Asuragen®

AmplideX®

DM1 Dx Kit

Instructions for Use

CE-IVD for US Export Only

For In Vitro Diagnostic Use

REF 76048 CE IVD

DI 863840000261

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Intended Use

The AmplideX® DM1 Dx Kit is an in vitro nucleic acid amplification kit for the analytical assessment of CTG repeats in the 3' untranslated region of the DMPK gene to aid in the diagnosis of myotonic dystrophy type 1 (DM1). The kit employs polymerase chain reaction (PCR) on extracted deoxyribonucleic acid (DNA) followed by capillary electrophoresis. The kit generates numerical values for alleles up to and including 200 repeats and a categorical value for alleles > 200 repeats to facilitate genotyping.

For size estimation beyond 200 repeats, the kit provides instructions to perform an optional PCR followed by agarose gel electrophoresis (AGE) for assessment purposes only.

Limitations

- This kit is intended for in vitro diagnostic use.
- This kit has been validated for use with genomic DNA (gDNA) isolated from whole blood collected in EDTA tubes and processed within 14 days.
- Buccal and chorionic villus sampling have also been evaluated. This kit has not been evaluated for use with other specimens including muscle tissues.
- This kit is designed to detect and size the CTG repeat in the 3' untranslated region of the *DMPK* gene. The kit does not detect any other sequence variations in the gene.
- Do not perform the optional AGE in the absence of the required CE steps, as false negative calls may result. The AGE is constrained by biological limitations (e.g. high somatic mosaicism) and technological limitations (e.g. occasional inability to resolve extremely large, highly mosaic expansion).
- Substances that may interfere with the PCR amplification of gDNA include certain drug compounds and heparin.

Warnings and Precautions

- Use appropriate personal protective equipment when working with these materials.
- **WARNING!** CHEMICAL HAZARD. Hi-Di™ Formamide. Causes eye, skin, and respiratory tract irritation. Possible developmental and birth defect hazard. Avoid breathing vapor. Use with adequate ventilation. Storage recommended based on manufacturer's requirements.
- Follow Universal Precautions in compliance with OSHA 1910:1030, CLSI M29, or other applicable guidance when handling human specimens.
- DNase contamination can cause degradation of the DNA samples. Use nuclease-free labware (e.g. pipettes, pipettes tips, reaction vials).
- PCR product carryover contamination can result in false positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Do not pool components from different reagent batches or lots.
- Do not use reagents after the labeled expiration date.
- Do not interchange the reagent tube caps, which may cause cross-contamination or degradation of reagents.
- The reagents have been verified for up to 6 total uses through volumetric and freeze-thaw studies. Additional uses are not recommended.

- Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible results. Ensure even distribution of master mix, which is viscous and can accumulate within the pipette tip. Ensure that micropipettors are validated, maintained, and calibrated according to the manufacturer's instructions.
- Prior to use, ensure that the thermal cycler and genetic analyzer are validated, maintained, and calibrated according to the manufacturer's instructions.
- When performing CE analysis on the 3730 instrument, run conditions differ between the AmplideX® PCR/CE *FMR1* Kit and the AmplideX DM1 Dx Kit. Refer to oven temperature guidelines provided within this document.
- Do not use the 3730*xl* (96-well capillary) for fragment analysis.
- Potential rare minor allele frequencies (MAF ≤ 1%) or unidentified polymorphisms that inhibit PCR or primer binding.
- A positive control must be run in the assay and compared to previously collected data to identify any drift in reference materials.
- Commercially available reference DNAs used as a calibrator could shift in performance (for example, from one lot to the next) that may result in miscalling repeats for unknown specimens.

Summary and Explanation

Myotonic dystrophies are autosomal dominant, multisystemic disorders with incomplete penetrance and variable expressivity characterized by progressive myopathy and myotonia. Myotonic dystrophy type 1 (DM1) is caused by CTG trinucleotide expansions in the 3' UTR of the myotonic dystrophy protein kinase gene (DMPK; OMIM 605377) on chromosome 19q13.3. The trinucleotide repeat mutations account for > 99% of disease causing mutations in DM1. DM1 is the most common inherited muscular dystrophy in adults.

DM1 is usually divided into four different clinical subtypes: mild, classical, juvenile, and congenital. The severity of disease and age at onset is roughly correlated with the size of the CTG repeats. Specimen results should be interpreted according to local clinical and regulatory guidelines. For example, Kamsteeg *et al.* (2012) defines four categories: no expansion (homozygous or compound heterozygous for a normal allele size), a heterozygous expansion in the size range of 36–50 repeats, a heterozygous expansion in the size range of 51–150 repeats, and a heterozygous expansion with a size over 150 repeats.

The AmplideX DM1 Dx Kit employs DNA amplification and fragment analysis to generate numerical repeat values in these ranges. For exploratory size estimation beyond 200 repeats, the kit facilitates an optional AGE analysis.

Principle of the Procedure

The AmplideX® DM1 Dx Kit can be used to amplify the *DMPK* trinucleotide CTG fragment from purified genomic DNA (gDNA). Amplified products are resolved by capillary electrophoresis (CE). Specimens flagged with > 200 repeats can optionally be assayed by agarose gel electrophoresis (AGE). Amplicon sizes determined during CE are converted to repeat lengths manually or by using the AmplideX PCR/CE *DMPK* Macro. The AmplideX PCR/CE *DMPK* Macro can determine size and mobility correction factors from batch run calibrators automatically or can use default values. Manual AGE size conversion relies on comparison to a DNA ladder with known fragment sizes.

PCR Methods

The kit includes reagents to perform *DMPK* CTG repeat-primed (RP) PCR with an option for gene-specific (GS) only PCR (**Figure 1**). GS PCR uses two primers that flank the $(CTG)_n$ repeat region and products represent full-length alleles (**Figure 1A**). CTG RP-PCR is primarily distinguished from the conventional two-primer, GS PCR by the addition of a third PCR primer complementary to the *DMPK* trinucleotide repeat region. The resulting electropherogram includes both full-length PCR products and CTG RP amplicons (**Figure 1B**). The CTG RP-PCR products correspond to individual PCR amplicons from each combination of the repeat primer with the GS reverse primer creating a “sawtooth” repeat pattern. These RP peaks are separated by ~3 bp, or 1 repeat unit, as expected.

The combination of the repeat profile and GS peaks provides two complementary data outputs. These peaks provide important confirmatory information of each specimen’s molecular features, resolve zygosity, and report the presence of large expanded alleles (> 200 repeats) through both RP peaks and a characteristic “pile-up” peak of amplicons larger than the linear resolution of CE (**Figure 1C**).

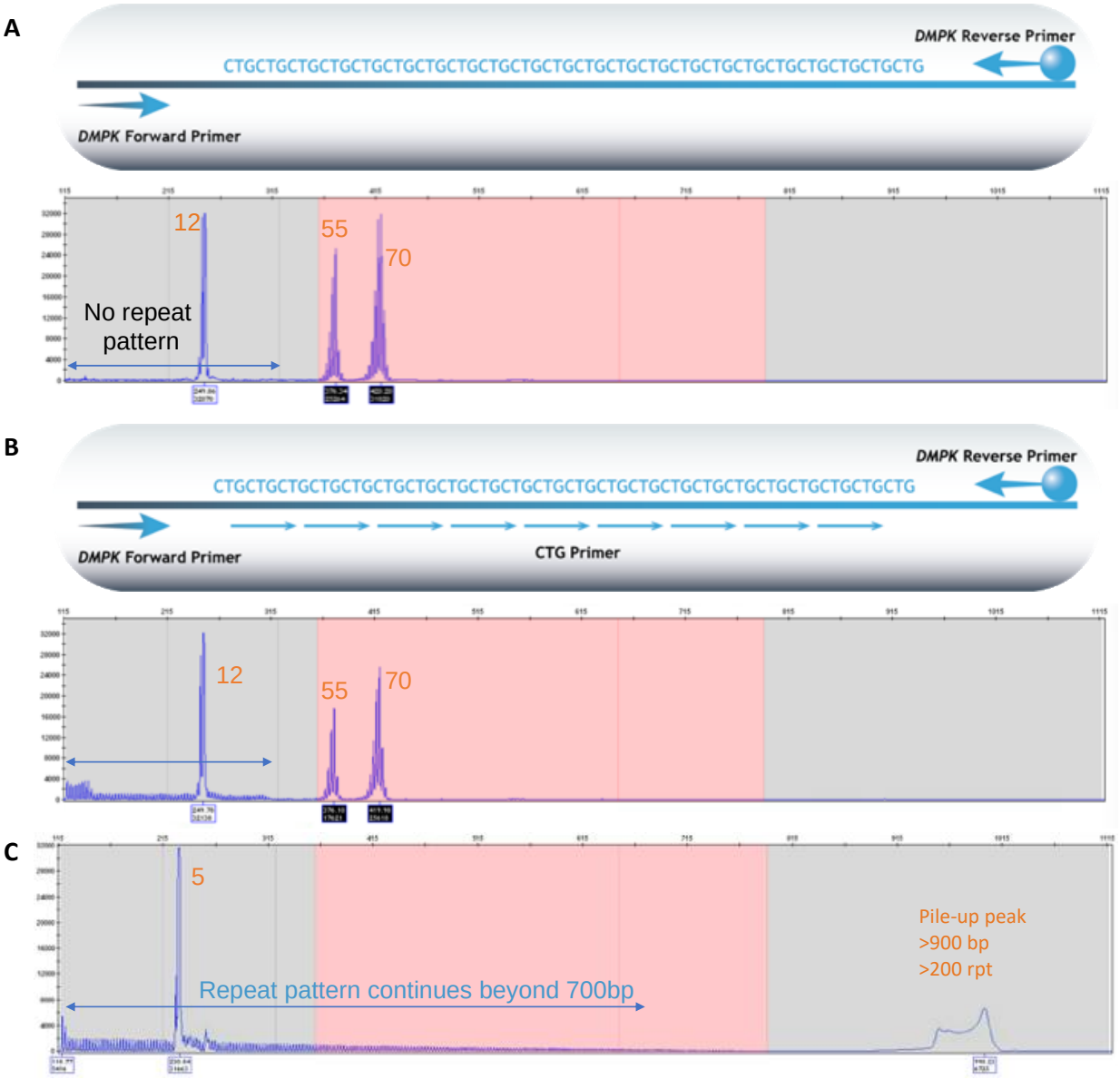


Figure 1. *DMPK* PCR Methodologies highlighting the features of **(A)** GS-only PCR with 2 primers, and **(B)** the CTG RP-PCR with 3 primers amplifying mosaic Coriell Cell Repository (CCR) sample NA06075 (12, 55, 70 repeats). **(C)** Specimens with alleles > 200 repeats can be identified by both the repeat pattern extending beyond 700 bp and the corresponding pile-up peak.

Workflow

The workflow for the test includes PCR master mix setup, thermal cycling, and analysis using either CE or AGE. Purified gDNA is first added to a PCR reaction well containing a master mix with the CAG/CTG PCR Mix, *DMPK* F,R FAM Primers, *DMPK* CTG Primer, and Diluent in a total of 10 µL. After thermal cycling, unpurified PCR products are directly mixed with Hi-Di™ Formamide and ROX 1000 Size Ladder. Following denaturation, amplicons are resolved on an Applied Biosystems® Genetic Analyzer running POP-7™ polymer (3130, 3730, or 3500 series). A schematic of the workflow is shown in **Figure 2**.

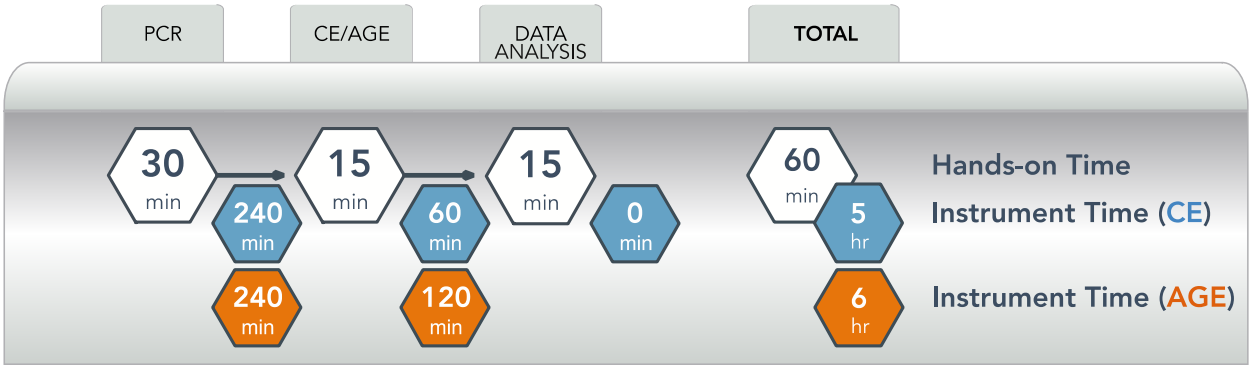


Figure 2. The workflow is streamlined from specimen to answer. The assay can be performed within a 7 hour work shift for either PCR/CE (for 4–24 specimens with per injection run times) or PCR/AGE (12 specimens). Total hands-on time for either workflow is approximately 1 hour. AGE analysis is intended as an optional reflex test for samples at >200 repeats.

After resolving PCR products by CE, the resulting electropherograms are analyzed by visual inspection to identify full-length GS product peaks and repeat peaks. These peaks are converted from size in base pairs to the number of CTG repeats using calibrator-derived batch run correction factors or verified default values appropriate for each instrument configuration.

GS peaks are detected within the linear range of the instrument up to 200 CTG repeats (~800 bp). Beyond 200 CTG repeats, the size of the PCR product exceeds the ability of the capillary polymer to adequately resolve and determine size of fragments. A characteristic “pile-up” peak is often observed instead (**Figure 1C**). The kit therefore provides a categorical interpretation of any peak > 200 repeats. In addition to CE sizing information, qualitative trace features can differentiate homozygous alleles from those with one large expanded allele, as well as sequence variability such as interruptions within the 3’ portion of the repeat region.

Sizing of *DMPK* PCR products exceeding 200 CTG repeats may be achieved from a PCR reaction containing only GS primers (*DMPK* F,R FAM-Primers). This PCR is cycled identically to the PCR/CE, but the PCR products are resolved by AGE. The performance at this step is sometimes constrained by biological limitations (e.g. high somatic mosaicism) and technological limitations (e.g. occasional inability to resolve extremely large, highly mosaic expansions). AGE should not be performed in the absence of the required CE steps, as false negative calls may result. AGE should also not be performed using the three-primer PCR configuration, only the two-primer configuration.

Use of Calibrators

Calibration is required. Refer to the Calibration section for additional information and recommended calibrator samples. A calibrator should be used initially to establish the mobility correction factors using the specific CE parameters in a given laboratory. However, calibration may not be required during each batch run.

Use of Batch Run Controls

Batch run controls (positive and negative) are assayed in every run. The Diluent provided within the reagent set may be used as a negative in the form of a no-template control (NTC) to assess for laboratory contamination. Cell lines or corresponding purified gDNA can be obtained from various repositories such as Coriell Cell Repository (CCR), and published reference materials are described in Kalman *et al.* (2013). Positive Controls such as CCR gDNA samples NA23378 (22, 76, 127 repeats), NA04648 (5, 1000 repeats), NA05164 (21, 325 repeats), and NA06075 (12, 55, 70 repeats) can be utilized. Note that the published allele repeat numbers and those observed with the AmplideX® DM1 Dx Kit assay may differ. Alternatively, gDNA extracted from other well-characterized cell lines with expanded *DMPK* alleles may be used for positive controls.

Reagents Supplied with the Kit

Item #	Description	Storage Temp
145586	CAG/CTG PCR Mix	-15 to -30 °C
145587	*DMPK* F,R FAM-Primers	-15 to -30 °C
145589	*DMPK* CTG Primer	-15 to -30 °C
145588	ROX 1000 Size Ladder	-15 to -30 °C
145183	Diluent	-15 to -30 °C

Note: The reagents have been verified for up to 6 total uses through volumetric and freeze-thaw studies. Do not perform additional uses.

Digital Files Supplied with the Kit

Digital files are provided for download.

- AmplideX DM1 Dx Kit Protocol Guide: https://asuragen.com/ceivd
- AmplideX PCR/CE *DMPK* Macro: http://software.asuragen.com
- AmplideX PCR/CE *DMPK* Macro User Guide: https://asuragen.com/ceivd

Required but Not Provided

Item Numbers, where provided, may vary by region. Please contact Asuragen Customer Solutions for assistance.

- Reagents for gDNA isolation are not included. DNA can be extracted via common, laboratory-validated sample preparation methodologies that ensure high-quality, intact DNA such as the Qiagen® QIAamp® DNA Blood Mini or equivalent kit.
- Genetic Analyzers running POP-7™ polymer: Applied Biosystems®, 3130, 3730, or 3500 series
- POP-7 Polymer: Applied Biosystems, P/N 4393708
- Hi-Di™ Formamide: Applied Biosystems, P/N 4311320
- DS-30 or DS-31 Matrix Standard Kit (Dye Set D), Applied Biosystems P/N 4345827 or 4345829
- General laboratory equipment and workspace to perform PCR and dilute buffers
- Thermal cycler: Applied Biosystems Veriti™ (not Veriti Fast or other models in the series) or 9700 (aluminum block)
- Centrifuge capable of spinning 96-well plates
- Multi-channel micropipette or repeat micropipettors capable of pipetting 1–10 µL are optional but recommended for accurate dispensing of samples and master mix.
- 96-well PCR Plates: Thermo Scientific™ P/N AB-0900 or equivalent
- Adhesive PCR Plate Seals: Thermo Scientific P/N AB-0558, Phenix LMT-0028 or equivalent
- PCR Compression Pad: Applied Biosystems P/N 4312639 or equivalent

Optional and Not Provided (AGE Step)

Item Numbers, where provided, may vary by region. Please contact Asuragen Customer Solutions for assistance.

- Quick-Load® Purple 1 kb Plus DNA Ladder, New England Biolabs® P/N N0550S
- Reliant™ Mini gels (1% SeaKem™ Gold Agarose, Ethidium Bromide, 12-well), Lonza™ P/N 54820
- Gel Loading Dye, Purple (6X), no SDS, New England Biolabs P/N B7025 (supplied with 1 kb Plus DNA ladder)
- 10X Bionic™ Buffer, Sigma® P/N B6185
- Gel Documentation: UVP GelDoc-It®2 or equivalent
- Power supply: PowerPac™ Basic, Bio-Rad® P/N 1645050 or equivalent
- Horizontal Agarose Gel Chamber: Owl Separation System D3 (wide), Thermo Scientific FB-SB-710 (mini), or equivalent

Number of Reactions

- The provided reagents are sufficient for up to 32 reactions (calibrators, controls, or specimens) of GS PCR or CTG RP-PCR.
- The reagents have been verified for up to 6 total uses through volumetric and freeze-thaw studies. Additional uses are not recommended.
- Master mixes can be prepared for the appropriate number of samples with a recommended total number of at least 5 reactions per run.

Reagent Stability

- The reagents are stable through the labelled date when stored under the specified conditions.

Storage & Handling

- Store the reagents in a non-frost-free freezer protected from light at -15 to -30 °C.
- Allow reagents to completely thaw at room temperature before use (10–15 minutes).
- **Vortex all reagents after thawing**.
- Prior to opening, briefly centrifuge each component vial to collect the solution at the bottom of the vial.
- Assay setup should be performed at room temperature (approximate range of 18 to 25 °C) within 30 minutes.

Procedural Steps

Pre-Analytical Steps

Genomic DNA (gDNA) extracted via common, laboratory-validated sample preparation methodologies from whole blood collected in EDTA is compatible with the AmplideX® DM1 Dx Kit. It is recommended that whole blood specimens are stored at 2–8°C prior to processing within 14 days of collection (Alexandre *et al.* 2016). Buccal swabs and chorionic villus sampling (CVS) are also acceptable specimens and should be processed following laboratory-validated procedures. Purified gDNA must be evaluated for concentration (e.g. via OD_{260}) and purity ($OD_{260/280}$ of 1.8 ± 0.3). Store DNA below -15°C. Use mass inputs between 20 to 80 ng in each reaction (for example, by adding 2 µL of DNA at 10 to 40 ng/µL).

Overview of AmplideX DM1 Dx Kit

The protocol involves three procedural steps:

1. PCR master mix setup and thermal cycling
2. Electrophoretic separation: Capillary electrophoresis (CE) or agarose gel electrophoresis (AGE)
3. Fragment sizing analysis

These instructions are written for the preparation and analysis of GS PCR or CTG RP-PCR products. There is only one difference between the two PCR protocols: GS (only) PCR is used for AGE but is performed without the *DMPK* CTG Primer in the master mix setup. The protocol is written for a single reaction; master mixes can be prepared for the appropriate number of reactions at each step of the protocol.

The workflow should proceed in a unidirectional manner starting with a dedicated pre-amplification area and moving to a segregated post-amplification area. Amplified product should remain in the post-amplification area to minimize the risk of amplicon contamination. Correction factors used to convert peak size (base pairs) to repeat length are specific to the CE instrument being used. Calibration is recommended to establish these correction factors when using this assay for the first time or after CE instrument service (such as replacing the capillary). Refer to the Fragment Sizing Analysis (PCR/CE or PCR/AGE) and Quality Control Procedures sections for guidance.

Note: All specimens must be profiled by PCR/CE. When sizing of longer alleles is desired, the PCR/AGE protocol may be utilized.

PCR Master Mix Setup and Thermal Cycling

1. Thaw all reagents for 10–15 minutes at room temperature. Vortex all tubes (3–5 times pulse vortexing) and briefly centrifuge to ensure contents are at the bottom of the tube. **Note**: The CAG/CTG PCR Mix may be occluded or have observable precipitation when cold. After completely thawing the tube till no ice remains, vortex to ensure homogeneity.
2. Assemble the components in the order listed below. Prepare a master mix with 10% overage. **Note:** The CTG/CAG PCR Mix is viscous; retract piston slowly to acquire proper volume of solution.

PCR Setup **per reaction**:

Reagent	Optional Gene-specific PCR (PCR/AGE only)	Repeat-primed PCR (PCR/CE only)
Diluent	2.0 µL	1.0 µL
CAG/CTG PCR Mix	5.0 µL	5.0 µL
DMPK F,R FAM Primers	1.0 µL	1.0 µL
DMPK CTG Primer	0 µL	1.0 µL
DNA Specimen, Calibrator, Control*	2.0 µL*	2.0 µL*
Total Reaction Volume	**10.0 µL**	**10.0 µL**

*Do not add to master mix

3. Thoroughly vortex the master mix (3–5 times pulse vortexing) and pulse-spin prior to adding to PCR plate or strip-tubes. **Note**: The master mix must be vortexed prior to dispensing to ensure adequate mixing of all reagents.
4. Dispense 8.0 µL master mix to each well. Use a repeater pipette if available. If using a standard pipettor, change the pipette tip at the start of every column on the plate.
5. Add 2.0 µL of DNA (calibrator, control, or specimen) to each well. Pipette up and down at least twice to ensure adequate mixing.
6. Seal the plate with an adhesive film seal; ensure that all the wells and the plate edges are properly sealed.
7. Gently vortex the plate.

8. Centrifuge the plate to collect the reaction at the bottom of the well (program the instrument for 1 minute at 1600 rcf).
9. Transfer the sealed PCR plate to a preprogrammed thermal cycler and run the following cycling protocol. **Note:** Thermal cycling requires about approximately 4 hours depending on ramp rates.

PCR/CE or PCR/AGE Cycling Protocol	
Description	**Duration**
1 hold	95 °C for 5 min
10 Cycles	97 °C for 35 sec
	64 °C for 35 sec
	68 °C for 4 min
18 Cycles	97 °C for 35 sec
	64 °C for 35 sec
	68 °C for 4 min + 20 s/cycle
1 hold	72 °C for 10 min
1 hold	4 °C forever

10. Transfer PCR products for CE analysis or store at -15 to -30 °C until analyzed. **Note**: PCR product stability on deck at 4°C has been verified up to 24 hours and stability at -15 to -30°C has been verified to 7 days.
11. After thermal cycling, proceed to CE or AGE. **Note**: Thermal cycling with both sets of primers is not compatible with AGE analysis.

Fragment Sizing with Capillary Electrophoresis Platforms Using POP-7™

For CTG repeat size analysis, several CE systems may be used (e.g. Applied Biosystems® Genetic Analyzers). Please refer to the specific instrument manuals or contact Technical Support (support@asuragen.com) for further platform specific information.

Note: The CE step must be performed. Use of the optional AGE step in the absence of the CE step may yield false negative results.

The following instructions are specific for Genetic Analyzers running POP-7 polymer.

1. Thaw the Hi-Di™ Formamide and ROX 1000 Size Ladder at room temperature.
2. Thoroughly vortex (3–5 times pulse vortexing or 15 seconds) and briefly centrifuge tubes before use.
3. Prepare a CE master mix solution by adding components in the order listed in the following table using 10% overage.

CE Setup **per reaction**:

Reagent	PCR/CE Mix
Hi-Di™ Formamide	11.0 µL
ROX 1000 Size Ladder	2.0 µL
PCR Reaction Product*	2.0 µL*
Total Reaction Volume	**15.0 µL**

*Do not add to master mix

4. Mix all added reagents (by pulse vortexing 3–5 times), and centrifuge briefly to collect the solution at the bottom of the tube.
5. Transfer 13.0 µL of Hi-Di™ Formamide/ROX solution to each well of a new CE analysis plate. **Note**: Reactions must be matched to the injection configuration of the Genetic Analyzer (e.g. A1–H2, A3–H4…A11–H12) in appropriate groups of 4, 8, 16, 24, or 48 capillaries. If insufficient samples will be run for any injection group, fill remaining wells with 15 µL of Hi-Di Formamide.
6. Transfer 2 µL of PCR products to the CE plate, pipetting up and down 2 to 3 times to mix. **Note**: A multi-channel pipette is recommended for transfer.
7. Seal the plate with an adhesive film seal. Ensure that all the wells and the plate edges are properly sealed.
8. Gently vortex the plate.
9. Centrifuge the plate to remove bubbles (program the instrument for 1 minute at 1600 rcf). **Note**: Ensure all bubbles are removed from the wells.
10. Denature for 2 minutes at 95°C followed by 4°C hold until ready for injection on the CE instrument. Alternatively, the products may be stored on ice and protected from light after the denaturation step. **Note**: The calibrator, control, or specimens must be denatured prior to CE analysis and may be run up to 24 hours after denaturation.
11. Prepare the genetic analyzer for data acquisition according to manufacturer directions. Use the injection and run conditions provided in **Table 1**. The following considerations apply:
    a. The instrument must be calibrated for the detection of both FAM and ROX fluorescent dyes.
    b. Use the factory-installed Fragment Analysis Protocol for POP-7™ polymer and capillary length for the instrument as a base protocol.
    c. Adjust the injection conditions and run time according to the particular instrument configuration and capillary length.

Table 1. Injection and Run Time adjustments to the default Fragment Analysis Protocols for different instrument classes and capillary lengths running POP-7 polymer

Instrument	Capillary Length	Injection	Run Time	Oven Temperature
3130*xl*	36 cm	2.5 kV, 20 s	15kV, 2400 s	60°C
	50 cm		15kV, 4200 s	
3730	36 cm	2.5 kV, 10 s	15kV, 2400 s	63°C
	50 cm	2.5 kV, 20 s	15kV, 4200 s	66°C
3500xL	50 cm	2.5 kV, 20 s	19.5kV, 2400 s	60°C

Optional Fragment Sizing with Agarose Gel Electrophoresis

For sizing of alleles with repeats larger than 200 CTGs, optional assessment by AGE may be performed. The PCR must be performed without the *DMPK* CTG Primer as previously described. The following instructions were established in the context of the Optional and not Provided (AGE step) section. A brief video detailing this procedure can be provided by contacting support@asuragen.com.

Note: The CE step must be performed. Use of the optional AGE step in the absence of the CE step may yield false negative results.

1. Dilute the Quick-Load Purple 1 kb Plus DNA ladder 10-fold by mixing 146.7 µL of Diluent + 33.3 µL 6X Gel Loading Dye + 20 µL Quick-Load 1 kb Plus DNA Ladder. Store unused ladder at 4°C. **Note**: Making 200 µL of 1:10 diluted ladder is sufficient for 10 gels loaded in two wells per gel at 10 µL per well.
2. Prepare fresh 1X Bionic™ Buffer from 10X Bionic Buffer Concentrate. Mix 100 mL Bionic Buffer 10X Concentrate with 900 mL deionized water. Scale as necessary. **Note**: Yields 1 L of 1X Bionic Buffer, enough for a wide format gel chamber such as the Owl D3.
3. Prepare samples by adding 2 µL of 6X Gel Loading Dye, Purple to the 10 µL PCR/AGE reaction directly in the PCR plate. Briefly vortex gently to mix taking care not to create bubbles, then centrifuge to collect. **Note**: 6X loading dye can be pre-added to 8-well strip cap tubes and transferred to the sample (2 µL per sample) using a multi-channel pipette.
4. Rinse gel electrophoresis chamber with deionized water to remove any buffer salts and then wipe the gel support surface with a lint free wiper to dry. **Note**: Rinsing the gel unit at this point is helpful to eliminate lint or dust particles that could stick to the gel and show up as non-specific background when imaging the gel. Depending on band intensities for a given sample, a gel may need to be imaged at exposure times up to 1000 ms. It is also recommended to rinse the powder off gloves when handling gels.
5. (Optional) Prepare a Reliant™ gel holding tray and the mount in gel electrophoresis chamber (**Figure 3**). Skip to step 6 if a modified tray has already been made and mounted in the gel electrophoresis unit. **Note**: It is recommended to use an empty Reliant gel tray for this step to avoid damaging the gel. Once mounted in the chamber, it can be rinsed and reused several times. If this step is not followed, gel run times should be increased 10–20%.

a. Lonza™ Reliant™ Gel system Tray modified procedure:
    i. Make vertical cuts to the corner nubs of an empty gel loading tray using scissors or a razor blade.
    ii. Cut the plastic at each short end off.
    iii. Peel the paper backing from the adhesive strips on the bottom of the gel tray.
    iv. Place the tray directly into the chamber platform. Align the wells so the DNA samples will run straight.
    v. Press the tray to stick to the chamber platform.

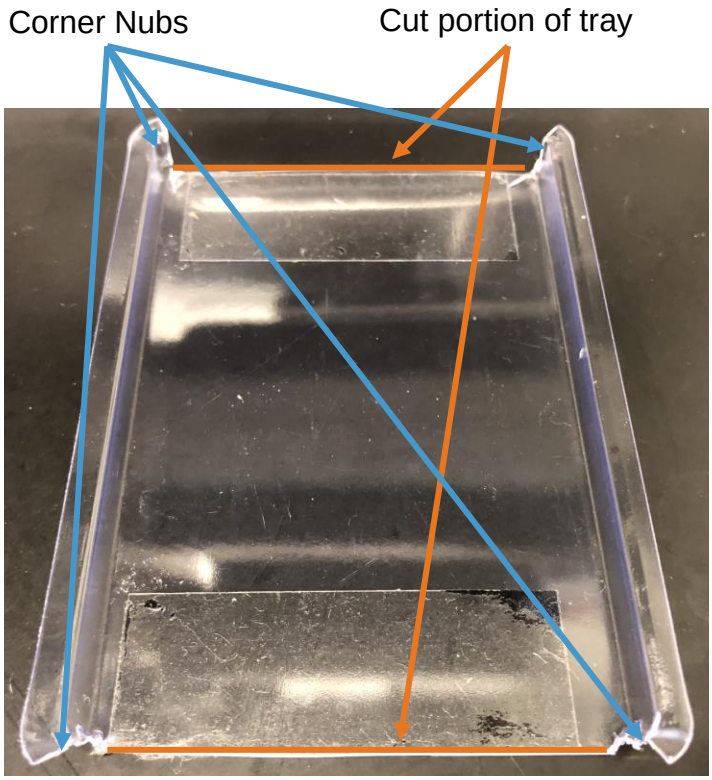


Figure 3. Gel Tray Processing.

6. Pour 1X Bionic™ buffer into the gel electrophoresis chamber to a depth of ~3 mm over the top edges of the gel tray.
7. Carefully, open the Reliant precast gel tray to avoid crushing the wells. **Note**: Well damage occurs easily if the gel tray is contorted when removing the foil cover. It is recommended to hold the gel tray flat on a solid surface while gently but firmly pulling off the foil to prevent twisting when opening.
8. Carefully, transfer the new Reliant precast gel into the process tray avoiding crushing the wells. **Note**: It is recommended to rinse the powder off gloves before handling the gel.
    a. Place the lid with foil side down over the top of the gel tray.
    b. Carefully flip the gel tray/foil lid to lay flat on bench surface.
    c. Using your thumb, apply gentle pressure on the bottom corner to push the gel out of the plastic gel tray to slide it onto the foil lid. Be careful not to crush the gel wells during this step.
    d. Once the gel comes out of the tray, discard or save the storage tray for future use to modify as described above.
    e. Use the foil to gently transfer the gel into the modified tray in the electrophoresis chamber. Slide it into place with the wells facing upwards and near the black (-) anode.

9. Ensure the gel is covered with ~2–3 mm of 1X Bionic™ Buffer. Adjust buffer volume with 1X Bionic buffer if necessary.
10. Load 10 µL of the diluted Quick-Load® Purple 1 kb Plus DNA ladder in lane 1 (and optionally in lane 12) of the gel. **Note**: Avoid making bubbles when adding the loading dye as it can make loading of samples to wells more difficult and less accurate.
11. Load 10 µL sample into separate wells using lanes 2–11. **Note**: It is recommended to load up to 10 samples and 2 ladders on each gel.
12. Electrophorese at 105V (constant voltage) until the purple dye in the 6X Gel Loading Dye has traversed about 3/4th (75%) of the gel. 100–120 minutes for larger gel chambers, 80–100 minutes for mini gel chambers.

    Notes:
    - It may be necessary to optimize run conditions specific to the lab setup, gel chamber and power supply.
    - The purple dye traverses near the 0.5 kb ladder band (~94 repeats). The normal allele bands run between 0.2 kb and 0.4 kb ladder bands.

13. Expose the gel using a gel documentation system with UV light box and ethidium bromide filter.

Tips for improving picture quality:

- Rinse powder off gloves before handling gels
- Use non-fluorescent object ruler or other object to hold gel in place
- Using a low f-stop number allows more light to enter the camera to enhance the ability to focus on details
- Other suggested settings if using the UVP GelDoc-It®2 gel documentation system:
    - Zoom = 68%
    - Focus = 67.9
    - Preview = 20%
    - 100% ROI
    - Typical exposure time for samples with > 200 repeats is about 400–1000 ms

Fragment Sizing Analysis (PCR/CE)

Fragment sizing analysis of GS or CTG RP-PCR data involves a series of steps using commercially available peak calling software to obtain the size of full-length product peaks and identify features in the run for interpretation of the data. These results are converted to repeat length as described in the Quality Control Procedures, Calibration section. The test has been verified for use with GeneMapper® and GeneMarker® commercially available peak calling software packages. An overview of the fragment sizing analysis workflow is shown in **Figure 4**.

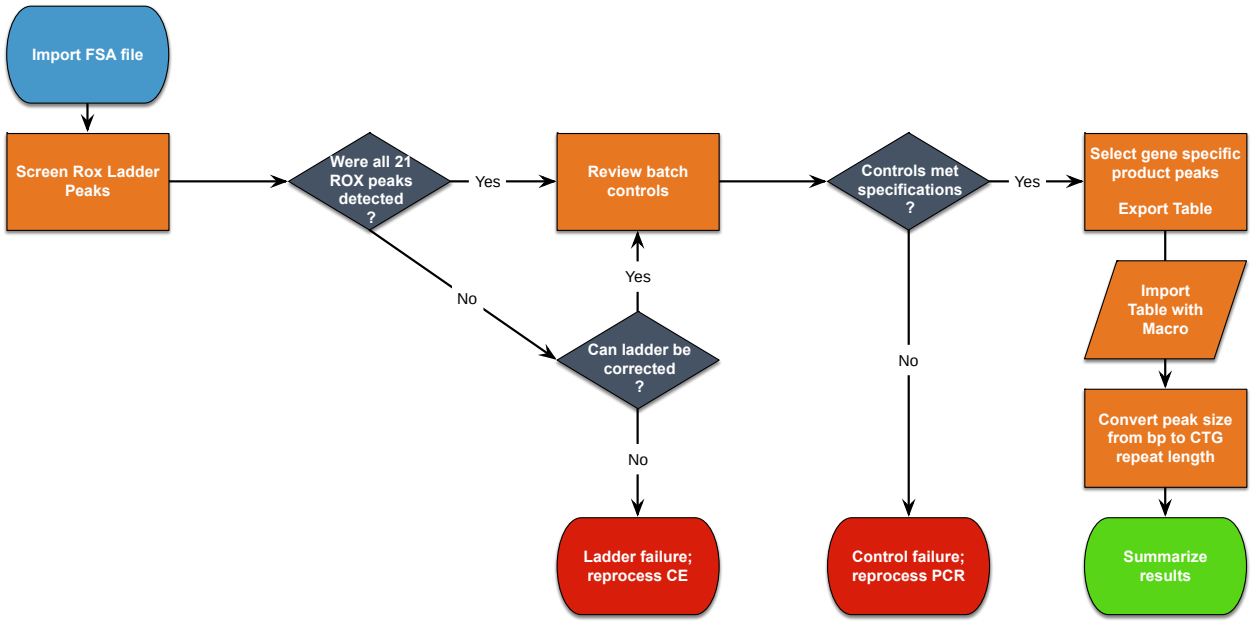


Figure 4. An overview of the fragment sizing analysis workflow including sample file processing, scoring the ladder peaks, qualifying the batch run, selecting GS peaks, and summarizing results.

Import Data and Process

1. Import the *.fsa files into the commercially available peak calling software.
2. Process files according to the methods, panels, and size standard settings established for *DMPK* PCR product analysis. These can be obtained by contacting support@asuragen.com.

Qualify the Run (Ladder Review)

1. Review the ROX 1000 Size Ladder peaks in each well.
2. Review size matches and size calling curve of the ROX 1000 Size Ladder for all samples. Identify any irregularities in the fit or any missing peaks for the ladder.

Notes:

- Ladders must be properly called before proceeding with analysis. Samples may need to be repeated if ladder peaks cannot be corrected.
- Spectral pull-up peak from the FAM channel may be observed. These peaks will generally not interfere with the sizing of the ladder. An example ROX 1000 Size Ladder size match and calling curve is shown in **Figure 5** using GeneMapper®. For assistance with ladder review in GeneMarker®, please contact Asuragen Technical Services.

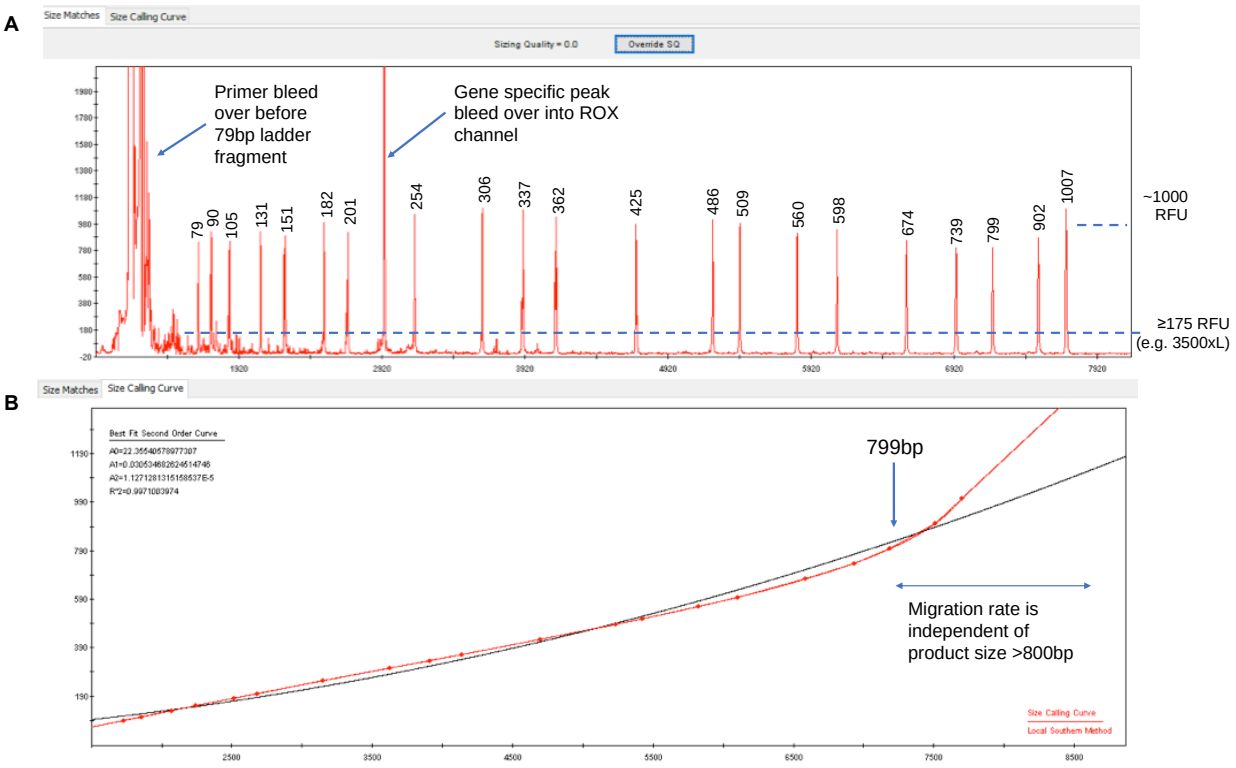


Figure 5. ROX 1000 Size Matches and Size Calling Curve on 3500xL using GeneMapper®. For assistance with ladder review in GeneMarker®, please contact Asuragen Technical Services. **(A)** The Size Match view highlights the 21 peaks of the ladder and includes an example spectral pull-up peak (arrow) from the FAM-labeled GS product peak that should be ignored in the ROX channel. Each ladder should have an RFU value ~1000 and will not be automatically called when < 175 RFU. **(B)** The Size Calling Curve view highlights the linearity of the platform.

Qualify the Run (Baseline Review)

Review all sample traces for appropriate baselines. For example, a known limitation in GeneMarker® peak calling software may lead to non-canonical baselines that prevent proper annotation of peaks. See **Figure 6**.

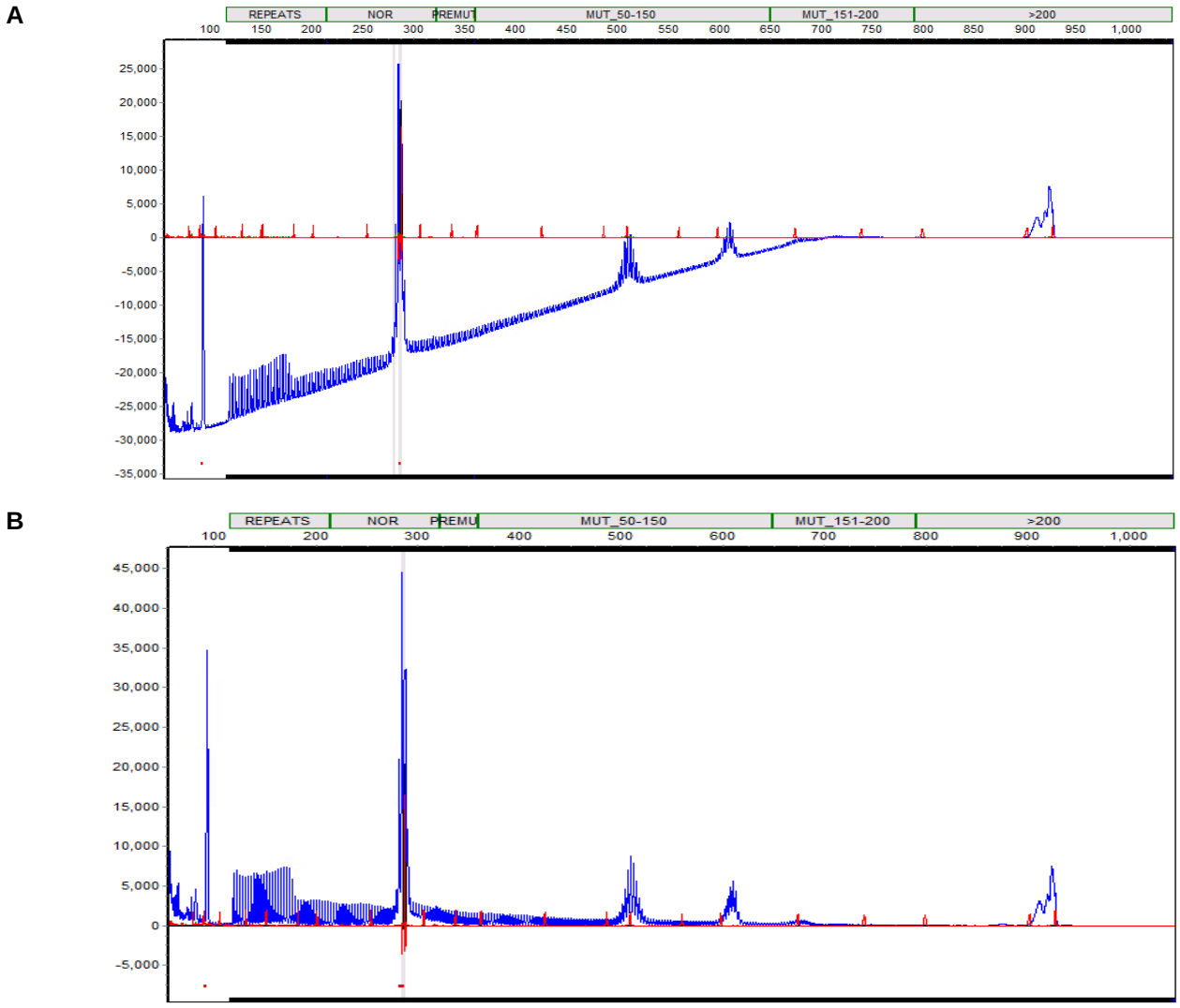


Figure 6. **Atypical baselines in GeneMarker.** The atypical baseline observed in (A) was corrected to the appearance in (B) by changing the setting for Raw Data Analysis frame start value from 0 to 1200.

Review Batch Run Calibrators

- If batch run calibrators were included, ensure that all 4 peaks< 200 repeats are identified.
- If the 4 expected peaks cannot be annotated, batch run calibration cannot be performed.

Review Batch Run Controls

- Ensure that a negative control included in the batch run meets specifications. See an example of a negative control in **Figure 16**.
- Ensure that positive control included in the batch run meets specifications. See **Table 9** and **Figure 20** for a list of example reference gDNA samples.

Perform Manual Peak Annotation

1. Deselect all peaks.
   a. By default, peaks exceeding an instrument-specific cut-off are automatically selected according to the following guidelines. Minor or low intensity peaks may be manually selected using a user-defined cut-off or 3-fold above baseline signal height. Due to the analytical sensitivity of the assay, mosaic alleles may be observed that were not previously identified in a given specimen.

Instrument	Cut-off (RFU)
3130, 3130*xl*	150
3730	175
3500, 3500xL	175

2. After deselecting all peaks, identify and select GS full-length product peaks using the guidelines illustrated in **Figure 7–11**.

- Review the electropherogram traces for peak selection criteria. For analysis of CTG RP-PCR products, the multiplicity of CTG RP peaks is deselected in order to simplify tracking, export, and conversion of the full-length GS PCR product peak to $(CTG)_n$ repeat length.
    - An example of this process is highlighted below (**Figure 7**) for both NA06075 and NA04648.
- Select highest peaks in the profile according to the characteristics throughout this guide.
- Gene-specific (GS) peaks in the normal range typically appear with very high signal (> 20,000 RFU on the 3500 series and 3730 or > 8,000 RFU for 3130*xl*).
- Mosaic specimens may have alleles with non-Gaussian distribution identified as a collection of GS peaks with similar intensities. These can be called as a range or as the centermost peak.
- Specimens appearing normal (GS peaks in the normal region without a pile-up peak indicating a large expansion) should be confirmed by ensuring that the repeat-primed (RP) peaks are present in a pattern that is concordant with the repeat length call. In **Figure 8**, Zygosity evaluation, panels A and B provide examples.
- **Note:** Peak-calling software may potentially prevent observation of pile-up peaks if they scroll off the right side of the screen. Carefully review traces for this behavior.

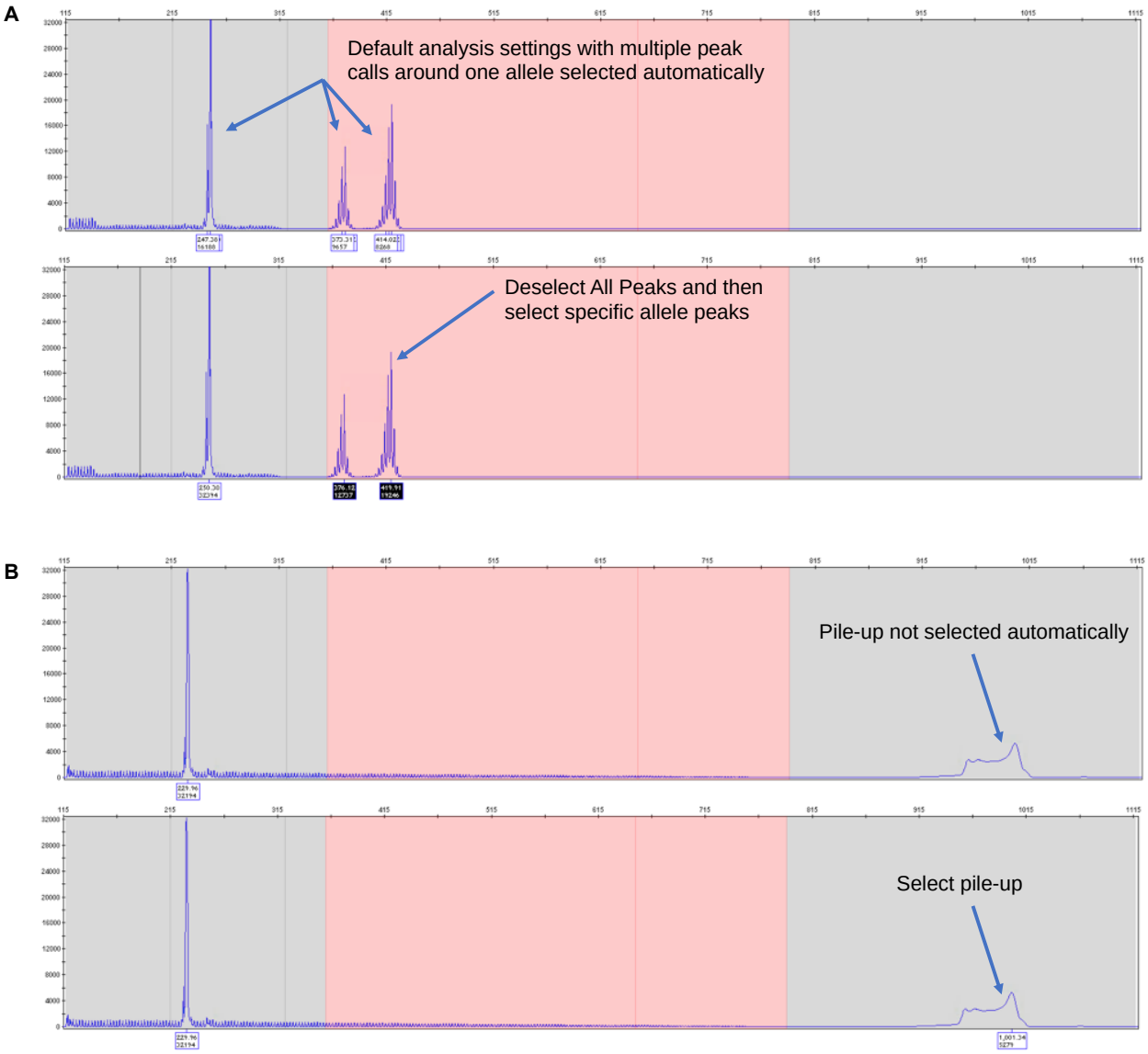


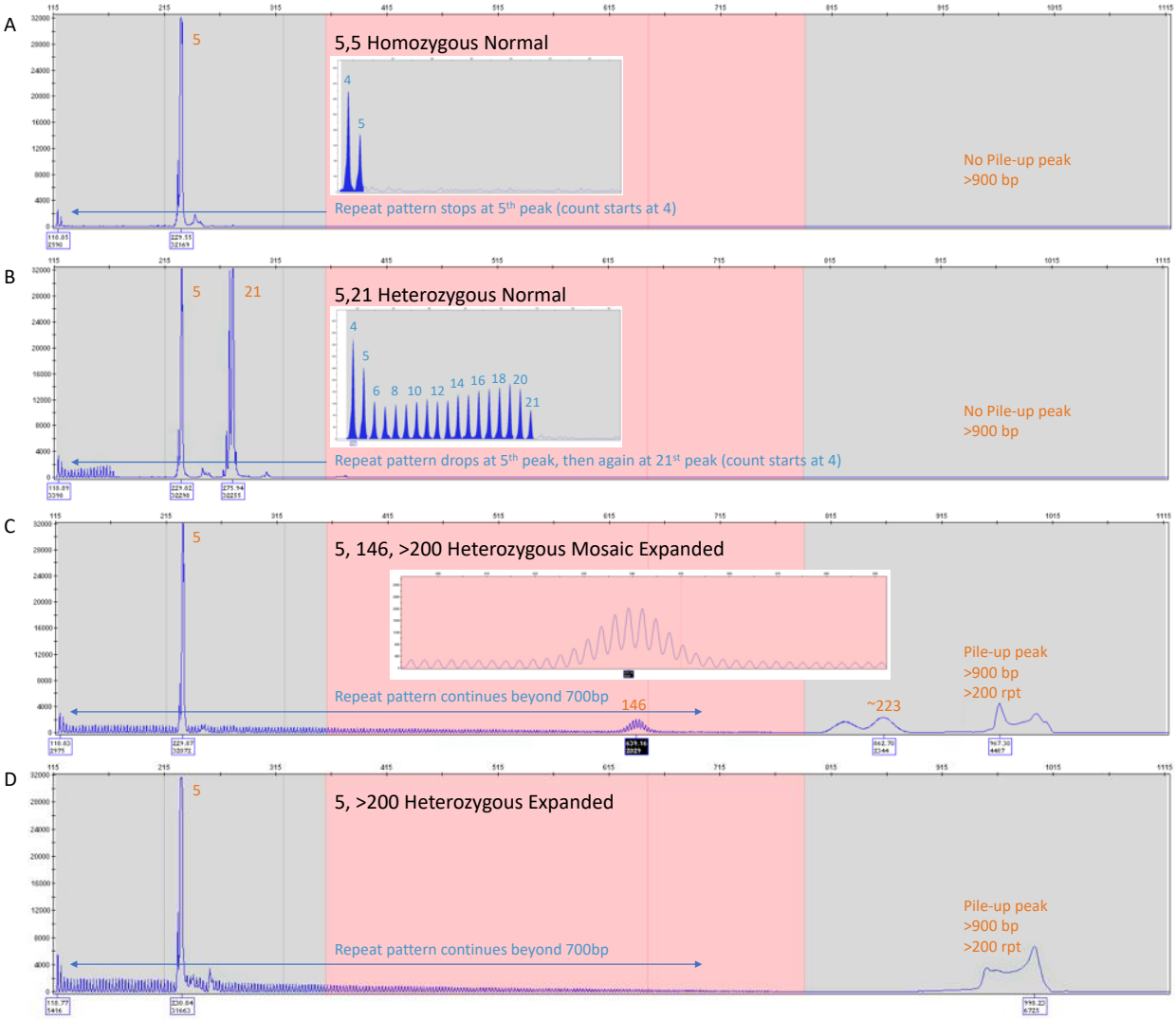
Figure 7. Peak selection guidelines based on size range and electropherogram features for samples run on a 3500xL CE instrument. **(A)** The default analysis settings for NA06075 with seven peaks selected automatically. After manual deselection and reselection of allele peaks, only the peaks with the maximum local signal intensity (RFU) are chosen. **(B)** Automatic peak selection of NA04648 failed to identify the pile-up peak. After manual deselection and reselection of allele peaks, the portion of the pile-up peak with maximum local signal intensity (RFU) is manually annotated by the analyst.

Refer to the Comparison to a Published Design in the Analytical Assessment section for a list of CCR samples and expected sizes by PCR/CE for reference.

Alleles are reported as whole-integer repeats associated with a specific genotype category. See the AmplideX® PCR/CE *DMPK* Macro User Guide for instructions on setting the limits of each genotype bin. The numerical range for PCR/CE is 5–200 repeats. Above 200 repeats, all alleles are identified categorically as > 200 CTG. If multiple peaks are identified in the region > 200, then the interpretation should be > 200, regardless of the number of peaks selected.

Refer to **Figure 8** and **Table 2** for data interpretation of a single or multiple GS allele peaks and repeat patterns. In samples with multiple alleles (mosaic), the longest allele may be reported. Lower-level minor alleles may be noted as well.

Identification of a single allele without a visible repeat pattern and pile-up peak is consistent with a homozygous specimen (**Figure 8**). Homozygous specimens contain a single GS peak and a discrete repeat pattern. Since only one major GS peak is observed in **Figure 8**, the genotype interpretation is a homozygous (5, 5), which is confirmed by the RP peak pattern (**Figure 8A**). The CTG RP-PCR repeat profile can indicate the presence of longer alleles in the amplification, irrespective of whether such alleles are detected as full-length products (**Figure 8B**) or a continuous repeat pattern and a collection of "pile-up peaks" beyond 900 bp, i.e. those that are too large to move through the polymer linearly due to size and therefore aggregate in the capillary (**Figure 8C** and **8D**). If a single normal allele is identified in combination with a repeat pattern and a pileup, this specimen is a heterozygous expanded with > 200 repeats (**Figure 8D**). Specimens with repeat interruptions at the 3' end of the repeat region may also be detected (**Figure 8E**). Consequently, the risk of a false negative result due to PCR dropout from interruptions in the repeat pattern, or of a very long allele, is reduced.



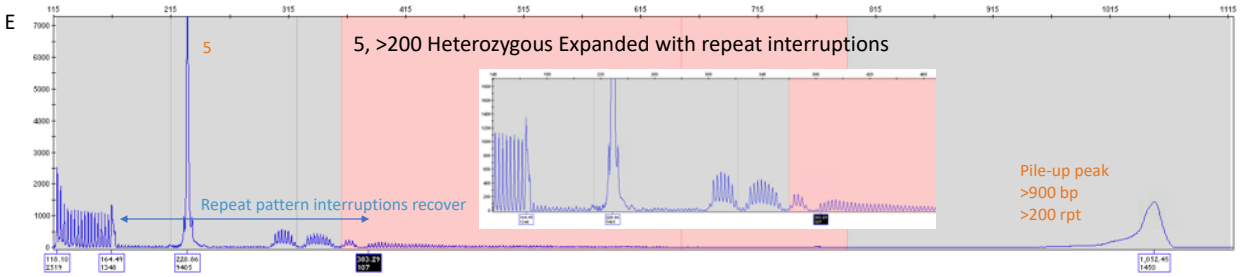


Figure 8. Zygosity evaluation. (A) This whole blood specimen was genotyped as normal homozygous (5, 5). The repeat peaks can be counted to confirm the GS allele sizing. Counting from 4, there are 2 peaks before the drop in repeat signal. **(B)** Presumed normal CCR sample NG12729 was genotyped as normal heterozygous (5, 21). Counting from 4, there are 21 allele peaks before the drop in repeat signal. **(C)** CCR sample NA23300 (5, 150–160, 550 mosaic) was genotyped as expanded heterozygous mosaic (4, 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. AGE identified a 230 repeat allele represented by the additional peak outside the linear range of the assay. The pile-up peak is due to the 550-repeat allele detected by AGE (not shown). **(D)** This whole blood specimen (5, 1200–1350 repeats) was genotyped as expanded heterozygous (5, > 200 repeats) by CE (shown) and 1100 by AGE (not shown). **(E)** This whole blood specimen (5, 480 repeats) was genotyped as an expanded heterozygous with repeat interruption (5, > 200 repeats). Each electropherogram utilized the 3-primer GS/RP PCR system.

An example of a homozygous specimen (11, 11) and heterozygous specimen (11, 12) is shown in **Figure 9**. A homozygous specimen will sometimes contain a smaller “pre-peak” that is 1 repeat smaller than the allele with a peak that is shorter in height (RFU) and narrower than the allele peak. A heterozygous specimen with alleles that differ by 1 repeat typically contains two peaks of similar height and width. In addition, a homozygous specimen will occasionally contain larger “post-peaks” that appear larger than the allele. This is depicted in **Figure 10**.

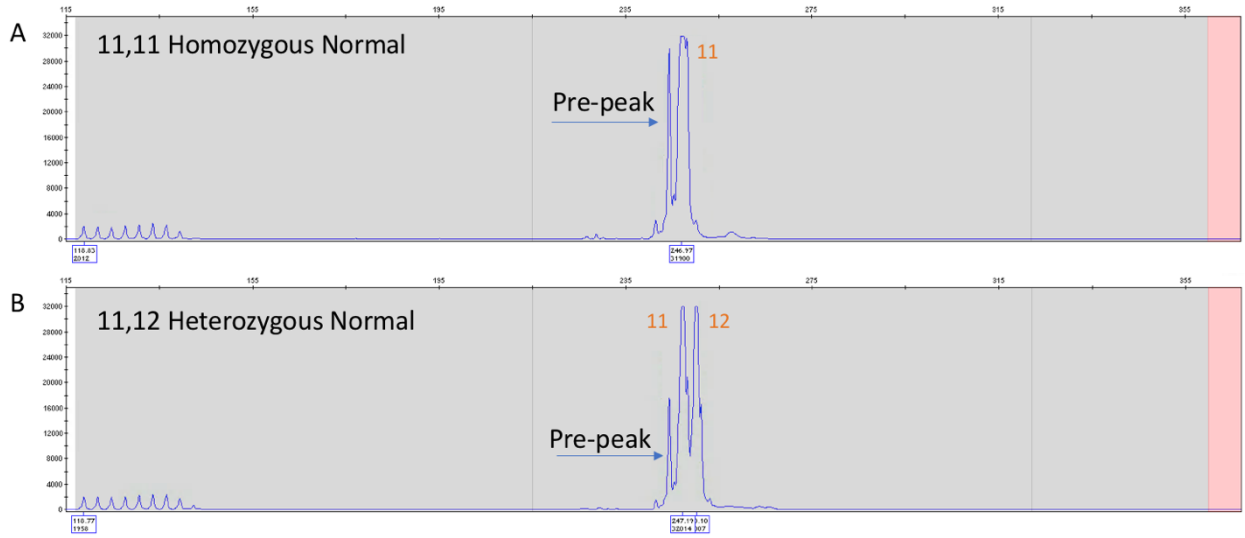


Figure 9. Differentiating homozygous and heterozygous specimens that differ by one repeat. The electropherograms were zoomed to 115–365 bp. **(A)** Whole blood specimen (11, 11) genotyped with a single peak corresponding to 11 repeats. **(B)** Whole blood specimen (11, 12) genotyped with two peaks corresponding to 11 and 12 repeats. In both electropherograms, a pre-peak is observed that is smaller (height and width) than the allele peak and should not be misinterpreted as an allele.

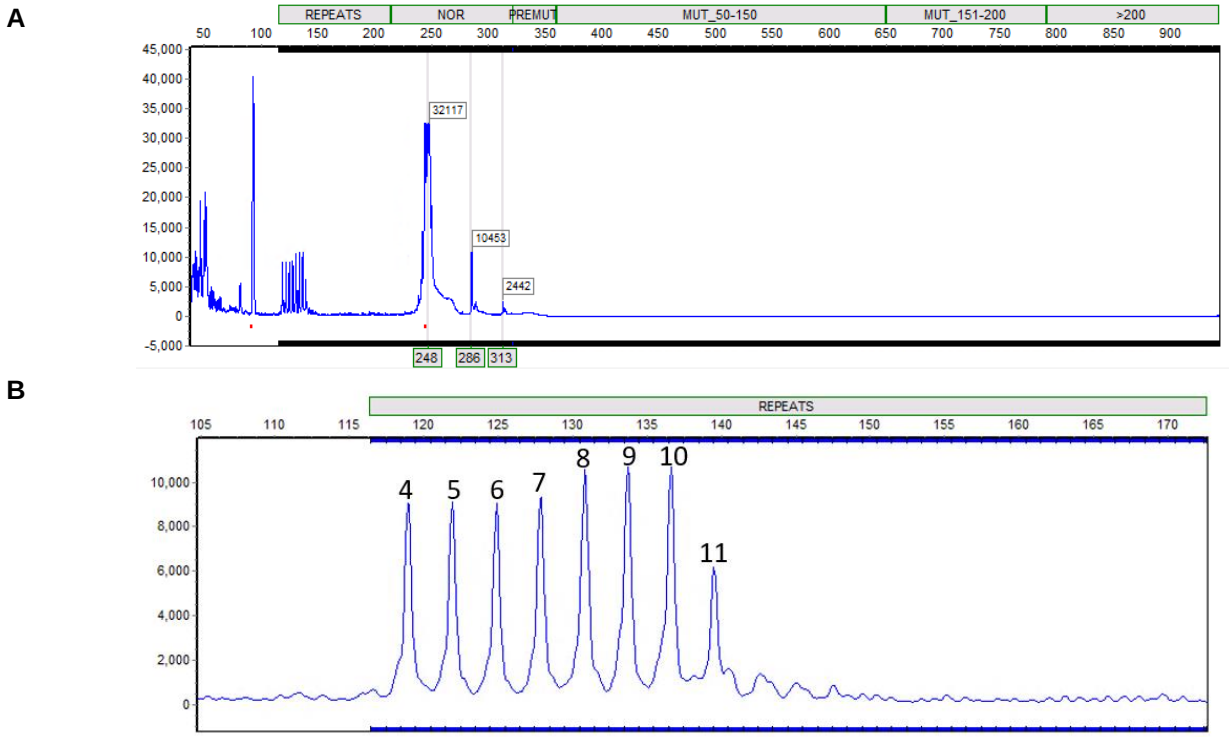


Figure 10. Post-peak evaluation. (A) In this normal specimen that used 80 ng DNA, a GS peak is observed with typically high signal, along with two apparent peaks migrating more slowly and at lower signals, potentially leading to ambiguous interpretation. This pattern is observed primarily in normal specimens. **(B)** Resolving this presentation of a normal specimen requires review of the GS peak's signal (should generally be > 20,000 RFU on the 3500 series or > 8,000 RFU for 3130*xl*), as well as reviewing the RP peaks against the guidance in **Figure 8,** Zygosity evaluation, panels A and B. For the specimen shown, only the tallest peak correlating to 11 repeats (248 bp) is consistent with these guidelines, so only it should be called. Following this and other guidance throughout this document yields an interpretation of homozygous (11, 11).

Please note that reaction wells that do not contain DNA may generate small, spurious peaks during CE analysis. This is a limitation of the capillary electrophoresis system. Therefore, a sample well that contained no DNA (e.g. due to operator or equipment error) bears the risk of being miscalled. **Figure 11** describes the analysis of such non-canonical traces. If the well is a no-template control, this is an expected behavior.

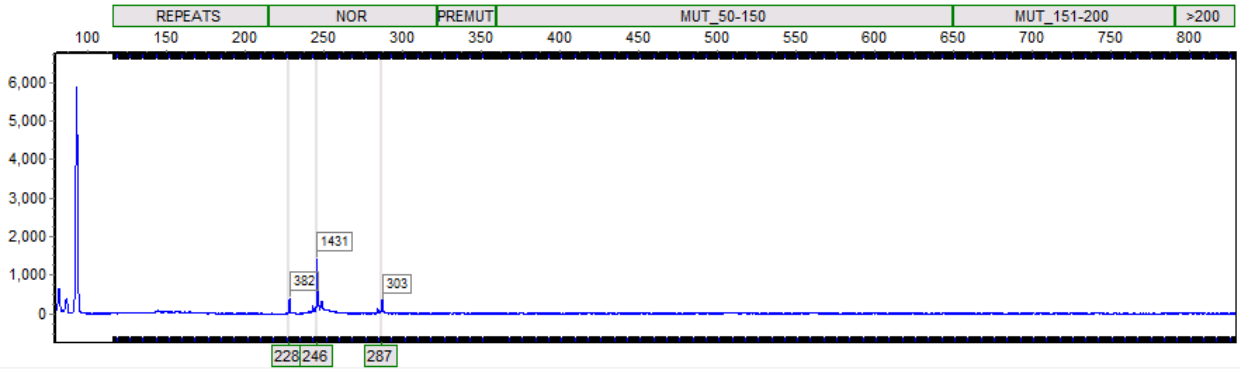


Figure 11. Spurious peaks in wells without template. Occasionally, peaks may be observed in wells into which no DNA was added during PCR. In the example shown, this observation can be distinguished

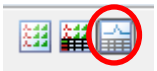
from valid specimen calls by visual inspection following the guidelines in this document (e.g. normal-range GS peaks typically > 20,000 RFU on 3500 series) and in **Figure 10** (i.e. no RP peaks are observed). Specifically, the marked peaks are seen in the normal range but the peak heights are more than 10-fold lower than the typical range. In addition, the RP peaks are missing, leading to an inability to call any valid peaks. The injection is therefore indistinguishable from a no-template control. If this occurs in a specimen well, the specimen should be flagged as failing and marked for repeating from the PCR stage.

Note: Once all peaks have been selected, save the project so the manual selections will be retained and available for future viewing after the software is closed.

Export Data Table

The full-length, GS product peaks may be converted from size in base pairs to the number of CTG repeats using predefined or within-assay conversion factors manually or through the use of the AmplideX® PCR/CE *DMPK* Macro. To use the Macro, a *.txt data table must be exported from the commercially available peak calling software. Otherwise, manual calculations can be made as described in the Quality Control Procedures, Calibration section. In addition, the RP profile can provide (CTG)n repeat quantification by directly counting the number of CTG repeat primed amplicon peaks from 4 up to 200 CTG in most samples.

1. GeneMapper®
   a. From the Samples Plot window, ensure Genotypes Table is showing by clicking **View**, selecting Tables, and clicking **Genotypes Table** or by clicking the radio button**.**



      i. Click **File** and click **Export Table**. Export file as Tab-delimited Text (.txt) to desired file location.
   b. From GeneMapper Software main window, select the Genotypes tab.
      i. Click **File** and click **Export Table**. Export file as Tab-delimited Text (.txt) to desired file location.
2. GeneMarker®
   a. Click the Report Settings button in the top banner of the Report panel.

   b. Select Allele List as the Report Style and click **Ok**.
   c. Select all allele calls in the Report panel, right click and click **Confirm Peaks**.
   d. Click the Save Report button and save as a Tab-delimited Text file (.txt) to desired file location.

Note: As an additional quality check, verify visually that the category determined by the AmplideX PCR/CE *DMPK* Macro corresponds to the bin settings in the electropherogram using the commercially available peak calling software.

Table 2. Data interpretation of GS allele and repeat patterns for PCR/CE. If the specimen's trace does not conform to one of the following sets of characteristics, please contact Asuragen Technical Services for support.

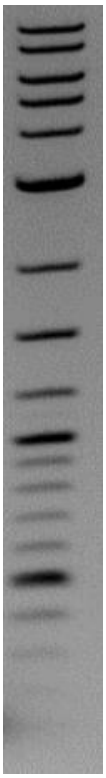
Gene-specific Pattern	Repeat Pattern	Zygosity	Category
2 different, normal-sized GS peaks, no pile-up peak	Repeat pattern corresponding to non-expanded repeat length	Heterozygous	Normal (no expansion)
2 peaks with 1 normal-sized GS peak, no pile-up peak	Repeat pattern with a plateau corresponding to the first allele and a second lower intensity pattern corresponding to the second allele	Heterozygous	Premutation or Expanded, depending on repeat length and current guidelines
1 normal-sized GS peak, no pile-up peak	Repeat pattern corresponding to non-expanded repeat length	Homozygous	Normal (no expansion)
1 normal-sized GS peak with pile-up peak	Repeat pattern extends through the expanded range	Heterozygous	Expanded
1 normal-sized GS peak, ≥ 1 additional GS peak with pile-up peak	Repeat pattern extends through the expanded range	Heterozygous Mosaic	Expanded
1 normal-sized GS peak, ≥ 1 additional GS peak, no pile-up peak	Repeat pattern with a plateau corresponding to the alleles with multiple plateau	Heterozygous, Mosaic	Premutation or Expanded, depending on repeat length and current guidelines

Fragment Sizing Analysis (PCR/AGE)

Fragment sizing analysis of GS bands on an agarose gel can be achieved following the Quick-Load Purple 1 kb Plus DNA ladder sizing in **Figure 12**. Sizing below 94 repeats (0.5kb) by AGE is not recommended as the ladder banding pattern is not easily identified. GS normal alleles will be below this ladder band and appear brightly just above a diffuse purple band. Sizing above 1000 repeats may not correlate with previously acquired Southern blot data. The optional PCR/AGE assay does not reliably detect alleles > 2000 repeats. These observations are likely due to both biological limitations (e.g. high somatic mosaicism) and technological limitations (e.g. inability to reliably amplify extremely large, highly mosaic expansions under assay conditions). AGE should not be performed in the absence of the required CE steps, as false negative calls may result.

The ladder band relationship between kb and repeats is defined by the following equation. The New England Biolabs® 1 kb Plus DNA ladder (**Figure 12**) has brighter bands (in red) corresponding to 928 repeats (3 kb), 261 repeats (1 kb) and 94 repeats (0.5 kb). When reading gel images, precision is unlikely to be maintained past two significant digits; for example, a band appearing near the 1261 repeat ladder band is likely to be indistinguishable from 1300 repeats. Refer to the Quality Control Procedures, Specimens section for a list of CCR samples and expected sizes by PCR/AGE.

$$band\ size\ (repeats) = \frac{(band\ size\ (bp) - 218)}{3}$$



Repeats	Kilobases	Mass (ng)
-3261	-10.0	-40
-2594	-8.0	-40
-1928	-6.0	-48
-1594	-5.0	-40
-1261	-4.0	-32
-928	-3.0	-120
-594	-2.0	-40
-428	-1.5	-57
-328	-1.2	-45
-261	-1.0	-122
-228	-0.9	-34
-194	-0.8	-31
-161	-0.7	-27
-128	-0.6	-23
-94	-0.5	-124
-61	-0.4	-49
-28	-0.3	-37

Figure 12. New England Biolabs® Quick-Load 1 kb Plus DNA Ladder Sizing conversion from Kilobases to Repeats.

Sizing of repeat expansions is in agreement (~80–90%) with Southern blot data between 200 and 1000 repeats for most specimens. Sizing agreement between 1000 and 2000 is dependent on the level of mosaicism. Agarose gel interpretation is performed by comparing the band or smear to the ladder (**Figure 13**). Strong amplicon bands that are observed on the gel near the 0.3 kb (27-repeat) ladder band represent the normal allele and provide a control for amplification when no other band or smear is present. Specimens with no or low mosaicism (S2, S6, S7, S8, or S9) are represented by well-defined bands on the gel.

Specimens with known mosaicism appear as a smear on the PCR/AGE assay, similar to Southern blot. The level of mosaicism can be low (S4, S5), medium (S1), or high (S3, S10) depending on the specimen. Specimens with high mosaicism should be listed as a range or sized by another method if required.

Alleles with > 2000 repeats are often represented by only a normal allele band.

Sample Name	Lab A Southern blot	Asuragen AGE (>200 allele)
S1	300–500	Smear, medium mosaic 300–500
S2	1200–1350	~1100
S3	1100–1500	Smear, high mosaic >500
S4	200–300	Smear, low mosaic ~260–350
S5	800–900	Smear, low mosaic ~650–700
S6	1050	~1000
S7	100–150	70, 130
S8	1900–2220	700, 1900
S9	300	Low mosaic, 160, 200, 260
S10	50–250	150, Smear, high mosaic >200

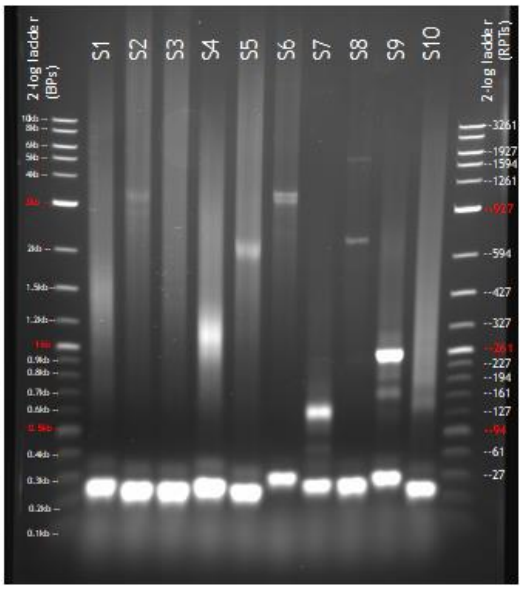


Figure 13. PCR/AGE results across 10 whole blood (S1-8, 10) or CVS (S9) donors. Southern blot sizing is provided along with predicted repeat size by AGE. Optional interpretation of mosaicism as subjectively determined from a collection of fragments closely sized and visualized as a smear or many discrete bands is shown in the table at left.

Quality Control Procedures

Calibration

The calibration procedure is used to derive the size and mobility correction factors specific to each set of CE parameters. After CE, the size (bp) of the target amplicon is derived from comparison to a co-injected size standard, e.g. the provided ROX 1000 Size Ladder. However, PCR products of triplet repeat regions have an anomalously faster migration than the general, non-repeat DNA of the size standard. This higher migration, attributed to the structure of repetitive GC-rich DNA, may result in underreporting of repeat length without the use of an appropriate correction factor. The AmplideX® DM1 Dx Kit recommends a correction factor for conversion of size in base pairs to the number of CTG repeats for each allele. The size of each peak is converted to repeat length according to the following equation:

$$CTG_n = \frac{Peak_i - C_o}{M_o}$$

Where $Peak_i$ is the size in base pairs of a given product peak; C_0 is the size correction factor (intercept); and M_0 is the mobility correction factor for each CTG repeat (slope). The size correction factor represents the common region of DNA included in the primers but omitting the CTG repeats and is close to 215 for gene specific peaks. The mobility factor accounts for the apparent mobility of each repeat unit and is approximately 2.9. **Table 3** provides default correction factors for the four recommended CE instrument configurations. The AmplideX PCR/CE *DMPK* Macro only provides one default option for the 3730 (e.g. the 50 cm capillary). If the 36 cm capillary was utilized on the 3730 and a calibrator was not run, the default values for the 3500xL may be used, or the correction factors listed in **Table 3** for the 36 cm capillary can be manually entered into the AmplideX PCR/CE *DMPK* Macro.

Table 3. Mobility (M_0) and size (C_0) correction factors for supported Applied Biosystems® genetic analyzers configurations

Instrument	Capillary Length	C_0	M_0	R^2
3130*xl*	36 cm	214.0153	2.8624	0.999999
3500, 3500xL	50 cm	214.9763	2.9174	1.000000
3730	36 cm	214.9743	2.9033	0.999999
3730	50 cm	215.2117	2.9259	1.000000

Note: Although the default correction factors may be used (**Table 3**), it is strongly recommended to use a calibrator sample to determine the correction factors specific to a given CE instrument. The correction factors identified using this calibrator should be consistent from one run to the next. The calibrator sample should be re-run periodically especially after instrument service (e.g. capillary change or spectral calibration). CCR samples NA04648 and NA06075 mixed at 1:1 ratio and assayed at 20 ng/reaction (**Figure 14**) are recommended for this calibration. The 4 expected numerical peak sizes (i.e. those ≤ 200 repeats) in repeats and base pairs are listed in **Figure 14**.

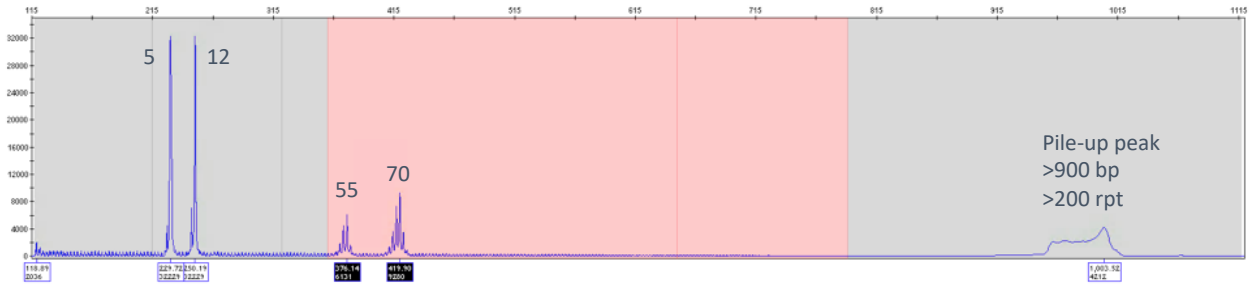


Figure 14. Recommended 4-point *DMPK* Calibrator admixture of NA04648 and NA06075, showing 4 numerical peaks and a categorical pile-up peak > 200.

Table 4. Recommended Calibrator and example manual determination of correction factors

Calibrator	Product Peaks (bp)	Repeat length (CTG)
DMPK Calibrator 1:1 mixture of NA06075 and NA04648	230	5
	250	12
	376	55
	420	70

Correction factors can be determined from the called peaks identified in the calibrator using the AmplideX® PCR/CE *DMPK* Macro or manually with Excel formulas. Using Excel manually, M_0 can be calculated with the SLOPE function in Excel: =SLOPE(known_ys, known_xs). C_0 can be calculated by the INTERCEPT function in Excel: =INTERCEPT(known_ys, known_xs).

If a run calibrator of known repeat size is used, the linear curve fit (R^2) should be determined to identify problems with the calibrator conversion that could lead to inaccurate repeat sizing.

Linear curve fit (R^2) can be calculated with the RSQ function in Excel: =RSQ(known_ys, known_xs). "known_ys" is the array or cell range of product peaks in base pairs and "known_xs" is the array or cell range of expected repeat lengths. The observed R^2 value should be ≥ 0.99.

Additional calibrators have been tested in the system and may be used as alternatives, if necessary (**Figure 15**). The calibrator should have 4 peaks in the numerical range with at least one allele in the expanded range (51–200).

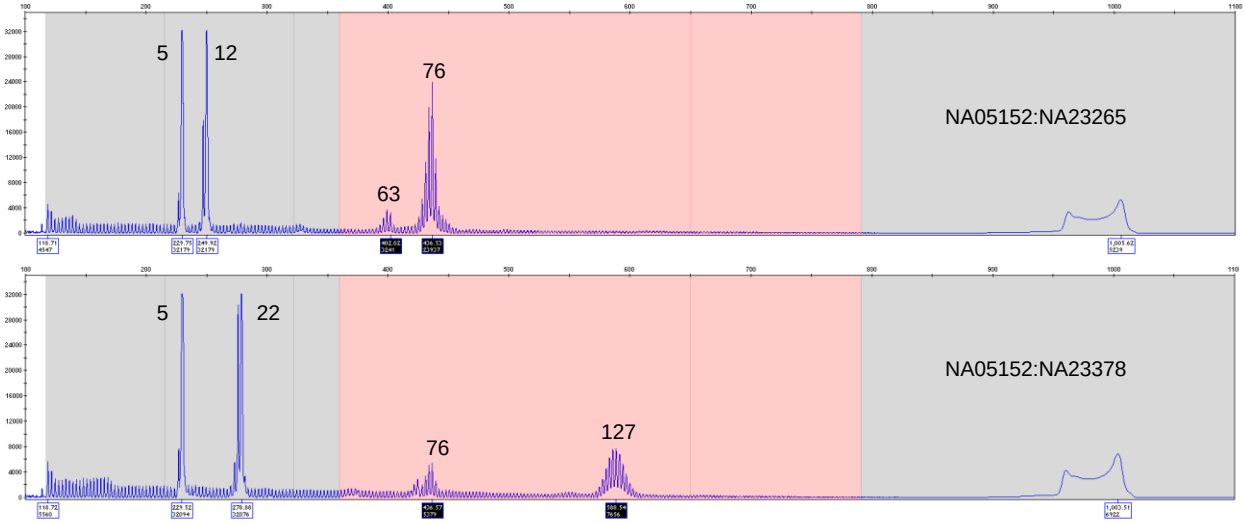


Figure 15. Alternate calibrator admixtures from CCR. NA05152 mixed with NA23265 will provide a 4-point calibrator of 5, 12, 63, and 76. NA05152 mixed with NA23378 will provide a 4-point calibrator of 5, 22, 76, and 127.

Batch Run Controls

Positive and negative controls are recommended in every run. The Diluent provided within the reagent set may be used as a negative no-template control (NTC). Ensure the negative control meets specifications (**Figure 16**).

Though a calibrator is not required for every run, a positive control is recommended to determine if the correction factors result in allele sizing that is out of specification (see the Single-site Precision section within Analytical Assessment). Any of the aforementioned calibrators, the samples listed in **Table 9** and **Figure 20**, or a well-validated sample can be used as a positive control.

If the positive or negative control fails, the batch run fails and should be repeated.

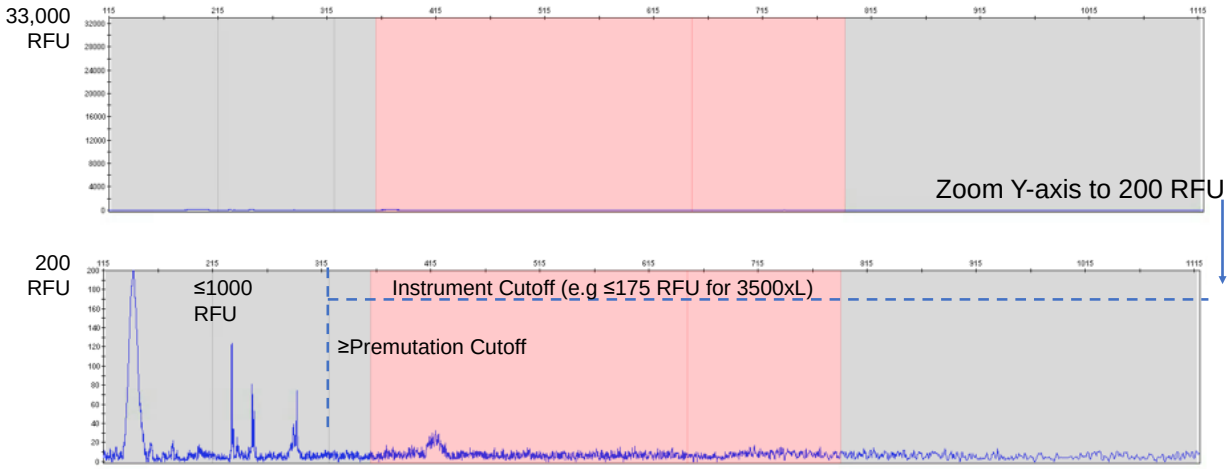


Figure 16. A typical NTC will have no peaks greater than the instrument cutoff (e.g. 175 RFU for 3500xL) within the range of alleles described as premutation or expanded (e.g. ≥ 316 bp or 35 repeats). There typically will not be a peak > 1000 RFU (3500 and 3730 series shown above) or > 330 RFU (3130 series) before this cutoff premutation cutoff. In some scenarios where this occurs, please review against the criteria provided in the Perform Manual Peak Annotation section.

Samples

A calibrator, control, or specimen is valid when its ROX 1000 Size Ladder trace passes (see the Qualify the Run (Ladder Review) section) and the sample trace displays a repeat pattern that is evenly spaced, well defined, and extends to the GS peak(s). A sample that does not display such a canonical trace, or the ROX 1000 Size Ladder does not pass, should be interpreted as failing. Such samples may be repeated.

Interpretation of Results

Specimen results should be interpreted according to local clinical and regulatory guidelines. For example, see “Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2” by Kamsteeg *et al*. (2012). To interpret the test results against such guidelines, bridging of the language used may be required. Using Kamsteeg *et al*. as an example, a result of “Normal” in the test may be interpreted as “No expansion (homozygous or compound heterozygous for a normal allele size).” A result of “Premutation” in the test may be interpreted as “A heterozygous expansion in the size range of 36–50 repeats.” A result of “Expanded” in the test with a numerical allele of 51–150 repeats may be interpreted as “A heterozygous expansion in the size range of 51–150 repeats.” A result of “Expanded” in the test with a numerical allele of 151–200 repeats or a categorical allele of > 200 may be interpreted as “A heterozygous expansion with a size over 150 repeats.” See the **Table 5**. Results should also be carefully interpreted in the context of biological and technical limitations. For example, rare or previously unidentified polymorphisms that inhibit amplification or detection may occur, leading to false negative results.

Table 5. Interpretation of results based on best practices

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).

Troubleshooting

Observation	Potential Cause	Action
Positive controls give unexpected value compared to previously collected data	Commercially available reference DNAs used as calibrator could shift in performance and result in miscalling repeats for unknown specimens.	• Identify and validate new reference material • Repeat batch run
	Correction coefficients were incorrect for the specific run	• Repeat batch run with a calibrator
PCR amplification artifacts such as a fragment with 25 bp periodicity in a positive or negative control	Failed amplification (Root cause unknown)	• Repeat batch run if observed
PCR amplification artifacts such as a fragment with 25 bp periodicity in a test specimen(s)	Failed amplification (Root cause unknown)	• Repeat specimen(s)
PCR artifacts present in no-template control specimens above specified minimum	Contamination of the no-template control	• Review against the guidelines in sections "Perform Manual Peak Annotation" and "Batch Run Controls" to ensure appropriate determination of failure has been made. • Repeat the batch run. Pay special attention to pipetting technique (e.g. aerosolization of samples) in a review of the laboratory's contamination control procedures.

Observation	Potential Cause	Action
GS peak is identified without corresponding repeat pattern	There may be a rare (MAF ≤ 1%) or unidentified polymorphism that inhibit PCR or primer binding	• Repeat specimen • Analyze specimen by another method
GS peak is identified without corresponding repeat pattern	Operator error in fragment analysis	• Ensure that any GS peak calls are consistent with the guidelines in this protocol (e.g. high RFU of GS peak(s) in normal specimens and presence of correlative number of RP peaks) • Repeat sample
Expanded repeat pattern observed without corresponding pile-up or expanded allele peak	There may be a rare (MAF ≤ 1%) or unidentified polymorphism that inhibit PCR or primer binding	• Repeat specimen • Analyze specimen by another method
No peaks identified in a sample	Sample was not added to reaction, compromised or degraded.	• Repeat specimen • Repeat DNA isolation
Widespread allele intensity saturation in all specimens	The wrong CE or thermal cycling protocol was used (e.g. FMR1 assay conditions).	• Ensure proper instrument settings are utilized • Repeat batch run
Ladder dropout partway through injection (e.g. 70–500 bp are detected, but > 500 bp ladder peaks are not detected)	Clogged capillary	• Repeat injection of sample in a different well • Replace capillary array
Difficult to decipher between background fluorescence and a true “smear” product by AGE	Specimen contains a mosaic collection of allele sizes that is observed as a smear by AGE typically > 1000 CTG repeats (~3.0 kb)	• Ensure consistent specimen loading or repeat the PCR with 20-40ng to prevent false positive from simply overloading the specimen. • Report a range of sizes (e.g. 1000–2000 or > 1000 repeats) • Reflex to Southern Blot. • Perform multiple exposure times to provide additional signal that can help with interpretation.
Non-canonical baseline of sample trace in GeneMarker®	GeneMarker baseline settings in analysis template are not optimal for the batch run. See **Figure 6** for an example.	• Revise GeneMarker baseline settings and repeat fragment analysis
Atypical sample trace observed	Potential causes must be interpreted in the context of other data	• Contact Asuragen Technical Services

Observation	Potential Cause	Action
PCR artifacts present in no-template control samples above specified minimum	Contamination of the no-template control. See **Figure 7** for an example.	• Ensure that any GS peak calls are consistent with the guidelines in this protocol (e.g. high RFU of GS peak(s) in normal specimens and presence of correlative number of RP peaks) • Repeat the batch run. Pay special attention to pipetting technique (e.g. aerosolization of samples) in a review of the laboratory's contamination control procedures.

Performance Characteristics – Analytical

Single-site Precision

Single-site precision was validated using cell line DNAs NA05164, NA06075, NA23256, NA23300, providing 4 genotype calls and 12 allele calls in aggregate. DNA input was 20 ng per reaction. The tolerances were set as depicted in **Table 6**. Study execution incorporated 3 kit lots, 12 batch runs, 12 days, 3 bench operators, 3 peak-calling analysts, 2 PCR instruments, and 2 CE instruments (both 3500xL Dx). All 3 analysts annotated peaks manually using GeneMapper v5.0 on all traces from the 3 bench operators. In other words, each FSA file was interpreted by 3 analysts in order to track bench operator and analyst as potential sources of variation. In this way, 864 possible genotypes were generated from 288 capillary traces, along with 1944 numerical alleles (those from 5–200 repeats) and 648 categorical alleles (those reported as "> 200").

Table 6. Target tolerance ranges for each range of CTG repeats.

CTG Repeat Range	Target Tolerance
< 50	± 1
50–120	± 2
121–200	± 5%
> 200	N/A

1941/1944 (99.8%) valid numerical alleles were obtained. All 1941/1941 (100%) 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". In addition, the greatest variability was seen in the allele with a mode of 135 repeats with an SD of 0.98.

As a supplemental study, one of the peak-calling analysts from the study described above re-analyzed the traces in all files using GeneMarker® v2.7. For each allele, the mode of the repeat lengths was determined from all valid calls across all conditions. The difference between the mode and individual

replicate measurements was then calculated. Then the percentage of valid allele calls that fell within the specifications laid out in **Table 6** was calculated. Target tolerance ranges for each range of CTG repeats, above. The results are shown in **Table 7**, below. Calling an allele shorter than the observed mode was rare (5/2572, 0.2%) and evenly spaced throughout the range of repeat lengths tested (21, 70, 101, and 145 repeats). However, calling an allele longer than the observed mode was more common (141/2572, 5%), but only observed for the 2 longest numerical repeat lengths tested (135 and 145 repeats). We note that all measurements were within the tolerance levels set for the system.

The single call of 20 repeats (instead of 21) and 2 calls of 100 repeats (instead of 101) were observed only in GeneMarker. Regardless, the use of either commercially available peak calling software was supported, with all calls falling within tolerance across the entire study. No individual replicate's repeat length was more than a single point away from the mode of repeat lengths.

Table 7. Total calls of each repeat length across the single-site precision study, aggregating data from both GeneMapper® and GeneMarker®. Three (3) different categorical alleles > 200 repeats are not shown. These all generated a result of >200 repeats (n = 856 valid measurements).

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	

Limit of Detection for Mosaicism

A preliminary range-finding assessment of the limit of detection (LOD) for mosaicism study utilized a challenge panel of 2 types of samples mixed with different allele levels (Moderate Expanded into normal and Moderate Expanded into Full Expanded). The assay detected ≥ 5% fractional mosaic alleles at ≥ 2-fold above background 7 repeats away from the called peak despite the presence of expanded peaks that are > 200 repeats (**Figure 17**). Further, the assay detected ≥ 1% fractional mosaic alleles at ≥ 2-fold above background in the absence of expanded repeat patterns representing > 200 repeat alleles (**Figure 18**).

Figure 17. Limit of detection for mosaicism tested by mixing NA23265 (12, 63, 76) at a mass allele fraction of between 1% and 50% into a background of NA04648 (5, > 200). Shown are both 5% and 10% samples. The * represents ±7 repeats from the called peak.

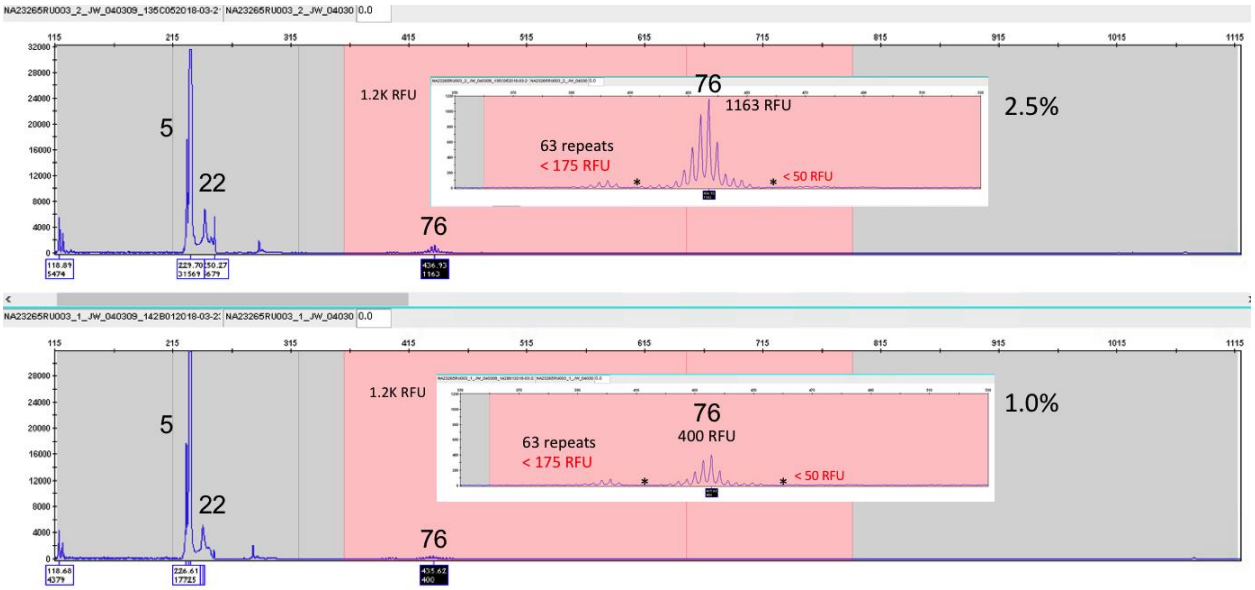


Figure 18. Limit of detection for mosaicism tested by mixing NA23265 (12, 63, 76) at a mass allele fraction of between 1% and 50% into a background of presumed normal (5, 5). Shown are both 1.0% and 2.5%. The * represents approximately ±7 repeats from the called peak.

For validation of LOD, the expanded sample NA23265 was diluted into a different expanded sample NA04648, to targets of 1, 3, 5, and 7% fractions. The DNAs were formulated at 20 ng/µL for a total reaction input of 40 ng. Only the mosaic allele of interest (near 76 repeats) was used in determination of LOD. Run execution incorporated 2 kit lots, 4 batch runs, 4 days, 2 operators, and 2 instruments (both 3500xL Dx). For each dilution level, a “hit rate” was computed as the ratio of the number of replicates

called positive for the allele of interest and the total number of replicates tested. These were then used for a probit analysis (**Figure 19**), which showed detection at the 95th percentile of just under 4%.

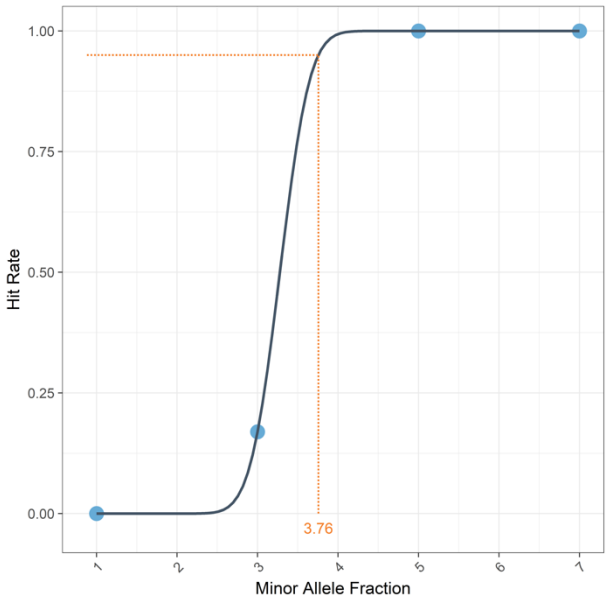


Figure 19. **Probit analysis for LOD.** The figure depicts a probit analysis of the hit rates (observed probabilities), shown in blue dots. The regression is shown as a solid black line. The 95th percentile is shown as a dotted orange line.

Analytical Specificity

Establishment of analytical specificity utilized a challenge panel of 4 *DMPK*-normal, *CNBP*-expanded (CCTG repeat) DNA samples from cell-line gDNA, each performed in triplicate. A total of 12/12 (100%) normal genotypes demonstrated specificity for CTG repeat measurements in the 3' UTR of *DMPK* without observed detection from CCTG repeats in intron 1 of *CNBP*.

Instrument Identity

The use of multiple thermal cyclers and CE instruments was established by using a challenge panel of DNA samples NA23256 (25, 101, 135, > 200 repeats), NA06075 (12, 55, 70 repeats), NA04034 (12, > 200 repeats), and NA04648 (5, > 200 repeats). The DNAs were amplified using two thermal cyclers (Applied Biosystems® Veriti and 9700). PCR products from both thermal cyclers were resolved on three different CE instruments (Applied Biosystems 3130*xl*, 3500xL, and 3730). In addition, two capillary lengths were used on the 3730 (50 cm and 36 cm). Allele calls (n=288) and genotypes (n=72) were indistinguishable across all measurements (i.e. within the tolerance set in the Precision section).

DNA Input Amount

DNA input levels were established using a challenge panel of 6 samples with DNA mass inputs corresponding to 5, 10, 20, 40, 80, 100, and 120 ng per reaction. Each reaction was performed in duplicate. After pooling of data across mass inputs, a mode of repeat length was determined for each allele. All (100%) of alleles generated repeat lengths within one repeat of the mode, including alleles up to 136 repeats. A contrived (admixed) panel sample with alleles at 12 and 13 repeats resolved both alleles throughout the input range. A second contrived panel sample with alleles at 21 and 22 repeats resolved both alleles throughout the input range. Reactions with less than 20 ng risked miscalling mosaic peaks as negative. Reactions with greater than 80 ng produced deleterious smearing on downstream agarose gel electrophoresis. Therefore, 20–80 ng per reaction is the acceptable DNA input range.

Specimen Type

Use of different specimen types was established using a challenge panel consisting of gDNA isolated from matched buccal swab and whole blood of 10 presumed normal donors. In addition, nine whole blood specimens, one neonatal buccal swab, and one prenatal CVS specimen from presumed *DMPK*-expanded donors were assayed. Each specimen was assayed in triplicate. All specimens generated valid results. All DNAs isolated from paired whole blood and buccal specimens yielded the same allele calls and genotypes within an individual across all replicates. An increase in peak intensity was observed for whole blood specimens compared to paired buccal swab specimens.

Carryover during CE

An assessment of carryover contamination during the CE step was carried out. All 96 wells of a PCR contained amplification reactions using positive control (NA23256, n=12), calibrator (NA06075 blended with NA04648, n=13), a homozygous normal DNA from a donor (n=11), and no-template controls (n=60) using a mix of checkerboard patterns. All 11 sample traces from the homozygous normal DNA performed at 80 ng per reaction were similar. A representative trace can be seen in **Figure 10**. In 37 traces from no-template controls, spurious peaks of very low signal were observed. A representative trace can be seen in **Figure 11**. These peaks were not due to carryover given their position on the plate (not shown). All 96 wells gave the expected allele call and genotype result.

Comparison to a Published Design

A comparison to a published method was carried out in triplicate on a subset of the samples reported by Kalman *et al.* (2013). All relevant alleles were called as expected with exceptions that included low-level mosaic allele detection or modest deviations in repeat size (**Table 8**). In all cases, the triplicates returned identical allele sizes, thus the table was collapsed to a single value for each sample. Two samples identified additional alleles as compared to the published design, suggesting increased sensitivity for mosaic alleles. Sample NA23265 yielded a difference of one repeat as compared to the publication in all three replicates. Sample NA23378 yielded a difference of 11 repeats (127 observed vs. 138 expected; 8.5%) as compared to the publication, though a note in the publication suggests one of the study sites detected both a 74 and 125 repeat by PCR instead of Southern blot, which would result in a difference of 2 repeats (2.7 and 1.6% respectively).

Table 8. Comparison to published design reported by Kalman *et al.* (2013)

	Allele 1			Allele 2			Allele 3		
Sample Identity	**Exp**	**Obs**	**Diff**	**Exp**	**Obs**	**Diff**	**Exp**	**Obs**	**Diff**
NA03132	5	5	0	> 200	> 200	0			
NA03696	12	12	0	> 200	> 200	0			
NA03697	12	12	0	> 200	> 200	0			
NA04567	21	21	0	> 200	> 200	0			
NA04648	5	5	0	> 200	> 200	0			
NA05152	5	5	0	> 200	> 200	0			
NA05164	21	21	0	> 200	> 200	0			
NA06075	12	12	0	55	55	0	70	70	0
NA23265	12	12	0	none	63	add	75	76	-1
NA23378	22	22	0	none	76	add	138	127	11

Exp = Expected repeat length of allele based on Kalman *et al.* (2013)
Obs = Observed repeat length of allele in the experiment
Diff = Difference in repeat lengths between publication and the experiment
add = Additional peak observed in the experiment as compared to the publication

Table 9 provides the expected peak sizing for normal and expanded alleles. Samples denoted with bold font represent independent testing by three laboratories. Example electropherograms from PCR/CE are shown in **Figure 20**. Example AGE banding patterns are shown in **Figure 21**.

Table 9. Expected peak sizing for normal and expanded alleles from Kalman *et al.* (2013). Example electropherograms in **Figure 20**. Bold samples were independently tested by three laboratories. Underlined allele sizes are major bands by AGE. Presence of more than two alleles suggests allele size heterogeneity (e.g. mosaicism).

CCR samples with reported genotypes in Kalman *et al*. (2013)	Peak sizes (bp) on 3500xL with 24 pin 50cm capillary	Repeat sizes by PCR/CE (Converted)	Repeat sizes by PCR/AGE (Converted)
NA03132 (5, 2078 ± 217)	230, 1020	5, > 200	Weak 700
NA03696 (12, 697 ± 13)	250, 1020	12, > 200	400, 450, 600
NA03697 (12, 412 ± 33)	250, 1020	12, > 200	400
NA03756 (450 Mosaic)*	253, 1020	13, > 200	300, 425
NA04034 (700)*	250, 1020	12, > 200	200, 600
NA04567 (21, 637 ± 33)	276, 1020	21, > 200	600
NA04648 (5, 1008 ± 49)	230, 1020	5, > 200	600, 1000
NA05152 (5, 1621 ± 30)	230, 1020	5, > 200	1500
NA05164 (21, 377 ± 53)	276, 1020	21, > 200	325
NA06075 (12, 56, 70 ± 0.9)	250, 376, 420	12, 55, 70	60-80
NA23256 (160-170, 775)*	288, 510, 611, 990	25, 101, 136, > 200	100, 125, 260, 430, 700
NA23265 (12, 75)	250, 400, 437	12, 63, 76	70
NA23378 (22, 138 ± 5)	279, 437, 586	22, 76, 127	70, 130
NA23299 (475 mosaic)*	279, 856, 1020	22, > 200	225, 275, 400, 450
NA23300 (550 mosaic)*	230, 639, 864, 1020	5, 146, > 200	150, 230, 550

* Normal alleles were not identified in published article

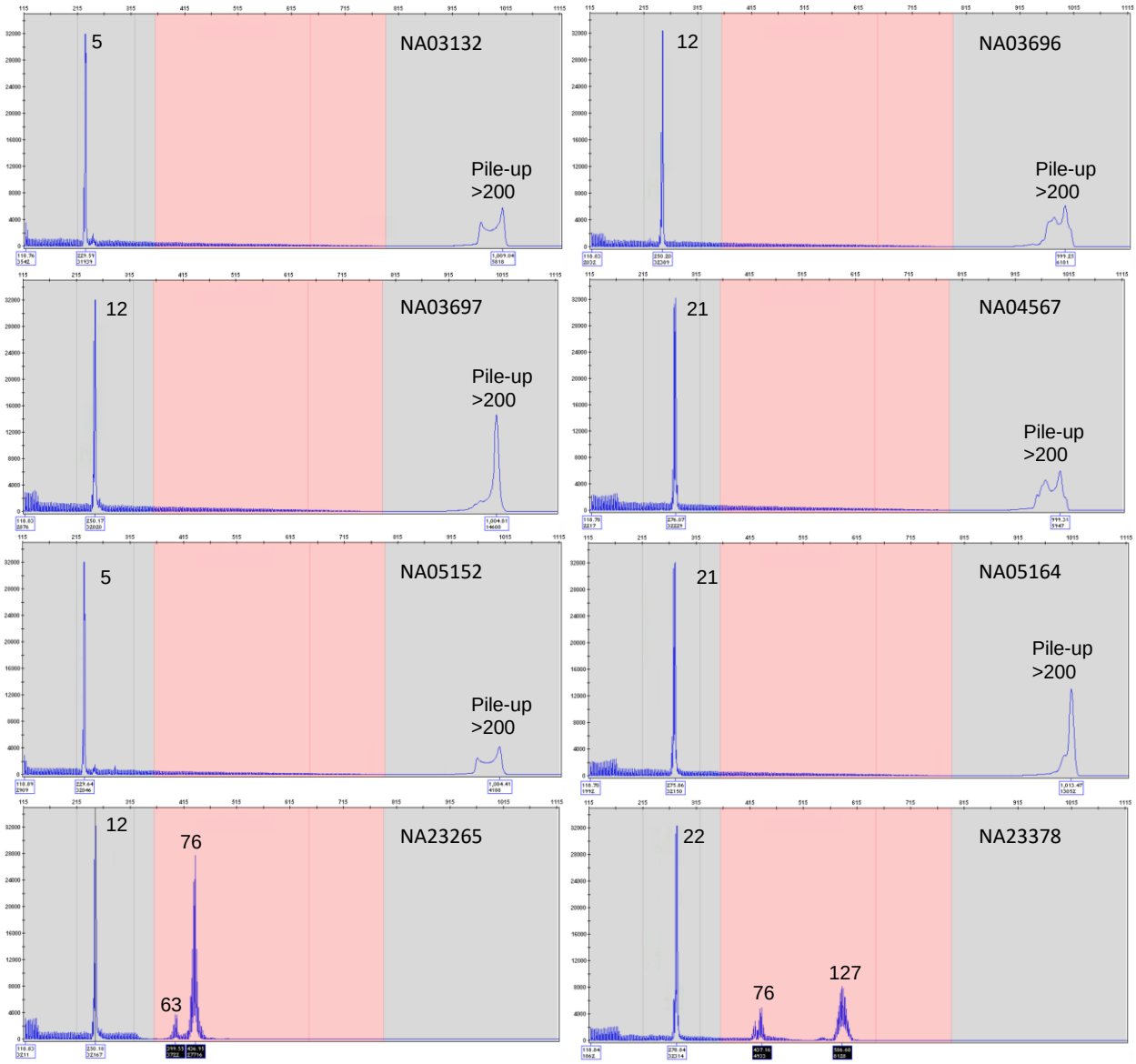


Figure 20. Electropherograms for reference gDNA samples listed in **Table 9**.

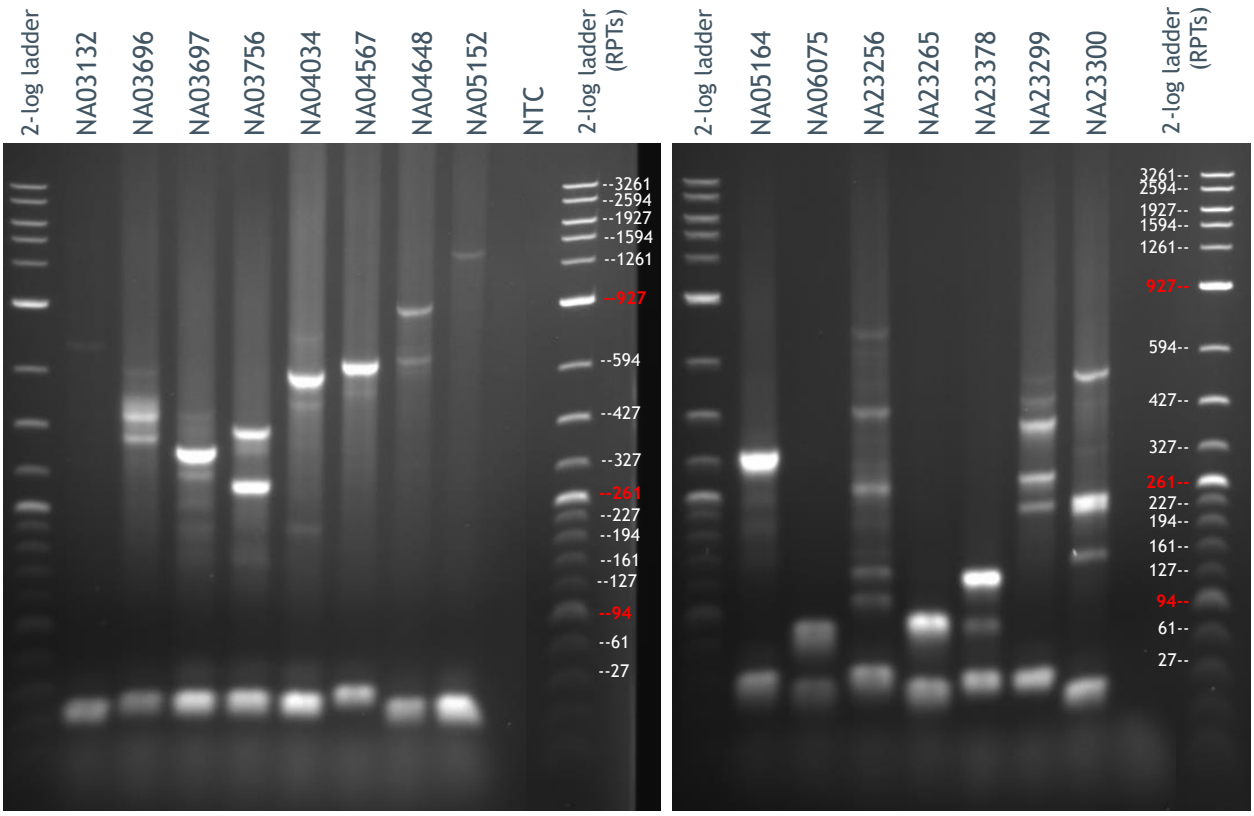


Figure 21. PCR/AGE results for reference gDNA samples listed in **Table 9**.

Performance Characteristics – Clinical Specimens

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. This specimen set was used to validate the test with the use of human DNA specimens. The specimens were assessed for concentration (total input range of 29 to 52 ng per reaction) and purity ($OD_{260/280}$ range of 1.7 to 1.9). All specimens generated valid results in the test. Nearly uniform bias was observed between the reference method and the test. Specifically, for all repeat lengths from 4 to 25 in the reference method, the test generated exactly +1 repeat. The exception was observed at 125 repeats, for which the test generated +2 repeats. These data were all within the target tolerances of the test and are reflected in the equation of correlation of y=1.01x+0.91. Mean bias was -1.1 repeats (not shown), confirming that the test generally reads 1 repeat greater than the reference method.

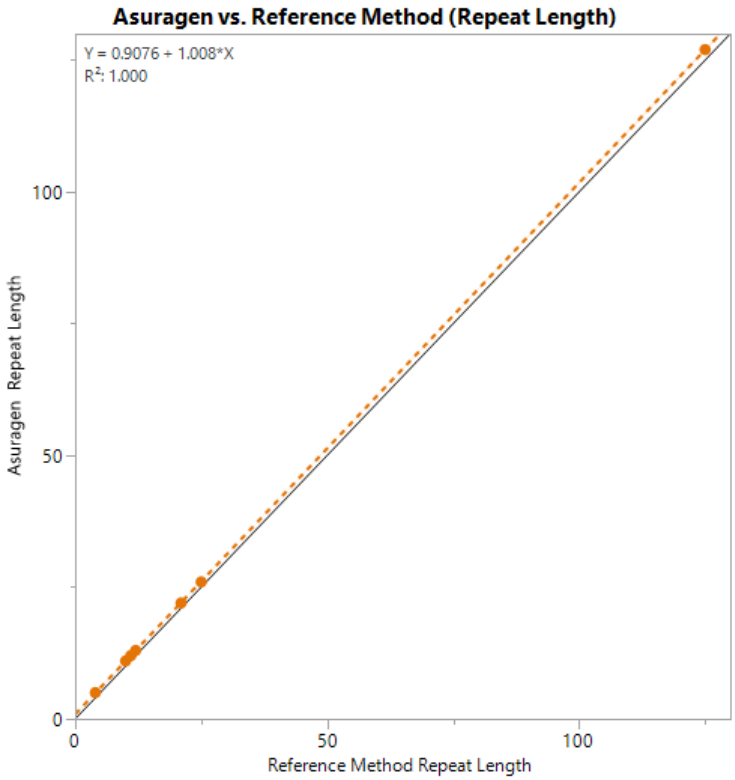


Figure 22. Correlation plot. A reference line for ideal response characteristics is shown as a black line. Observed measurements are shown in orange. The dotted orange line is the linear regressions.

Conclusion

The AmplideX® DM1 Dx Kit is a precise and accurate test that aids in the diagnosis of myotonic dystrophy type 1 (DM1) using human DNA. The kit generates numerical values for alleles up to and including 200 repeats and a categorical value for alleles > 200 repeats to facilitate genotyping.

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References

1. Alexandre B, *et al.* Blood DNA yield but not integrity of methylation is impacted after long-term storage. Biopreservation and Biobanking 2016; 14(1): 1–10.
2. Kalman L, *et al.* Development of a genomic DNA reference material panel for myotonic dystrophy type 1 (DM1) genetic testing. Journal of Molecular Diagnostics 2013; 15: 518–525.
3. Kamsteeg *et al*. Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2. European Journal of Human Genetics 2012 Dec; 20(12): 1203.

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Change History

Revision	Change Description	Effective Date
1	Initial Release	2018-11-26