Development of a unified *DMPK* and *CNBP* PCR workflow for determining repeat expansions relevant to myotonic dystrophies

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Summary

- Myotonic dystrophy (DM) is the most common adult-onset muscular dystrophy. Type 1 is caused by an expansion of CTG in *DMPK* while Type 2 is caused by an expansion of CCTG in *CNBP*.
- Both diseases have similar presentation making differentiation important.
- The repeat regions of both *DMPK* and *CNBP* are extremely challenging to amplify and resolve using PCR.
- Here we demonstrate a unified workflow to genotype at least 200 CTG repeats in *DMPK* and flag expansions of >75 CCTG repeats in *CNBP*.

Introduction

Myotonic dystrophy type 1 and 2 (DM1 and DM2) are the most prevalent adult-onset muscular dystrophies. DM1 is caused by a trinucleotide expansion of CTG in the 3' untranslated region (UTR) of myotonic dystrophy protein kinase (*DMPK*). Clinical phenotypes manifest in patients who have >50 repeats with classic disease occurring in those with >100 repeats. DM2 is caused by a quadruplet nucleotide expansion of CCTG in intron 1 of the cellular nucleic acid-binding protein (*CNBP*). Pathogenicity is observed in those with >75 repeats. Expansions in the DM2 disease state can be between 75-11,000 repeats; however, the average is 5,000. In both cases, expansions often include hundreds to thousands of repeat units. DM1 and DM2 molecular diagnosis relies on a complex combination of PCR and Southern blot analysis. Due to a set of overlapping symptoms, DM1 and DM2 can be part of the same differential diagnosis. Current methods fail to identify expansions in *DMPK* and *CNBP* using a streamlined assay format. Thus we have proposed a workflow that unifies the determination of an expansion occurring in either *DMPK* or *CNBP*.

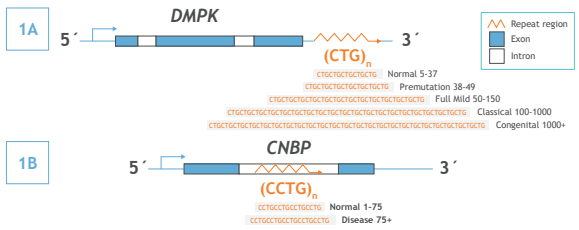

Figure 1. *DMPK* Gene Map and *CNBP* Gene Map. A) DM1 is caused by the expansion of the trinucleotide repeat CTG in the 3' UTR of the *DMPK* gene which is found on chromosome 19. As shown, 5-37 repeats is considered normal. Anticipation is observed when the repeat length reaches between 38-49 and a set of mild disease phenotypes occurs when the expansion reaches between 50-150 repeats. Classical DM1 is associated with more than 150 repeats and full congenital DM1 beyond 1000 repeats which can result in more severe symptoms and early death. **B)** DM2 is caused by an expansion of CCTG in intron 1 of *CNBP* on chromosome 3. Unlike DM1, DM2 has less specificity in the diagnostic applicability of the number of repeats. Normal individuals can carry up to 75 repeats but often far less (between 1-30). Disease occurs when an individual has 75 or more repeats.

Materials and Methods

A streamlined *DMPK*/*CNBP* assay and workflow was developed with prototype AmplideX® PCR/CE reagents optimized for the repeat-primed amplification of both *DMPK* and *CNBP* repeats using distinct dye-tagged primers. Sample gDNAs were amplified in separate PCR reactions for *DMPK* and *CNBP* on the same PCR plate. Amplicons were resolved on a 3500xL Genetic Analyzer CE instrument (Thermo Fisher) using POP7 polymer, with 2.5 kV, 20 sec injection and 40 min run time. Samples for Figure 6 were multiplexed post PCR and run on the CE. Genotyping for *DMPK* was achieved following sizing analysis using a ROX 1000 size ladder (Asuragen) and a four-point calibrator. Sanger sequencing was accomplished by cloning purified gene-specific *DMPK* PCR product into a vector and then screening for inserts via colony PCR. Vectors containing inserts were then sequenced.

*Proof-of-concept data only
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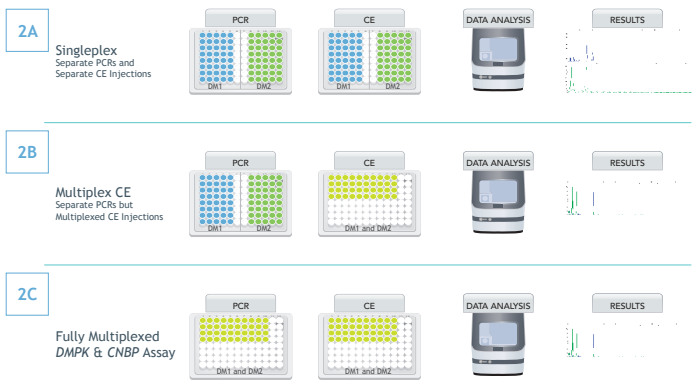

Figure 2. *DMPK*/*CNBP* PCR/CE Workflows. A) The majority of data for Figure 3, Figure 4, and Table 1 were generated using the framework depicted. This singleplex workflow allows samples to be run on the same plate in the thermocycler and by CE. **B)** The assay can also be performed in separate thermocycling reactions but on the same plate, yet multiplexed during CE injection. This methodology was used to generate Figure 6. **C)** Further, it may be possible to fully multiplex this assay into a single-tube PCR and single CE injection.

Results

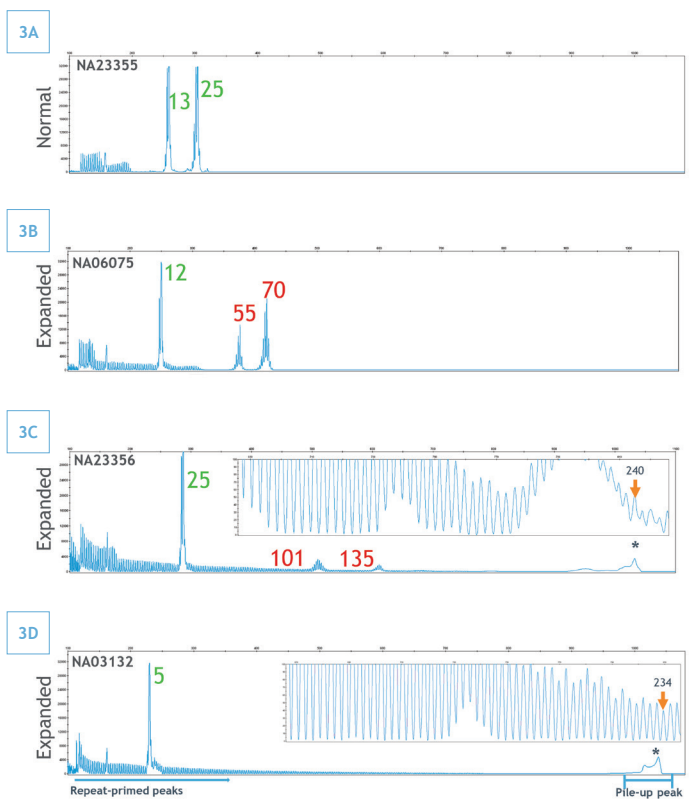

Figure 3. Representative *DMPK* PCR/CE Results. A) Coriell sample NA23355 with normal genotype (13 and 25 CTG repeats shown in green text by gene-specific amplicons). **B)** NA06075 with *DMPK* genotype containing a normal allele plus mosaic expanded alleles greater than 50 repeats (12, 55, 70). **C)** NA23356 with *DMPK* genotype of 25, expanded include mosaic alleles at 101 and 135 repeats plus evidence of an expanded allele with >200 repeats. **D)** NA03132 with *DMPK* full classic genotype (5, expanded) without mosaicism detectable >200 repeats.

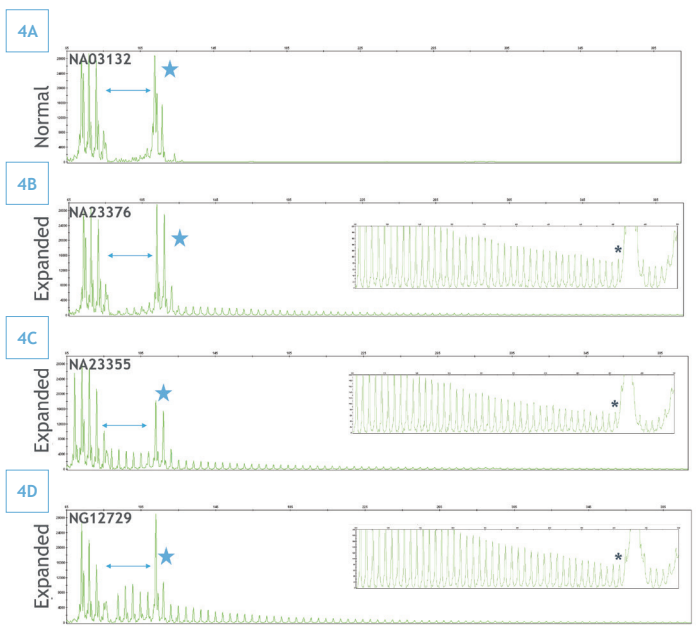

Figure 4. Representative *CNBP* PCR/CE Results. A) Coriell sample NA03132 with a normal *CNBP* genotype. The observed $(CCTG)_n$ repeat peaks show combined readout for both alleles. Non-expanded alleles typically manifest as taller peaks in the leftmost region of the profile. The largest allele detected in this region is indicated with a star. The blue arrow indicates an interruption pattern (typically seen in normal samples). **B-D)** Samples with expanded *CNBP* genotypes. Assay output for these samples show $(CCTG)_n$ repeat peaks for both the normal and expanded allele in each case where the last detectable normal peak is indicated with a star, and the 100th repeat peak is indicated with an asterisk for expanded alleles.

Sample	*DMPK* Allele 1	*DMPK* Allele 2	*CNBP* Status
NA03132	5	>200	Normal
NA03696	12	>200	Normal
NA03697	12	>200	Normal
NA03756	13	>200	Normal
NA03759	14	>200	Normal
NA03991	14	110, >200	Normal
NA04567	21	>200	Normal
NA04648	5	>200	Normal
NA05152	21	>200	Normal
NA05164	5	>200	Normal
NA06075	12	55,70	Normal
NA23256	25	101, 135, >200	Normal
NA23265	12	63, 75	Normal
NA23299	22	>200	Normal
NA23300	5	>200	Normal
NA23378	22	75, 127	Normal
NA23259	12	22	Expanded
NA23355	13	25	Expanded
NA23376	20	22	Expanded
NG12729	5	21	Expanded

Sample	*DMPK* Allele 1	*DMPK* Allele 2	*CNBP* Status
RS03127	5		Normal
RS03128	12	13	Normal
RS03129	12		Normal
RS03130	5	13	Normal
RS03131	5	29	Normal
RS03132	14		Normal
RS03133	5	14	Normal
RS03134	5	13,14	Normal
RS03135	5	12	Normal
RS03136	5	15	Normal
RS03137	12	14	Normal
RS03138	5	13	Normal
RS03139	13	14	Normal
RS03140	5	15	Normal
RS03141	5	14	Normal
RS03142	12		Normal
RS03143	5	12	Normal
RS03144	12	14	Normal
RS03145	5	13	Normal
RS03170	15	22	Normal

Table 1. *DMPK* and *CNBP* Genotypes for 40 Samples. Samples in bold orange text are also presented in Figure 3 or 4. The prototype *DMPK* assay identified 16/16 previously-reported expansions, 13 of which had expansions of 100 or more repeats. The prototype *CNBP* assay correctly flagged 4/4 previously-characterized samples and identified 36 samples as normal genotype including 16 Coriell *DMPK* samples and 20 normal healthy volunteers. Samples with greater than 75 repeats are reported as expanded.

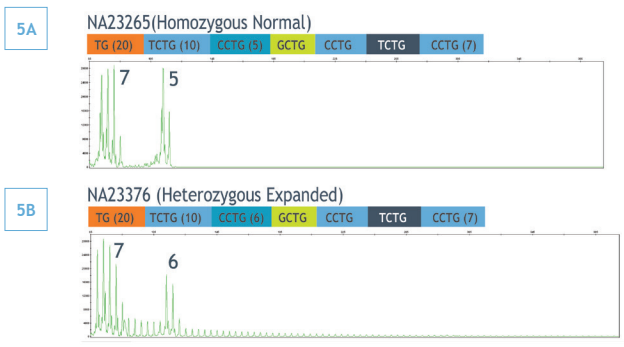

Figure 5. Comparison of *CNBP* PCR/CE Electropherograms Traces with Sanger Sequencing Results. A) CE trace for NA23265, a homozygous normal sample. Above the CE trace is the consensus sequence we obtained from Sanger sequencing the *CNBP* locus. This sequence contains the standard interruption pattern that is characteristic of normal samples. **B)** CE trace for NA23376, a heterozygous expanded sample. The normal allele contains the standard interruption pattern whereas the expanded allele can be observed extending well beyond the primary peak pattern.

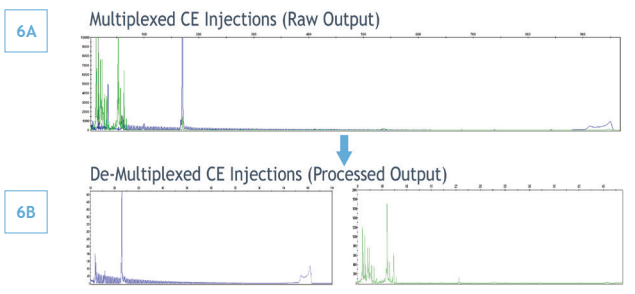

Figure 6. Unified *DMPK*/*CNBP* Workflow Output. A) CE trace for NA03132, a sample with unexpanded *CNBP* but expanded *DMPK*. **B)** The bottom two figures depict the deconvolved CE results with the *DMPK* expansion in the FAM (blue) channel and unexpanded *CNBP* in the HEX (green) channel.

Conclusions

- Prototype *DMPK* and *CNBP* PCR/CE assays demonstrated highly resolved repeat-specific signals, repeatable results, and accurate categorical classifications.
- The *DMPK* PCR/CE assay detected 16/16 samples with repeat expansions in agreement with published data, as well as expected results with presumed normal samples.
- The *CNBP* PCR/CE assay identified 4 expanded samples and 36 unexpanded samples, consistent with expectation.
- The *CNBP* PCR/CE assay revealed interruption patterns in both normal and expanded samples.

References

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