Asuragen®

AmplideX®

PCR/CE *HTT* Kit

Protocol Guide

Research Use Only. Not for use in diagnostic procedures.

REF 49657

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Purpose

The AmplideX® PCR/CE *HTT* Kit is an *in vitro* nucleic acid amplification kit for the analytical assessment of CAG repeats in exon 1 of the *HTT* gene. The kit employs PCR on extracted genomic DNA followed by capillary electrophoresis (CE) performed on a laboratory-validated thermal cycler and an Applied Biosystems® genetic analyzer, respectively. The kit generates numerical values for alleles up to and including 200 repeats and a categorical value for alleles > 200 repeats.

The AmplideX PCR/CE *HTT* Kit is for research use only (RUO) and is not for use in diagnostic procedures.

Limitations

- This kit is intended for Research Use Only. Not for use in diagnostic procedures.
- This kit has been evaluated for use with genomic DNA (gDNA) isolated from whole blood collected in K_2EDTA tubes and processed within 14 days.
- This kit has not been evaluated for use with other sample sources including muscle tissues.
- This kit is designed to quantify the trinucleotide repeat region in exon 1 of the *HTT* gene. Nonsense, frameshift, or missense mutations that lead to loss of function will not be detected.

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 samples.
- Substances that may interfere with the PCR amplification of gDNA include certain drug compounds and heparin. Highly lipemic samples, hemolyzed samples, icteric samples, or samples with proteinemia should not be used.
- DNase contamination can cause degradation of the DNA samples. Use nuclease-free labware (e.g. pipettes, pipettes tips, reaction vials).
- PCR carry-over 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 pipettors are validated, maintained, and calibrated according to the manufacturer's instructions.

- Prior to use, ensure that the thermal cycler and genetic analyzers are validated, maintained, and calibrated according to the manufacturer's instructions.
- When performing CE analysis on the 3730 instrument, injection conditions differ slightly between the AmplideX® PCR/CE *DMPK* Kit (49655) and the AmplideX PCR/CE *HTT* Kit. A 10 second injection is recommended for the AmplideX PCR/CE *HTT* Kit; however, a 20 second injection may be used.
- The 3730*xl* (96-well capillary) is not recommended for fragment analysis by the manufacturer.
- Signal saturation on genetic analyzer, especially when using 3730 platform, may result in extraneous peaks above background; ensure repeat profile precedes all peaks selected. See Troubleshooting section for additional information.
- Performance of commercially available reference DNAs, used as a calibrator may vary from lot to lot which may result in miscalling repeats for unknown samples
- Potential rare minor allele frequencies (MAF ≤ 1%) or unidentified polymorphisms that inhibit PCR or primer binding are possible and may affect results. Exercise caution with sample genotype results at a boundary condition (e.g. 38–42 CAG repeats).

Principle of the Procedure

The AmplideX PCR/CE *HTT* Kit can be used to PCR-amplify the *HTT* trinucleotide CAG fragment from purified genomic DNA (gDNA). Amplified products are resolved by CE and converted to CAG repeat length manually or by using the AmplideX PCR/CE *HTT* Macro. The AmplideX PCR/CE *HTT* Macro can automatically determine size and mobility correction factors from within-run calibrators or, alternatively, default values may be used. Commercially available reference DNA samples are recommended for use as a calibrator and control. The kit also includes Diluent, which may be used as a negative control or no-template (NTC).

Background Information

Huntington disease (HD) is a progressive brain disorder that causes uncontrolled movements, emotional problems, and loss of cognition. Adult-onset HD, the most common form of this disorder, usually appears in a person's thirties or forties. Individuals with the adult-onset form of HD typically live about 15 to 20 years after signs and symptoms begin. A less common form of HD known as the juvenile form begins in childhood or adolescence. It also involves movement problems and mental and emotional changes. Additional signs of the juvenile form include slow movements, clumsiness, frequent falling, rigidity, slurred speech, and drooling. School performance declines as thinking and reasoning abilities become impaired. Seizures occur in 30 percent to 50 percent of children with this condition. Juvenile HD tends to progress more quickly than the adult-onset form; affected individuals usually live 10 to 15 years after signs and symptoms appear.

Expansions of a CAG trinucleotide repeat in exon 1 of the *HTT* gene are associated with HD. Normal alleles contain less than 27 CAG repeats. Mutable normal alleles have 27 to 35 CAG repeats, often referred to as the meiotic instability range, or "intermediate alleles" or "mutable normal alleles." These alleles have yet to be convincingly associated with an HD phenotype; however, they can be meiotically unstable in sperm. With repeats between 36 and 39 individuals may or may not develop the signs and symptoms of HD (i.e. HD alleles with reduced penetrance), whereas those with 40 or more almost always develop the disorder (Bean L and Bayrak-Toydemir P (2014)). The largest known case reported was 250 repeats; however, CAG expansions are rarely observed to be larger than 120 in number.

Table 1. Mutation category based on CAG repeat length in exon 1 of the *HTT* gene

Category	CAG repeats
Normal	≤ 26
Intermediate	27–35
Reduced Penetrance	36–39
Expanded	≥ 40

PCR Methods

The kit includes reagents to perform a two-primer, anchor-primed PCR of the *HTT* CAG repeat region followed by detection by CE (**Figure 1**). The resulting electropherogram includes both full-length PCR products and CAG repeat-primed (RP) amplicons, creating a repeat pattern. These RP peaks are separated by ~3 bp, or 1 repeat unit, as expected. The true alleles are distinguished as the highest fragment peaks; stutter peaks extending from the smaller allele and terminating with the larger allele represent products from the reverse primer hybridizing within the $CAG_{(n)}$ region. When an allele is too large to be resolved by CE, a stutter peak of decreasing amplitude extends to the end of the electropherogram, often accompanied with a pile-up peak representing fragments too large to separate in the linear range of CE.

Figure 1. *HTT* 2-primer PCR assay configuration

Workflow

The workflow for the test includes PCR master mix setup, thermal cycling, and fragment analysis using CE. Purified genomic DNA derived from human whole blood is first added to a PCR reaction well containing a master mix with the HTT PCR Mix, and HTT F,R FAM Primers, in a total reaction volume of 10 µL. After ~4 hours of 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 (e.g. 3130, 3730, or 3500 series). A schematic of the workflow is shown in **Figure 2**.

After resolving PCR products by CE, the resulting electropherograms are analyzed to identify full-length gene-specific (GS) product peaks and repeat peaks. These peaks are converted from size in base pairs to the number of CAG repeats using calibrator-derived correction factors, or default values provided in the AmplideX® PCR/CE *HTT* Macro, appropriate for each instrument configuration.

GS peaks are detected within the linear range of the instrument up to ~200 CAG repeats (~800 bp). Beyond ~200 CAG repeats, the size of the PCR product exceeds the ability of the POP-7 polymer to adequately resolve fragments and the migration rate is independent of product size. A characteristic “pile-up” peak is often observed with fragments ≥ 1000 bp that cannot be resolved within the linear range of CE.

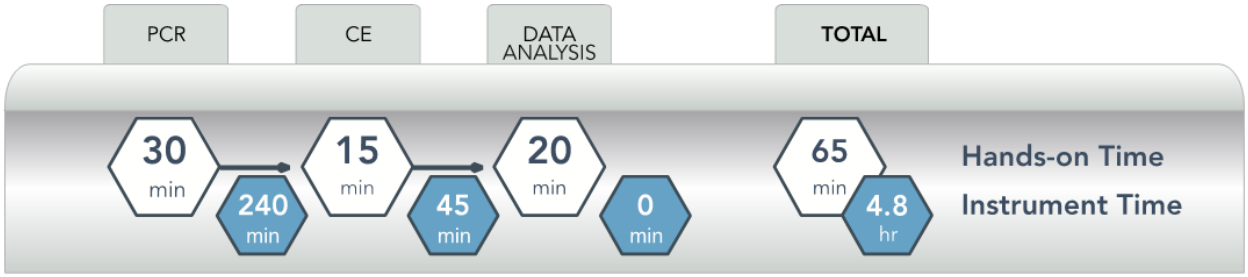


Figure 2. The workflow for the AmplideX® PCR/CE *HTT* Kit (RUO), assuming 24 samples (1 injection).

Use of Controls

Positive and negative controls are recommended in every run. The Diluent provided within the reagent set may be used as a negative or NTC. Cell lines or corresponding purified gDNA may be obtained from various repositories such as Coriell Cell Repository (CCR), and may include published reference data as described in Kalman *et al.* (2007). Additionally, a Standard Reference Material is available through the National Institute for Standards and Technology (NIST, SRM2393).

Positive controls such as NIST Component A (15, 29), Component B (17, 36), Component C (15, 40), or Component D (39, 50) may be utilized using the established genotypes. Alternatively, 1 of the 14 CCR gDNA samples, as referenced by Kalman *et al.* (2007), may be utilized using the published genotypes. Note that the published allele sizes and the size observed with the AmplideX PCR/CE *HTT* Kit may differ slightly. Allele size may differ from published values with subsequent passages of the CCR samples.

Use of Calibrators

Calibration of the assay to the laboratory specific genetic analyzer is recommended. A calibrator should be used initially to establish the mobility correction factors using the specific CE parameters in a given laboratory. An admixture of NIST SRM2393 Component E and Component F at 1:2 ratio or CCR materials at the same 1:2 ratio (NA20210 + NA20251) may be utilized. A total input of 10 or 20 ng of these admixtures is recommended. Refer to the Calibration section for additional information and recommended calibrator samples.

Reagents Supplied with the Kit

Item #	Description	Storage Temp
145590	HTT PCR Mix	-15 to -30 °C
145591	HTT F,R FAM Primer Mix	-15 to -30 °C
145427	ROX 1000 Size Ladder	-15 to -30 °C
145388	Diluent	-15 to -30 °C

Digital Files Supplied with the Kit

Digital files provide for download.

- AmplideX® PCR/CE *HTT* Kit Protocol Guide: https://asuragen.com/ruo
- AmplideX PCR/CE *HTT* Macro: http://software.asuragen.com
- AmplideX PCR/CE *HTT* Macro User Guide: https://asuragen.com/ruo

Required but not Provided

Item Numbers, where provided, may vary by region. Please contact Asuragen Technical Service for assistance.

- Reagents for DNA isolation are not included in the kit. 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 Kit or similar kit.
- Thermal cycler: Applied Biosystems® Veriti™ 96-well Thermal Cycler, GeneAmp® PCR System 9700 (aluminum block), Bio-Rad® C1000 Touch™ Thermal Cycler, Eppendorf® Mastercycler® nexus 96-well Thermal Cycler, or equivalent
- Genetic Analyzer: Applied Biosystems 3130, 3500, or 3730 series
- Thermo Scientific™ NanoDrop™ Spectrophotometer or equivalent
- Hi-Di™ Formamide: Applied Biosystems P/N 4311320 or equivalent
- Dye set calibrators for FAM and ROX. DS-30, DS-31 Matrix Standard Kit (Dye Set D): Applied Biosystems P/N 4345827, 4345829 or equivalent
- POP-7™ Polymer, as required for specific genetic analyzer
- Anode Buffer, as required for specific genetic analyzer
- Cathode Buffer, as required for specific genetic analyzer
- Centrifuge capable of spinning 96-well plates
- General laboratory equipment and workspace to perform PCR and dilute buffers
- Micro-centrifuge or benchtop centrifuge: Eppendorf® 5810 or equivalent
- Vortex
- Pipettes: Units with an accuracy range between 0.2–2 µL, 2–20 µL, 20–200 µL, and 100–1000 µL
- Multi-channel pipette unit capable of pipetting 1–10 µL
- 96-well PCR semi-skirted plates for use with the Applied Biosystems Veriti 96-well, GeneAmp 9700 (gold block), or Eppendorf Mastercycler nexus 96-well Thermal Cycler, Phenix Research Products, P/N MPS-3580, or equivalent
- 96-well PCR fully-skirted plates for use with the Bio-Rad® C1000 Touch Thermal Cycler, Bio-Rad, P/N HSP9631 or equivalent
- Adhesive Foil Seals (Beckman Coulter® P/N 538619 or equivalent) or Plastic Seals (VWR P/N 89134-428 or equivalent)
- MicroAmp™ Optical Film Compression Pad: Applied Biosystems P/N 4312639 or equivalent

Number of Reactions

- The provided reagents are sufficient for up to 32 reactions, including master mix overage.
- The kit supports up to 6 freeze-thaw cycles.
- Master mixes can be prepared for the appropriate number of samples with a recommended total number of at least 4 reactions per run. 10% overage is recommended for master mixes.

Reagent Stability

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

Storage & Handling

- Store reagents in a non-frost-free freezer protected from light at -15 to -30 °C.
- Allow reagents to completely thaw at room temperature (approximate range of 18 to 25 °C) before use (up to 15 minutes for unopened components).
- **Vortex all reagents after thawing.**
- Prior to opening, briefly centrifuge each component to collect the solutions at the bottom of the vials.
- Assay setup should be performed at room temperature. Individual kit components are stable for up to 45 minutes at room temperature. Once assembled, master mixes are stable for up to one hour at room temperature.

Procedural Steps

Pre-Analytical Steps

Genomic DNA (gDNA) extracted via common, laboratory-validated sample preparation methodologies from whole blood collected in K_2EDTA is compatible with the AmplideX® PCR/CE *HTT* Kit. It is recommended that whole blood samples are stored at 2–8 °C prior to processing within 14 days of collection (Alexandre *et al.* 2016). 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 10 to 80 ng in each reaction (for example, by adding 2 µL of DNA at 5 to 40 ng/µL). **Note**: Higher inputs may be required for accurate quantitation of > 75 CAG repeats.

Overview of AmplideX PCR/CE *HTT* Protocol

The protocol involves three primary procedural steps:

1. PCR master mix setup and thermal cycling
2. CE using a genetic analyzer
3. Fragment sizing analysis

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 may be specific to the CE instrument being used (**Table 7**). It is also recommended that an internal run calibrator sample with at least 4 GS peaks be used to determine these correction factors when using this assay for the first time or after CE instrument service (such as replacing the capillary). Refer to the Calibration section for guidance.

PCR Master Mix Setup and Thermal Cycling

1. Thaw the HTT PCR Mix, HTT F,R FAM Primer Mix, and Diluent (if NTC is to be included) at room temperature until each reagent is a uniform liquid (up to 15 minutes, depending on volume). Vortex all tubes for approximately 5 seconds at maximum speed and briefly centrifuge to ensure contents are at the bottom of the tube. **Note**: The HTT PCR Mix may be occluded or have observable precipitation when cold. After completely thawing the tube, vortex to ensure complete mixing.
2. Assemble the reagents in the order listed below. Prepare a master mix with 10% overage. Inclusion of a reaction with Diluent in place of DNA as an NTC is optional. **Note**: The HTT PCR Mix is viscous; retract pipette piston slowly to draw up solution.

   PCR Master Mix:

Reagent	Volume/reaction (µL)
HTT PCR Mix	5.0
HTT F,R Primer Mix	3.0
Sample, calibrator, control or NTC*	2.0*
Total Volume	**10.0**

   * Do not add to master mix

3. Thoroughly vortex the PCR master mix for approximately 5 seconds at maximum speed and briefly centrifuge prior to aliquoting to PCR plate. **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 or tube. Use a repeater pipette if available.
5. Add 2.0 µL of the appropriate sample (sample, calibrator, control, or NTC) to each well. Pipette up and down at least three times to ensure adequate mixing.
6. Seal the plate with an adhesive film seal (plastic or foil). Ensure that all the wells and the plate edges are properly sealed. **Note**: When using foil seals, use of a roller (Beckman Coulter® P/N 538618 or equivalent) is recommended to prevent evaporation during thermal cycling.
7. Gently vortex the plate.
8. Centrifuge the plate to remove bubbles (1 minute at 1600 relative centrifugal force (rcf)).
   Note: Ensure all bubbles are removed from the bottom of the plate wells.

9. Transfer the sealed PCR plate to a pre-programmed supported thermal cycler, cover the plate with a thermal PCR pad (as recommended by manufacturer) and run the following cycling protocol using the default ramp speed. **Note**: The AmplideX® PCR/CE *DMPK* Kit uses the same parameters.

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 sec/cycle
1 hold	72 °C for 10 min
1 hold	4 °C forever

10. Transfer PCR products for CE analysis. **Note**: Once the PCR cycling is complete, PCR products are stable for up to 48 hours at 2 to 8 °C or at -15 to -30 °C for up to 14 days.

Capillary Electrophoresis using a Genetic Analyzer

1. Thaw the Hi-Di™ Formamide and ROX 1000 Size Ladder at room temperature until each reagent is a uniform liquid.
2. Thoroughly vortex each reagent (max speed for 5 to 10 sec) and briefly centrifuge to collect the contents.
3. Prepare a CE master mix solution by adding reagents in the order listed in the following table using 10% overage.

   CE Master Mix:

Reagent	Per Reaction (µL)
Hi-Di™ Formamide*	11.0
ROX 1000 Size Ladder	2.0
Total Volume	**13.0**

   * Not provided with the kit

4. Mix all added reagents (vortex at max speed for 5 seconds), and briefly centrifuge to collect the contents.
5. Aliquot 13.0 µL of CE master mix to each well of a new CE analysis plate. **Note**: Samples must be matched to the injection configuration of the genetic analyzer in appropriate groups of 4, 8, 16, 24 or 48 capillaries (e.g. A1–H2, A3–H4…A11–H12). If running less than the number of samples for any injection group, fill empty wells subject to injection with 15.0 µL of Hi-Di™ Formamide.
6. Transfer 2.0 µL of each PCR product to the CE plate, pipetting up and down at least 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 (1 minute at 1600 rcf). **Note**: Ensure all bubbles are removed from the wells.
10. Denature for 2 minutes at 95 °C followed by a 4 °C hold until ready for injection on the CE instrument. Alternatively, PCR products may be stored and protected from light prior to denaturation at 4 °C for up to 48 hours or at -20 °C for up to 14 days. **Note**: The samples must be denatured prior to CE analysis.
11. Prepare the genetic analyzer for data acquisition according to the manufacturer instructions. Final injection and run conditions must be validated by the end user and may differ between instruments. 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 or to support laboratory needs. Recommended starting values are listed in **Table 2**.

Table 2. Recommended Genetic Analyzer Injection Conditions using the POP-7 Polymer

Instrument	Capillaries	Capillary Length	Injection	Run Time
3130 / 3130*xl*	16	36 cm	2.5 kV, 20 sec	15 kV, 2400 s
3500 / 3500xL	8 or 24	50 cm	2.5 kV, 20 sec	19.5 kV, 2400 s
3730*xl*	48	50 cm	2.5 kV, 10 or 20 sec	15 kV, 4200 s

Fragment Sizing Analysis (using GeneMapper)

Fragment sizing analysis of GS or CAG RP-PCR data involves a series of steps to obtain the size of GS peaks and identify features in the run for interpretation of the data. These results are converted to CAG repeat length as described in the Data Interpretation section. An overview of the fragment sizing analysis workflow is shown in **Figure 3**.

Note: The terms used for analysis refer to GeneMapper® Software v4.1 and v5.0 features. When using GeneMarker® 2.7 or greater for fragment analysis, please refer to the user manual.

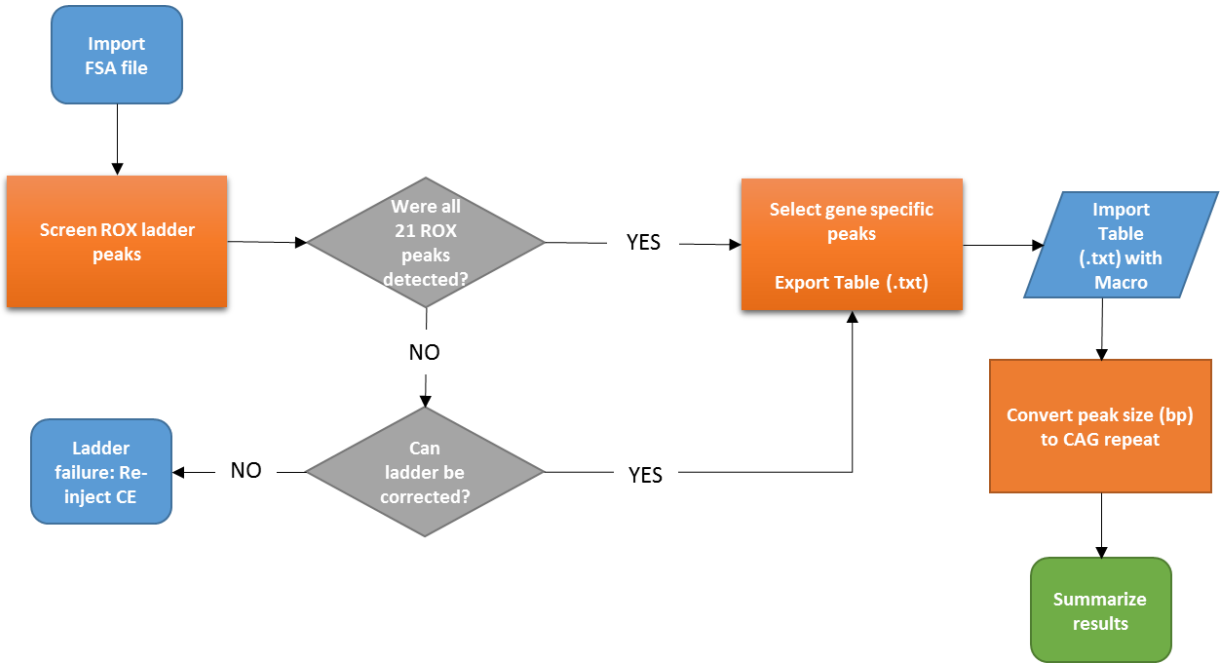


Figure 3.Overview of fragment sizing analysis workflow

Import Data and Process

1. Open fragment analysis software and import .fsa files into a new project.
   a. Click **File**.
   b. Click **Add Samples to Project**.
   c. Browse to the desired folder(s).
   d. Click **Add to List**.
   e. Click **Add & Analyze**.
2. Select the following settings. Contact Asuragen Technical Services (support@asuragen.com) for additional support for available settings.
   a. Analysis Method
   b. Panel
   c. Size Standard
   d. Table
   e. Plot
3. Click **Analyze** to initiate peak calling analysis.

Qualify the Run (Ladder review)

Confirm the ROX 1000 Size Ladder peaks are identified correctly for each sample.

1. Select all the samples in the Sample File column.
2. Click **Size Match Editor** under the Analysis menu to review the ROX 1000 Size Ladder.
3. If any ladder is missing expected peaks, or if any peaks have shifted, manually call the proper peaks, apply the changes, and click **Analyze** (if changes were made).
    a. If necessary, zoom between 150–850 bp (x-axis) and 0–2000 RFU (y-axis). All 21 peaks (79–1007 bp) should be visible with ≥ 500 RFU at each peak (example in **Figure 4**).

Notes:

- Spectral pull-up peaks should be ignored. Peaks between the 105 and 131 ladder peaks may be mis-assigned due to spectral pull-up from FAM channel.
- A total of 21 peaks, ranging from 79–1007 bp, are expected.

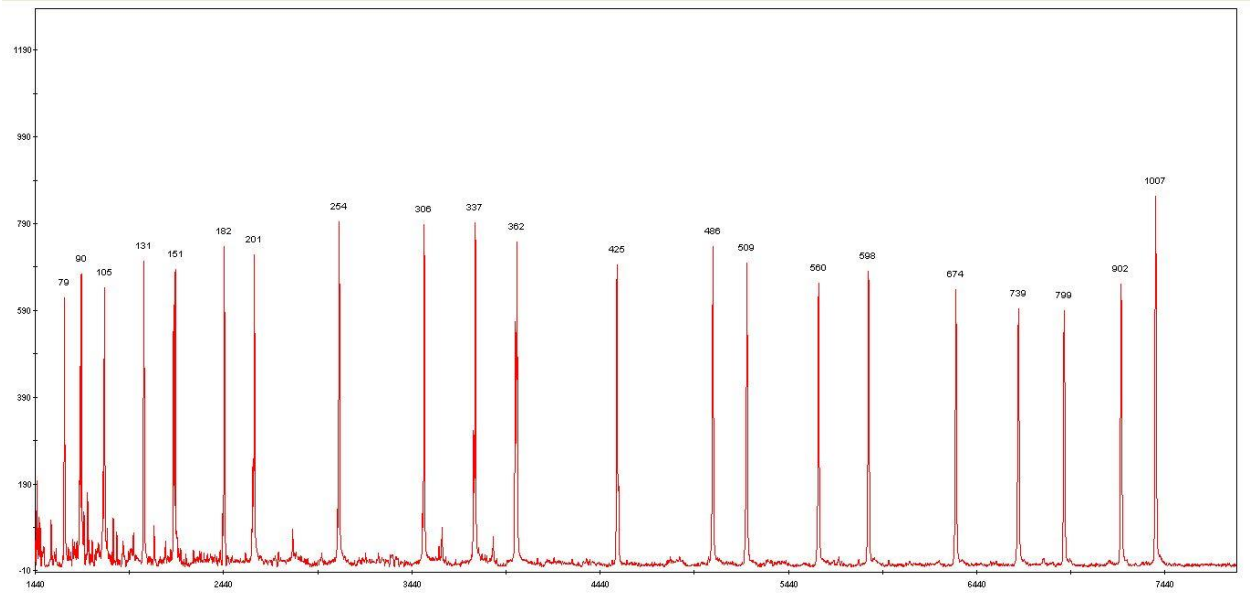


Figure 4. ROX Ladder Peak Identification; representative results using the Applied Biosystems® 3500 Genetic Analyzer

Review Sample Data

Verify the peaks have been correctly identified:

1. Select all samples.
2. Click **Display Plots** under the Analysis dropdown menu.
3. Delete all alleles automatically called by the software.
    a. Click **Alleles**.
    b. Select **Delete Alleles**.
    c. Click **Delete All Alleles**.
    d. Click **OK**.

Note: For analysis of *HTT* PCR/CE products, the multiplicity of CAG RP peaks is deleted in order to simplify tracking, export, and conversion of the full-length GS PCR product peak to $(CAG)_n$ repeat length.

4. Select all the GS peaks for each sample using the examples below. Right click on the peak and select **Add Allele Call** for each sample (**Figure 5**).
   a. Zoom to ~50–1150 bp (x-axis) and ~3500–32000 RFU (y-axis) for an overall view of the electropherogram for the sample.
   b. Identify, but do not call, the initial repeat peak at ~83 bp, corresponding to 5 CAGs, as it is not necessary for the exported results.
   c. Identify highly expanded samples. Highly expanded samples will have a RP profile ("sawtooth") extending beyond 300 bp and/or $(CAG)_n$ repeat length > 100. A pile-up peak or multi-peak allele will be selected as a GS allele for these samples.

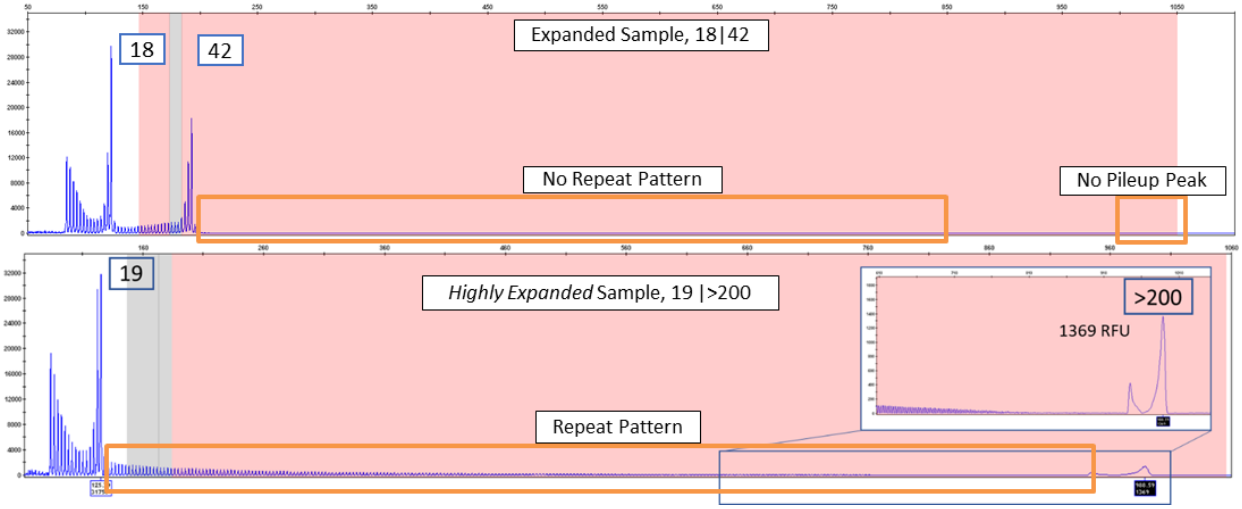


Figure 5. Example electropherograms of an expanded genotype sample with two GS peaks (top: 18, 42) and a highly expanded genotype sample (NA14044: 19, > 200) with one GS peak, a continuous repeat peak pattern, and a pile-up peak

   d. Identify up to 2 GS peaks for each sample. GS peaks characterized by local RFU maxima in the FAM channel, and 1–2 pull-up peaks preceding them.

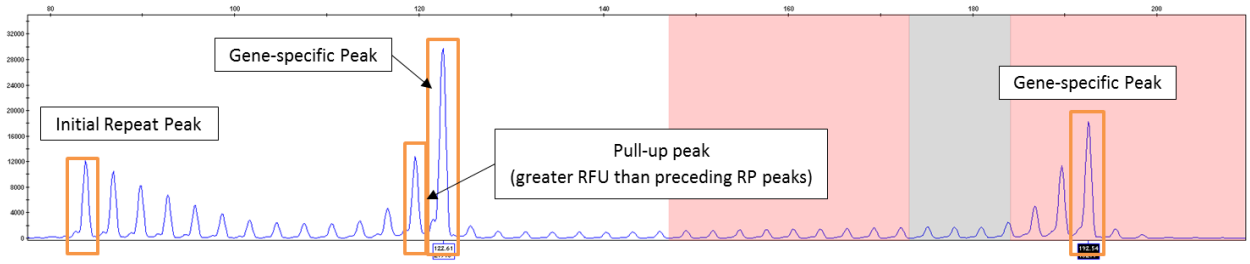


Figure 6. Overview of sample electropherogram characteristics using the AmplideX® PCR/CE *HTT* Kit

   e. For identified highly expanded samples, zoom to the expanded range on the x-axis (~184–1150 bp) and an appropriate RFU range (y-axis) to enlarge CAG RP peaks. Identify and select the high molecular weight pile-up peak at > 900 bp range, if present.
   f. Look for a multi-peak GS allele in the zoomed window, characterized by 4–6 consecutive peaks at greater fluorescent intensity than the preceding repeat pattern. Select the center peak of this group, or the one with the highest intensity, as the GS allele.

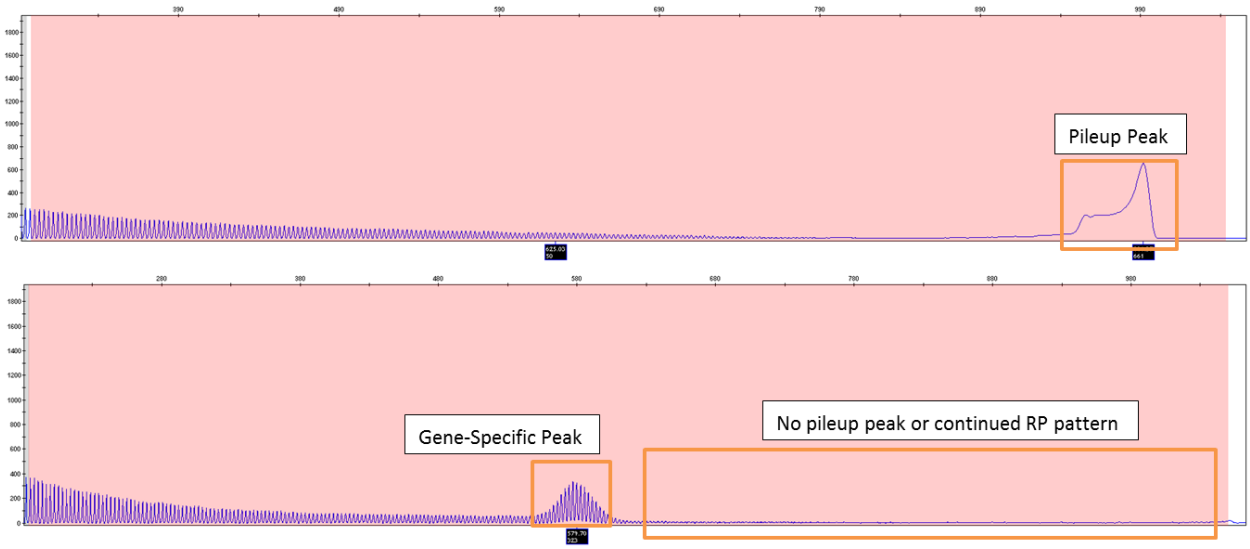


Figure 7. Example electropherograms of an expanded genotype sample with a pile-up peak (top) and an expanded genotype sample with a multi-peak gene-specific allele

Notes:

- A CAG repeat pattern will precede full-length GS alleles. The repeat pattern will dissipate quickly after a GS allele peak.
- If there is only one apparent GS peak and trace lacks a RP profile, i.e. “sawtooth”, following the allele, the sample is homozygous. Use methods in the Troubleshooting section to assist in confirmation, if necessary.
- When using the recommended calibrator (CAL) sample (see Appendix: Derivation of Mobility and Size Correction Factors), 4 GS peaks will be present. Select all 4 GS Peaks.
- Inspection of negative or NTC controls is recommended; GS peaks will have heights of at least 10-fold greater than background noise.

Export Data Table

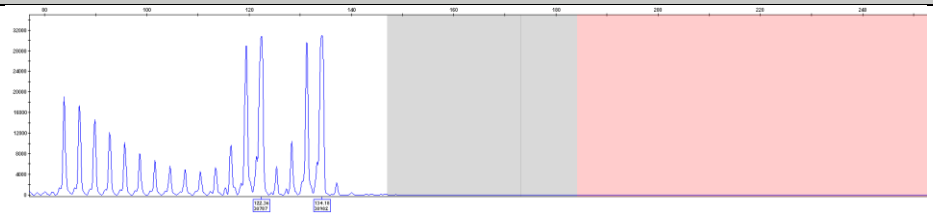
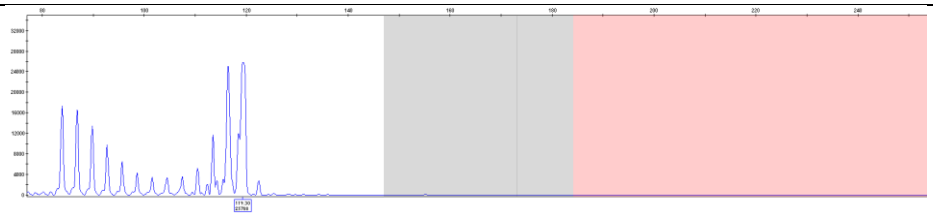
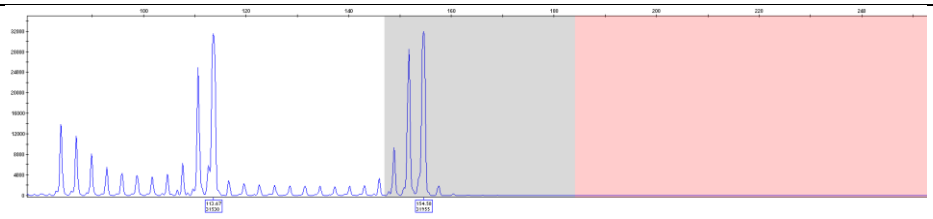
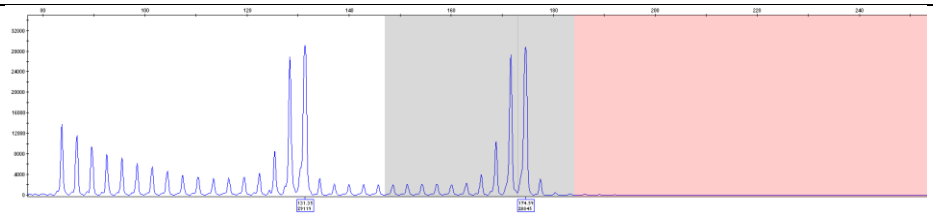
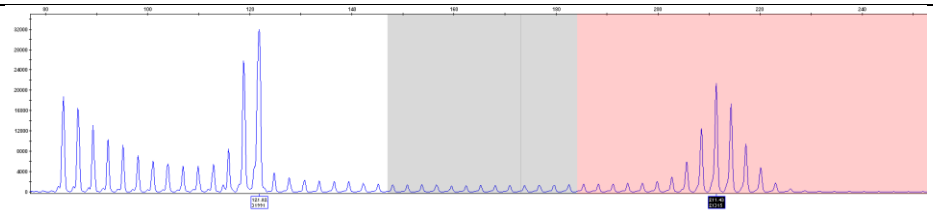
1. Once all the alleles are selected, close the Display Plot window.
2. Select all samples in the Genotypes tab.
   a. Click **File**.
   b. Select **Export Table**.
3. Save the report as a text file (*.txt) for import into the Macro (see AmplideX® PCR/CE *HTT* Macro User Guide for instructions). Samples with their respective peak heights and sizes will be listed in a Genotypes Table file.

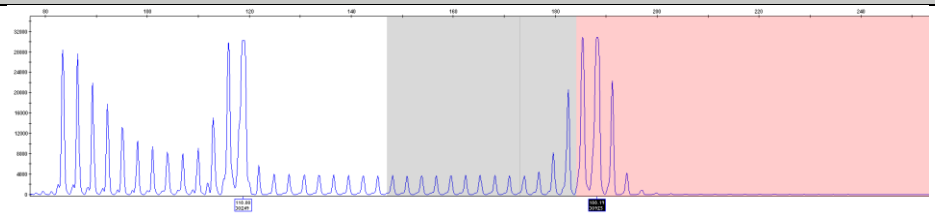
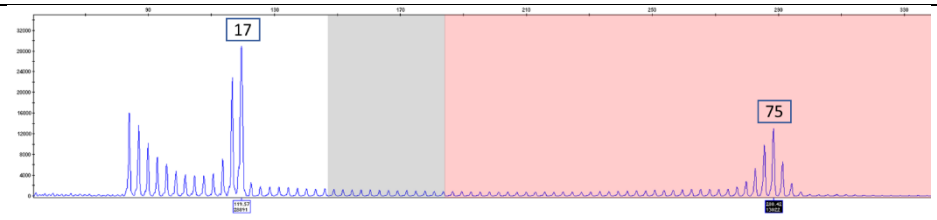
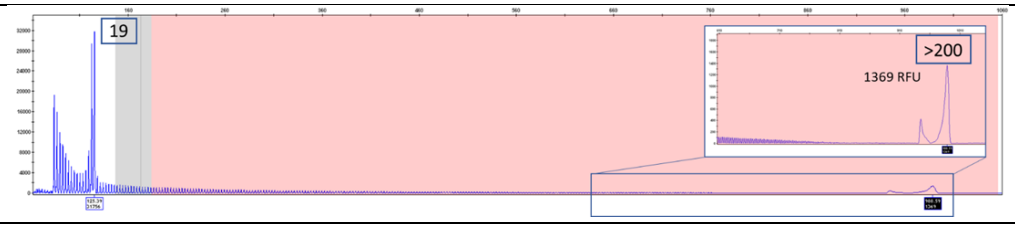
Data Interpretation

Data analysis and interpretation is conducted using the fragment analysis software (i.e. GeneMapper® or GeneMarker®) and an Excel-based analysis tool, AmplideX® PCR/CE *HTT* Macro. Download the analysis tool at http://software.asuragen.com. The AmplideX PCR/CE *HTT* Macro User Guide provides detailed instructions for using the tool and is provided separately with the download package.

Alleles are reported as CAG repeats, in integers. The largest allele size determines genotype category: normal, intermediate, reduced penetrance, or expanded. Up to 200 CAG repeats are reported; > 200 repeats are identified as "> 200". Genotype examples are listed in **Table 3**.

Table 3. Example traces representing *HTT* genotypes

Genotype / Zygosity	Example
Normal (18, 22) / Heterozygous	
Normal (17, 17) / Homozygous	
Intermediate (15, 29) / Heterozygous	
Reduced Penetrance (21, 36) / Heterozygous	
Expanded (18, 49) / Heterozygous	

Genotype / Zygosity	Example
Expanded (17, 41) / Heterozygous	
Expanded (17, 75) / Heterozygous	
Expanded (19, > 200) / Heterozygous	

Quality Control Procedures

Calibration

GC-rich PCR products of repeat regions, such as the *HTT* $(CAG)_n$ element, have a faster migration than the DNA composition of the size standard (ROX 1000 Size Ladder, supplied) during CE. In the absence of correction factors, the higher migration rate of the *HTT* $(CAG)_n$ element may result in underreporting of repeat length. Mobility (M_0) and size (C_0) correction factors are dependent on the instrument, polymer type, capillary length, and run conditions used during CE, and may vary slightly from laboratory to laboratory.

Use of commercially available, cell line-derived genomic DNA with known CAG repeat length is recommended to establish laboratory specific correction factors.

Refer to the Appendix: Derivation of Mobility and Size Correction Factors section for derivation and default values provided with the AmplideX® PCR/CE *HTT* Kit.

Controls

Positive and negative controls are recommended in every run. The Diluent provided within the reagent set may be used as an NTC. An NTC should not contain peaks > 1000 RFU (3500 or 3730 series Genetic Analyzer) or > 330 RFU (3130 series Genetic Analyzer).

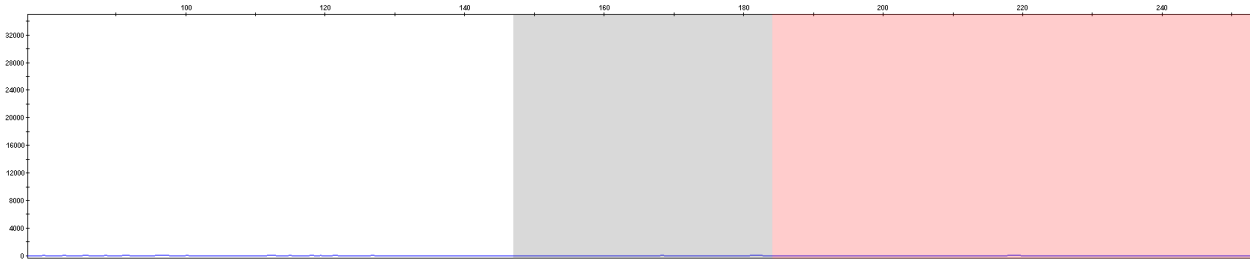


Figure 8. Example electropherogram of negative control using 3500 series Genetic Analyzer

Though a calibrator is not required for every run, a positive control is recommended to determine if the selected correction factors result in allele sizing that is out of specification. A well-validated sample with previously determined genotype, such as NIST SRM2393 Component A (15, 29), Component B (17, 36), Component C (15, 40), or Component D (39, 50), may be used as a positive control.

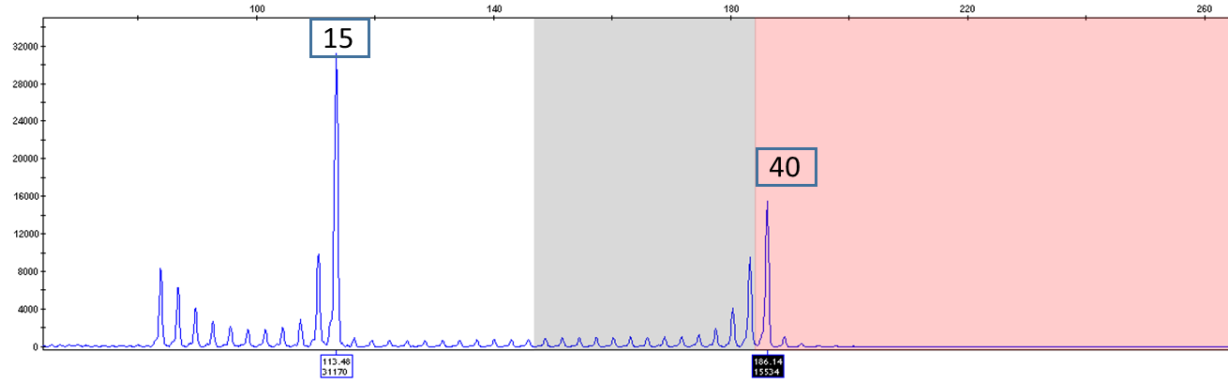


Figure 9. Example electropherogram of NIST SRM2393 Component C, used as positive control using 3500 series Genetic Analyzer

If a positive or negative control fails predetermined specifications, the run should be repeated prior to allele sizing of unknown samples.

Samples

A calibrator, control, or sample is valid when the ROX 1000 Size Ladder is correctly identified (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 cannot be adjusted, should be interpreted as failing and accurate allele sizing cannot be performed. Such samples may be repeated.

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 that may result in miscalling repeats for unknown specimens	Identify and validate new reference material
	Correction coefficients were incorrect for the specific run	Repeat PCR with calibrator sample
PCR artifacts present in NTC samples above specified maximum RFU	Contamination of the NTC	Repeat the batch run. Pay special attention to pipetting technique (e.g. aerosolization of samples) in a review of the laboratory contamination control procedures
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 PCR Analyze specimen by another method
Expanded repeat pattern observed without corresponding pile-up or expanded allele peak	There may be a potential rare (MAF ≤1%) or unidentified polymorphism that inhibit PCR or primer binding	Repeat PCR Analyze specimen by another method
No peaks identified in a sample	Sample was not added to reaction, compromised or degraded	Repeat PCR Repeat DNA isolation
Widespread allele intensity saturation	The wrong CE or thermal cycling protocol was used	Ensure proper instrument settings are utilized Repeat PCR and or injection
GS peaks are oversaturated	Input too high CE run conditions not optimal	Suggested actions: • Repeat injection with same PCR product, using shorter injection time • Repeat PCR with lower input (< 20 ng)
A single GS peak observed; repeat pattern does not continue	Sample is homozygous Sample alleles are n, n+1	If greater resolution is desired, perform the following steps*:* • Repeat injection with same PCR product, using a shorter injection time • Repeat PCR with lower input (< 20 ng)

Analytical Assessment

For analytical performance assessment of the AmplideX® PCR/CE *HTT* Kit, the tolerance around measurements of CAG repeats depends on the length of the CAG repeat region. All categories described by ACMG and EMQN guidelines (Bean L and Bayrak-Toydemir P (2014) & Losekoot *et al*. (2013)) have a consensus size of ± 2 CAG repeat, up to 49 CAG repeats. The analytical performance was assessed using the target tolerance for CAG repeat length of ± 1 repeats up to 49 repeats as listed in **Table 4**. Additional performance testing of larger expansions, such as > 75 CAG repeats, was been demonstrated using CCR samples NA14044 (19, > 200) and NA09197 (18, 178).

Table 4. Target tolerance of Kit for each range of CAG repeat lengths

Categorical Bin	CAG Repeat Length	Target Tolerance	ACMG Consensus
Normal	≤ 26	± 1	± 2
Intermediate	27–35		
Reduced Penetrance	36–39		
Expanded	40–49		
	50–75	± 2	± 3
	76–100	± 3	± 4
	> 100	± 10%	n/a

Precision

Precision studies at a single-site were conducted to establish the repeatability of CAG repeat sizing and genotype determination. A 7 sample panel representing each categorical genotype (see **Table 1**) was assessed to determine the allele size across multiple factors, including 5 days, 2 operators, 2 peak-calling analysts, and 3 CE platforms (3130, 3500 or 3730 series). Peak annotation was performed using either GeneMapper Software v4.1 or v5.0.

The expected alleles for each sample (*n* = 128–132 each) were determined using the mode of all measurements. For all samples analyzed, all alleles observed were as expected, within the kit target tolerance.

Table 5. Total calls of each repeat length across single-site precision study

Sample	Expected Repeat Length ± Tolerance	Observed Repeat Length	Frequency	% Within Tolerance
Sample 1	15 ± 1	15	132	100
Sample 2	17 ± 1	17	131	100
	39 ± 1	39	131	100
	50 ± 2	49	2	100
		50	129	
	75 ± 2	74	6	100
		75	125	
Sample 3	15 ± 1	15	132	100
	40 ± 1	40	132	100
Sample 4	17 ± 1	17	132	100
	33 ± 1	33	132	100
Sample 5	21 ± 1	21	132	100
	36 ± 1	36	132	100
Sample 6	15 ± 1	15	131	100
	42 ± 1	42	131	100
Sample 7	15 ± 1	14	1	100
		15	127	

DNA Input Amount

Recommended mass input of DNA was established using a 5 sample panel representing each categorical genotype (see **Table 1**) at 10, 20, 40, or 80 ng total input per reaction. At least 10 replicates of each sample and input level were assessed. CAG repeat alleles were 100% concordant with the expected alleles for each sample, determined using the mode of all measurements. All alleles, regardless of input level, produced peaks with intensities at least 10-fold higher than background signal based on an NTC; peak morphology at the highest input level (80 ng) appeared oversaturated in all cases but no incorrect calls were observed. Therefore, 10–80 ng per reaction is an acceptable DNA input range, with a recommended input of 10 or 20 ng to reduce risk of oversaturation.

Instrument Identity

The AmplideX® PCR/CE *HTT* Kit was tested across multiple thermal cycler models, using default ramp rates established by manufacturer. Genotype and CAG repeat allele concordance (100%) was achieved using a representative panel of seven unique samples with a maximum CAG repeat length of 75. Any laboratory-validated thermal cycler may be used.

Resultant PCR products of the representative panel of 7 samples, as mentioned, were analyzed by various CE genetic analyzers; Applied Biosystems® 3500xL and 3500 Dx, 3130*xl*, and 3730*xl* (48-capillary). All platforms used the POP7™ polymer and the parameters listed **Table 2**. Genotype and CAG repeat allele concordance (100%) was achieved across all platforms tested using samples with CAG repeat lengths of 15 to 75. Since electropherograms generated by each platform differ slightly with

respect to peak size and intensity, take care to ensure platform-specific calibration and control specifications are used prior to allele sizing of unknown samples.

Comparision to a Published Design

A reference set of 14 cell line samples with associated genotype data (Kalman, *et al.* 2007) was tested by the AmplideX® PCR/CE *HTT* Kit, in singlicate, and analyzed using both GeneMapper or GeneMarker. The SRM2393 (NIST) components with multi-laboratory defined genotype was also evaluated. In all cases, the reported alleles were observed (**Table 6**). Two samples, NA20253 and NA20252, identified the presence of an additional lesser allele in each sample, as compared to published reports, suggesting an increased sensitivity for mosaic alleles. An allele of 63 was identified in NA20252 (**Figure 10**) while an allele of 128 was also observed in NA20253 **(Figure 11**).

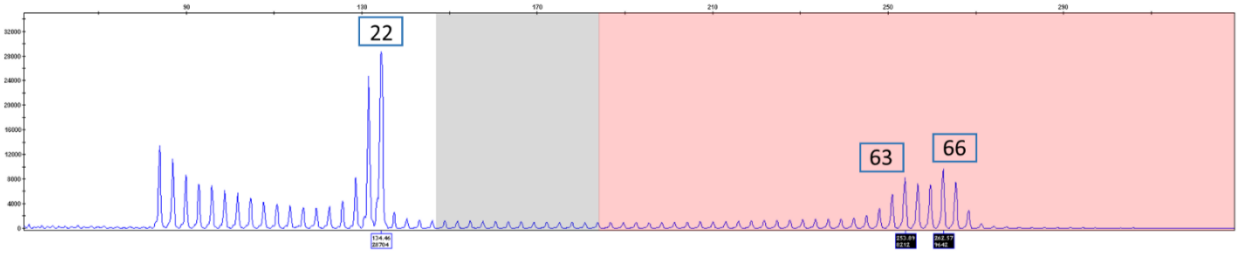


Figure 10. Electropherogram of NA20252 (CCR) depicting CAG repeats determined by the AmplideX PCR/CE *HTT* Kit (22, 63, 66)

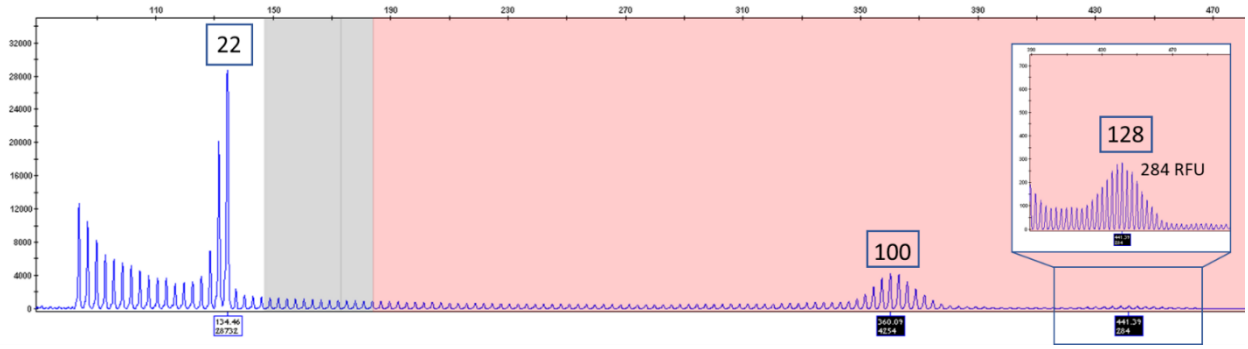


Figure 11. Electropherogram of NA20253 (CCR) depicting CAG repeats determined by the AmplideX PCR/CE *HTT* Kit (22, 100, 128)

Table 6. Comparison of CAG repeat alleles in commercially available samples, as reported by Kalman *et al.* (2007)

Sample ID	Sample Type	Input (ng)	Genotype	Reported Alleles	Observed Alleles
NA20206	Cell line	20	Normal	17, 18	18, 18
NA20207		20	Normal	19, 21	19, 21
NA20208		20	Expanded	35, 45	35, 45
NA20209		20	Expanded	45, 47	45, 47
NA20210		20	Expanded	17, 74 or 75	17, 75
NA20245		20	Normal	15, 15	15, 15
NA20246		20	Normal	15, 24	15, 24
NA20247		20	Intermediate	15, 29	15, 29
NA20248		20	Reduced Penetrance	17, 36	17, 36
NA20249		20	Reduced Penetrance	22, 39	22, 39
NA20250		20	Expanded	15, 40	15, 40
NA20251		20	Expanded	39, 50	39, 50
NA20252		20	Expanded	22, 65 or 66	22, 63*, 66
NA20253		20	Expanded	22, 101	22, 100, 128*
SRM 2393 A		20	Intermediate	15, 29	15, 29
SRM 2393 B		20	Reduced Penetrance	17, 36	17, 36
SRM 2393 C		20	Expanded	15, 40	15, 40
SRM 2393 D		20	Expanded	35, 45	35, 45
SRM 2393 E		20	Expanded	39, 50	39, 50
SRM 2393 F		20	Expanded	17, 75	17, 75
* Additional lesser peak identified with Kit					

Limit of Detection (LOD) or Analytical Sensitivity

A preliminary range-finding assessment of the limit of detection (LOD) for mosaicism study evaluated 2 samples with 4 distinct alleles mixed together at 4 target ratios. Sample 1 (NA20210) was diluted into sample 2 (NA20251, 17,75) at 7%, 5%, 3% and 1% target fractions, maintaining a total concentration of ~10 ng/µL (20 ng input). The allele of interest is expected to be ~75 repeats from NA20210.

For the cell line DNA mixture, a 100% hit rate was observed in all LOD dilution levels except at the 1% level, where the 75 CAG repeat allele was detected in only 40% of replicates. Fitting these hit rates to a regression model, the calculated LOD for the longest CAG allele in the cell line mixture is 1.63% (see **Figure 12**, left). As the 17 CAG repeat allele was co-diluted with the 75 CAG repeat allele, only the 1% allele fraction tested observed < 100% hit rate for the 17 CAG repeat allele. Fitting the probit to the regression model, the calculated LOD for the 17 CAG repeat allele is 1.49% (see **Figure 12**, right).

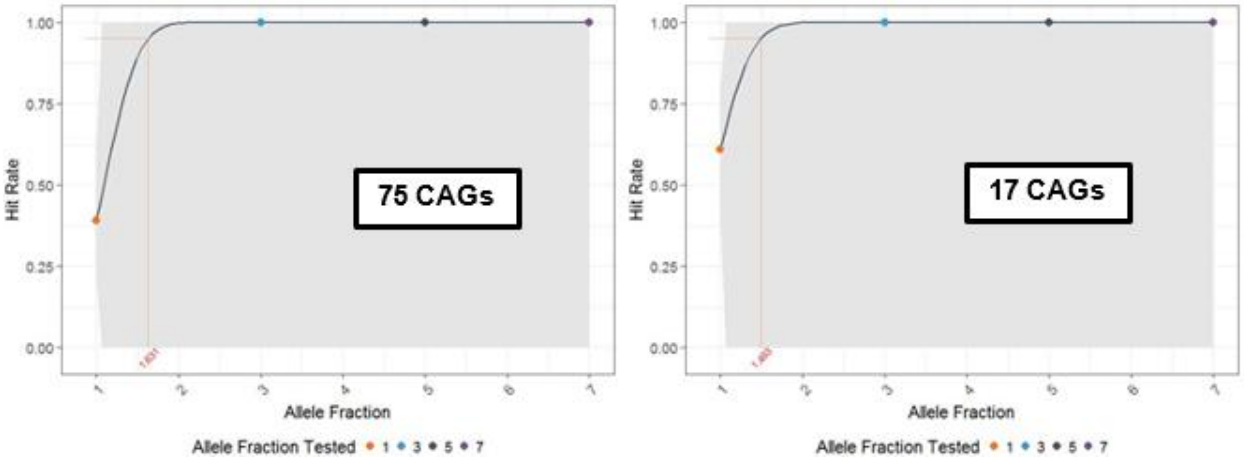


Figure 12. Computed probit of 75 CAG repeat allele (left) in a cell-line sample mixture and corresponding 17 CAG repeat allele (right)

Analytical Specificity

As described in the ACMG Technical Standards and Guidelines for HD, “polymorphisms surrounding or within the CAG tract have been identified and have a collective frequency of > 1%...” (Bean L and Bayrak-Toydemir P (2014)). Two A->G SNPs (rs473915 and rs76533208) occur within the 12 base pair spacer region of *HTT* and result in a loss of interruption between the CAG and CCG tracts.

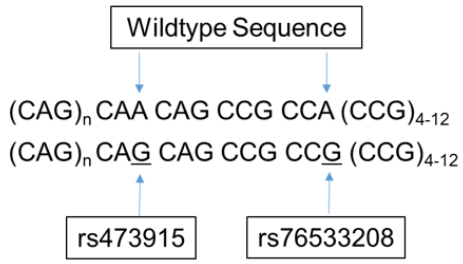


Figure 13. Location of common polymorphisms in CAG repeat tract of *HTT*

To evaluate the effect of these polymorphisms on CAG repeat sizing, synthetic ssDNA samples were designed to contain the SNPs of interest and evaluated using the AmplideX® PCR/CE *HTT* Kit. Synthetic samples containing one of the SNPs of interest were compared to wild type (wt) synthetic sample with an

expected CAG repeat allele of 19. As shown in **Figure 14**, the CAG detection and repeat size of 19 was accurately maintained in the presence of the SNPs with minimal loss in signal or alteration of peak morphology.

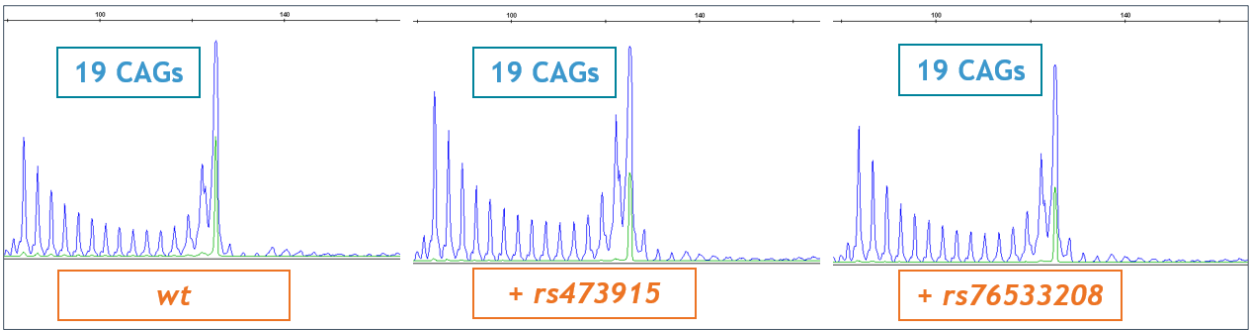


Figure 14. Evaluation of common polymorphisms using synthetic DNA samples

Disclaimers

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References

1. Kalman L, *et al*. Development of genomic reference materials for Huntington disease genetic testing. Genet Med. 2007 Oct; 9(10):719–23.
2. 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.
3. Bean L, Bayrak-Toydemir P: Technical Standards and Guidelines for Huntington Disease (2014 Edition). American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories.
4. Losekoot M, van Belzen MJ, Seneca S, Bauer P, Stenhouse, SAR, Barton DE: EMQN/CMGS Best Practice Guide for the Molecular Genetic testing of Huntington Disease. European Journal of Human Genetics (2013); 21, 480–486.

Appendix: Derivation of Mobility and Size Correction Factors

A calibrator sample composed of CCR samples NA20251 (39, 50) and NA20210 (17, 75) mixed at a 1:2 ratio with a total input of 10–20 ng per reaction is recommended (Kalman L., *et al.* 2007). Alternatively, NIST SRM2393 Component E (39, 50) and Component F (17, 75) may be mixed at the same 1:2 ratio and 20 ng input. Together, this mix has expected GS peaks corresponding to repeats of 17, 39, 50, and 75; these peaks may be used to establish a linear calibration curve between observed peak size (in bp) and expected CAG repeats. As shown in **Figure 15**, the slope (M_0) and intercept (C_0) of the linear regression fit line is calculated using the known CAG repeat values (x-axis) and peak size (bp, y-axis) of the mixture.

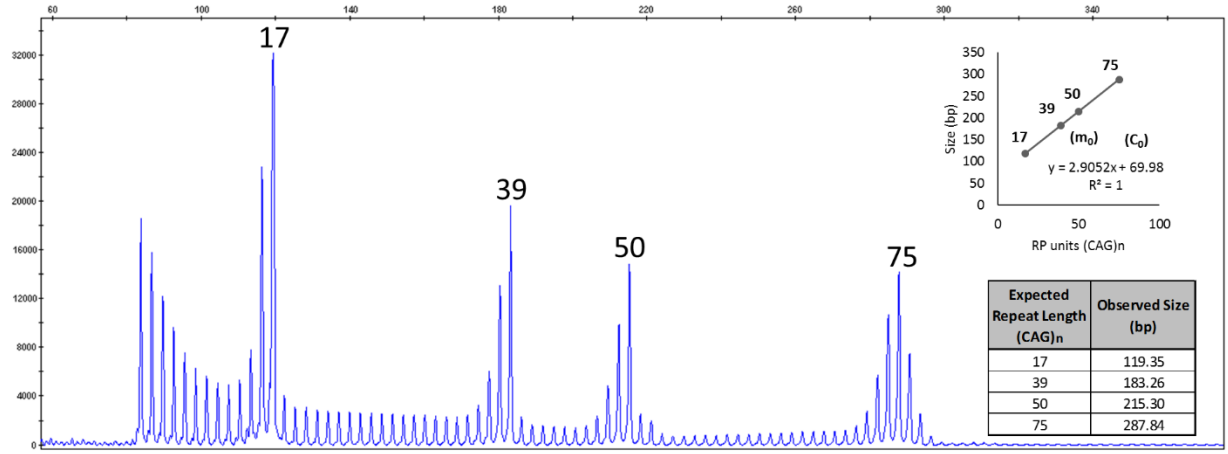

Figure 15. Derivation of a linear calibration curve to determine mobility (M_0) and size (C_0) correction factors using sample mixture of NIST SRM2393 Component E (39, 50) and Component F (17, 75)

The proposed correction factors (M_0 and C_0) for supported Applied Biosystems® genetic analyzers configurations are listed in **Table 7**. Correction factors for other configurations of instrument, capillary length, polymer type, and/or run condition are not provided but may be determined using the procedures previously described.

Table 7. Mobility (M_0) and size (C_0) correction factors for supported Applied Biosystems genetic analyzers configurations

Instrument	Capillaries	Capillary Length	C_0	M_0
3130*xl*	16	36 cm	70.4290	2.8588
3500, 3500xL	8 or 24	50 cm	70.4032	2.8967
3730*xl*	48	50 cm	70.0129	2.9137

Glossary of Symbols

Symbol	Description
REF	Catalog number
LOT	Batch code
Σ	Contains sufficient for <n> tests
	Consult instructions before use
	Temperature limitation
	Use by
	Date of Manufacture
	Manufacturer

Asuragen, Inc.
2150 Woodward St.
Suite 100
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