled at the top of each trace reflect independent OSU results (by SB). **B)** Representative sample 54409 with 134 CTG repeats. **C)** Sample 61033 with a 149 repeat amplicon and pile-up peak. **D)** Sample 55263 with 220 repeat peak and a pile-up peak. **E)** Sample 63015 with substantial pile-up peak. In total, 70/113 samples contained more than 350 repeats (by SB), and 10 contained interruptions. **F)** AGE was performed on a subset of samples containing expansions up to 2300 repeats (sized by SB) with repeat sizing by AGE listed. The samples near and above the congenital border are shown. NM, No mosaicism. LM, low mosaicism. HM, high mosaicism (as previously determined by SB at OSU).

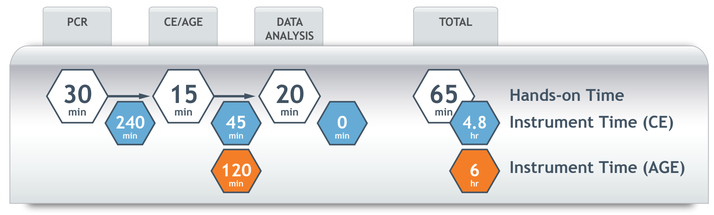


Figure 6. The Prototype *DMPK* PCR/CE Workflow is Streamlined from Sample-to-Answer. The assay can be performed within a 7 hr work-shift for either PCR/CE (for 4-24 samples with per injection run times) or PCR/AGE (12 samples). Total hands on time is ~1 hr.

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 is able to differentiate normal, mosaic and expanded samples by sizing 4 to at least 200 repeats, flagging >200 repeats, and identifying interruptions.
- The PCR/AGE assay is able to size expansions up to 1600 repeats, well past the boundary associated with congenital cases.
- Using both PCR/CE and PCR/AGE assays, OSU demonstrated 100% sensitivity for clinical samples with up to 500 repeats, and accurately flagged larger expansions across all 113 samples.
- The PCR based workflow significantly reduces the burden of SB testing for *DMPK* expansions by substantially reducing the cost, reagents, labor, and time from sample to answer.

References
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