Analytical Validation of a Sensitive Myotonic Dystrophy Type 1 (DM1) Diagnostic Test That Provides Precise Repeat Sizing and Resolves Zygosity in a Single PCR

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Summary

- Myotonic Dystrophy type I (DM1) is an inherited muscular disorder that is caused by CTG repeat expansions in the *DMPK* gene.
- We developed a single-tube PCR test that combines gene-specific and repeat-primed modes to genotype DM1 repeats and resolve zygosity.
- We analytically validated this test for precision and sensitivity, and evaluated it against an independent method.
- The test, which is available as the AmplideX® DM1 Dx Kit, provides a streamlined procedure that reduces the labor and turnaround time required for Southern blot and may help standardize results across laboratories.

Introduction

Myotonic dystrophies are autosomal dominant, multisystemic disorders with variable expressivity characterized by progressive myopathy. Myotonic dystrophy type 1 (DM1) is caused by CTG expansions in the 3' UTR of the *DMPK* gene on chromosome 19q13.3. The severity of disease and age at onset is roughly correlated with the number of the trinucleotide repeats. Reliable assessment of CTG expansions is therefore critical for diagnosis of DM1. We developed and validated the AmplideX® DM1 Dx Kit*, which generates numerical values for alleles ≤200 repeats and a categorical report for alleles >200 repeats.

Genotype Report[1]	Numerical Report[1]	Number of Repeats[2]	Stability of Repeat[2]	DM1 Phenotype[2]
Normal	a number 1-35	5-35	Stable	No DM
Premutation	a number 36-50	36-50	May be unstable	No DM
Expanded	a number 51-150	51-150	Unstable	No, minimal, or classical DM
Expanded	a number 151-200 or a categorical report of "> 200"	> 150	Unstable	Classical, juvenile, or congenital DM

[1]Generated by the test. Values are inclusive unless marked with ">".
[2]According to Kamsteeg et al (2012).

Materials and Methods

The kit enables repeat-primed PCR (RP-PCR) of genomic DNA isolated from whole blood, followed by capillary electrophoresis (CE), manual peak annotation, and automated repeat length calculations. For exploratory size estimation beyond 200 repeats, the kit facilitates an optional analysis by agarose gel electrophoresis (AGE).

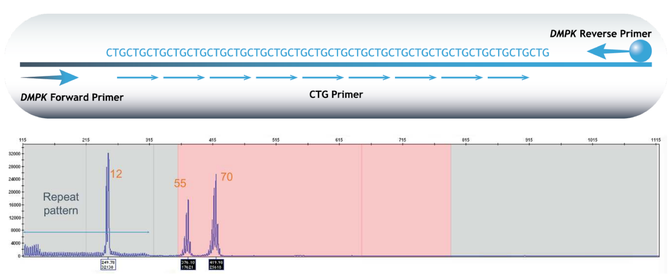

Figure 1. Three Primer Repeat-Primed PCR Methodology. This figure highlights the features of the CTG RP-PCR with 3 primers amplifying a sample (Coriell Cell Repository NA06075) previously characterized as expanded with mosaicism.

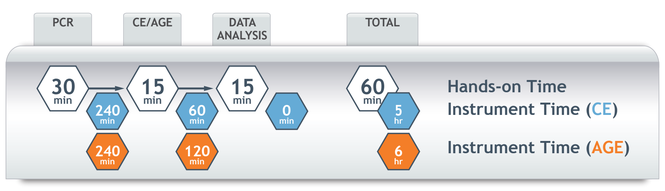

Figure 2. Assay Workflow. The process is streamlined from specimen to answer. The assay can be performed within a 7 hour work shift for either PCR/CE (for 4-24 specimens with per injection run times) or PCR/AGE (12 specimens).

*CE marked. For US export only.
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Results

Table 1. Single-site Precision. A) Target tolerance ranges. **B)** 1941/1944 (99.8%) valid numerical alleles were obtained. All 1941/1941 (100%) allele repeat lengths were within target tolerance when compared to the mode of measurements. 858/864 (99.3%) valid genotypes were obtained. All (858/858, 100%) matched expectations by providing a result of "Expanded" (>50 CTG). The greatest variability was seen in the allele with a mode of 135 repeats with an SD of 0.98.

A

CTG Repeat Range	Target Tolerance
< 50	± 1
50-120	± 2
121-200	± 5%
> 200	NA

B

Mode of Repeats ± Tolerance	Measured Repeat Length	Difference	Count	% Within Tolerance
5 ± 1	5	0	284	100
12 ± 1	12	0	288	100
21 ± 1	20	-1	1	100
	21	0	287	
25 ± 1	25	0	284	100
55 ± 2	55	0	288	100
70 ± 2	69	-1	1	100
	70	0	287	
101 ± 2	100	-1	2	100
	101	0	282	
135 ± 5%	135	0	167	100
	136	1	117	
145 ± 5%	144	-1	1	100
	145	0	259	
	146	1	24	

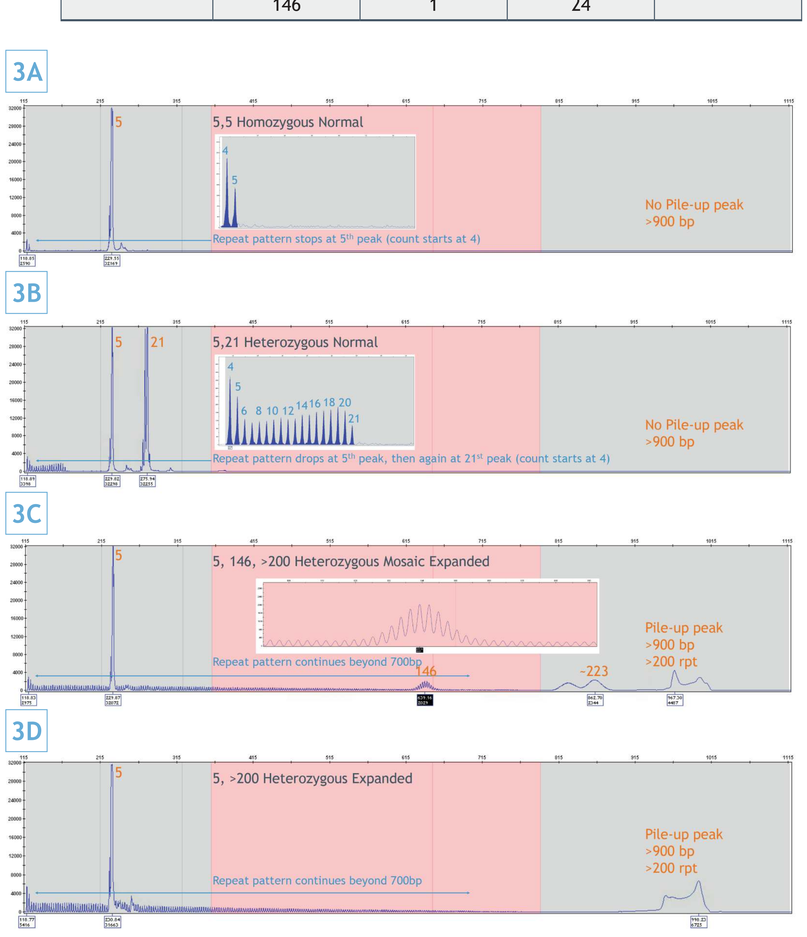

Figure 3. Zygosity Resolution. A) A whole blood specimen genotyped as normal homozygous (5, 5). The repeat peaks can be counted to confirm the gene-specific (GS) allele sizing. **B)** Presumed normal Coriell sample NG12729 genotyped as normal heterozygous (5, 21). **C)** Coriell sample NA23300 (5, 150-160, 550 mosaic) genotyped as expanded heterozygous mosaic (5, 146, > 200 repeats) based on the presence of multiple peaks identified with GS primers. The specimen is expanded based on the extended repeat pattern and pile-up peak. **D)** A whole blood specimen (5, 1200-1350 repeats) genotyped as expanded heterozygous (5, > 200 repeats).

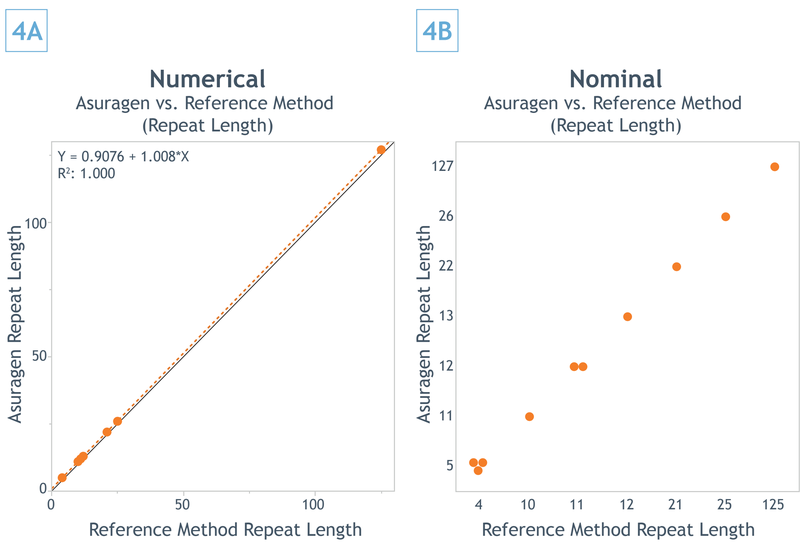

Figure 4. Method Comparison. A panel of 10 residual clinical DNA specimens were de-identified and provided by an external CLIA-certified laboratory along with the results obtained in their independently developed test. A method comparison on expanded samples yielded 100% concordance of genotypes and highly correlated repeat lengths (see chart). **A)** A reference line for ideal response characteristics is shown as a black line. Observed measurements are shown in orange. The dotted orange line is the linear regression. **B)** Nominal values are plotted categorically to clarify overplotting.

5A

Dilution Level	Positive	n	Hit Rate
1%	0	60	0%
3%	10	59	17%
5%	60	60	100%
7%	60	60	100%

5B

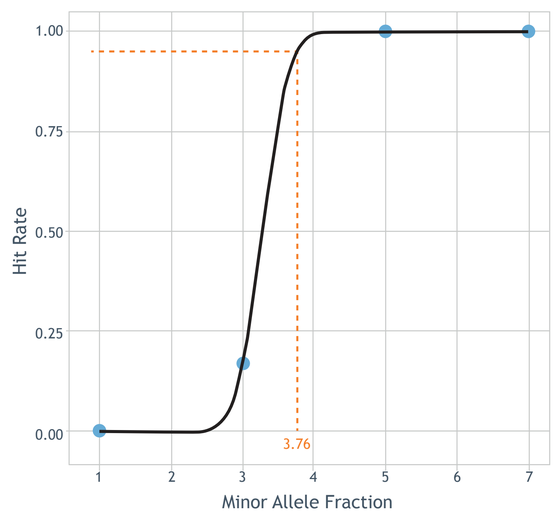

Figure 5. Probit Analysis for Limit of Detection (LOD). A) A specimen containing alleles of 12, 63, and 76 repeats was diluted into an expanded specimen (5 and >200 repeats) at 1, 3, 5, and 7% mass fraction. Traces were reviewed for the presence or absence of the allele of interest (76) at two-fold above background repeat pattern observed ±7 repeats away. **B)** The limit of detection was 3.76% fractional mosaic by probit analysis. Hit rates (observed probabilities) are shown in blue dots. The regression is shown as a solid black line. The 95th percentile is shown as a dotted orange line.

6A

Sample Name	Site 2 SB Comparator	AmplideX® PCR/CE	AmplideX® PCR/AGE
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

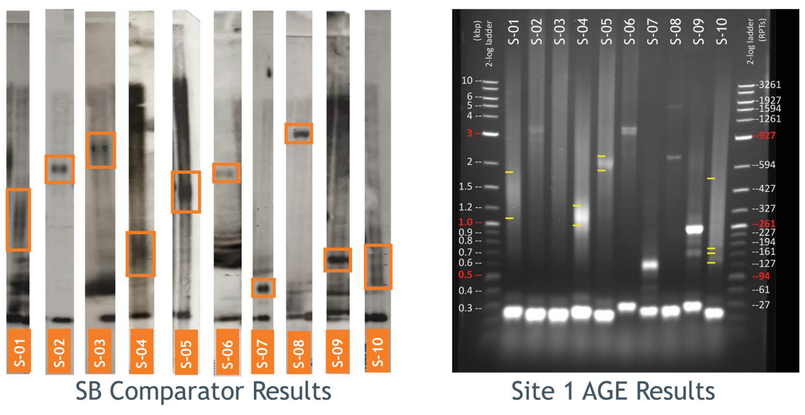

Figure 6. PCR/AGE and Southern Blot (SB) Comparison. A) Sizing by SB, PCR/CE, and PCR/AGE for a representative set of 10 residual clinical samples. Sizing of repeat expansions, listed in CTG repeats (RPTs), using PCR/AGE were consistent with SB for the majority of samples with low mosaicism (e.g. S-02, S-05, S-06, S-07, S-08, S-09). **B) and C)** Samples with mosaicism by SB appeared as a smear on the PCR/AGE assay (e.g. S-01, S-03, S-04, S-10) and in some cases could not easily be interpreted by AGE (e.g. S-03, S-10). Yellow lines indicate sizing of banding patterns. Asuragen was Site 1 and Comparator was an external clinical laboratory.

Conclusions

- Analytical validation of the test spanned 2192 valid measurements, 1588 genotypes, and 730 unique CE files.
- Single-site precision demonstrated that alleles with 5 to 145 repeats were sized within 1 repeat of the mode.
- The limit of detection was <4% fractional mosaic.
- Expanded alleles were accurately sized ≤200 repeats. Larger expansions were amplified and categorically detected with the test, and then investigationally assessed by AGE with similar results to Southern blot.

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