AmplideX® PCR/CE *C9orf72* Kit

Protocol Guide

For Research Use Only.
Not for Use in Diagnostic Procedures.

REF	**49581**
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Background Information

Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are neurodegenerative disorders that are now believed to be part of the same clinical continuum of diseases. FTD is a progressive dementia syndrome associated with atrophy of the frontal and anterior temporal lobes, which may result in changes in personality, decision-making skills, impulse-control, behavior, and language but leave memory and perception relatively unaffected. In people under 65 years of age, it is the second most common form of dementia after Alzheimer disease. ALS is characterized by a loss of upper (i.e., brain) and lower (i.e., brainstem and spinal cord) motor neurons, which leads to progressive weakness of the voluntary muscles. Clinical studies have shown that approximately 15% of patients with FTD also have ALS [1]. In addition, while approximately 15% of patients with ALS have FTD, up to 50% of patients with ALS also exhibit frontal lobe impairment but do not meet strict criteria for FTD [1-3].

At the molecular level, an expansion of a hexanucleotide repeat element (G_4C_2) in intron 1 of the Chromosome 9 open reading frame 72 gene (*C9orf72*; NM_001256054.2) has been linked to FTD and ALS [4, 5]. Unaffected individuals have <20 hexanucleotide repeats, while affected individuals have >30 repeats and often >1,000 repeats. The range of 20-30 repeats is considered to be an intermediate range with no clinical evidence of disease manifestation. The expansion appears in approximately 25% of familial FTD patients and 20-67% of familial ALS patients depending on the population studied, making this the most prevalent genetic mutation in both diseases. The expansion also appears in approximately 6% of sporadic FTD patients and 7% of sporadic ALS patients [6]. In addition, intermediate repeat lengths (20-30 repeats) have been reported to be a significant risk factor for Parkinson Disease (PD) [7].

Screening studies have revealed C9orf72 expansions at an incidence of ~1:700 in the general population [8], highlighting the importance of understanding the influence of these expansions in both frank disease as well as the disease risk for unaffected individuals. To this point, *C9orf72* expansions have been observed in multiple neurodegenerative syndromes, including Alzheimer disease, sporadic Creutzfeldt-Jakob disease, and Huntington disease-like syndrome. Accurate patient diagnoses will also be vital to target appropriate therapies to patients with various forms of FTD - ALS spectrum disorders as well as test family members of patients for their risk of developing FTD or ALS before symptoms manifest.

Currently, researchers rely on low-sensitivity and low-resolution Southern blot analysis and/or one or multiple low-performance repeat-primed PCR assays to detect and quantify *C9orf72* hexanucleotide repeat expansions. However, these assays are inadequate to profile the *C9orf72* locus at high resolution. For example, genotyping accuracy using current PCR methods is widely known to be suspect beyond about 30 repeats [9]. In addition, previously described methods require multiple reactions to genotype unexpanded repeats and flag large repeat expansions. Due to these technical limitations, laboratories bin results into broad categories, such as >30 repeats, and endure burdensome procedures.

To address these limitations, Asurage developed the AmplideX® PCR/CE *C9orf72* Kit (RUO). This kit has been verified to provide accurate capillary electrophoresis (CE) sizing of alleles up to 145 G_4C_2 repeats and identify expanded alleles >145 G_4C_2 repeats in a single-tube PCR.

Test Principle

The AmplideX® PCR/CE *C9orf72* Kit (RUO) is used to PCR-amplify the *C9orf72* hexanucleotide fragment from purified genomic DNA using a three-primer G_4C_2-Repeat Primed (RP)-PCR configuration, followed by fragment sizing on an Applied Biosystems Genetic Analyzer. The PCR reagents include gene-specific and G_4C_2 repeat primers, diluent, a polymerase mix and buffer for amplification of the $(G_4C_2)n$ repeat region in the *C9orf72* gene, and a ROX 1000 Size Ladder for sizing by CE. The size of the PCR products are converted to the number of G_4C_2 repeats using size and mobility conversion factors.

PCR Methods

The kit includes reagents to perform G_4C_2 RP-PCR with an option for gene-specific PCR (Figure 1). Gene-specific PCR uses two primers that flank the $(G_4C_2)_n$ repeat region. PCR products from the gene-specific primers represent full-length alleles with up to ~145 repeats (Figure 1, right pane). G_4C_2 RP-PCR is primarily distinguished from the more conventional two-primer, gene-specific PCR by the addition of a third PCR primer that is complementary to the *C9orf72* hexanucleotide repeat region.

The resulting electropherogram includes both full-length PCR products and G_4C_2 repeat-primed amplicons (Fig. 1, left pane). The G_4C_2 RP-PCR products correspond to individual PCR amplicons from each combination of the repeat primer with the gene-specific reverse primer. These RP peaks are separated by 6 bp, or 1 repeat unit, as expected. The profile of these peaks provides important confirmatory information of each sample's molecular features, resolves zygosity, and reports the presence of large expanded alleles (>145 repeats).

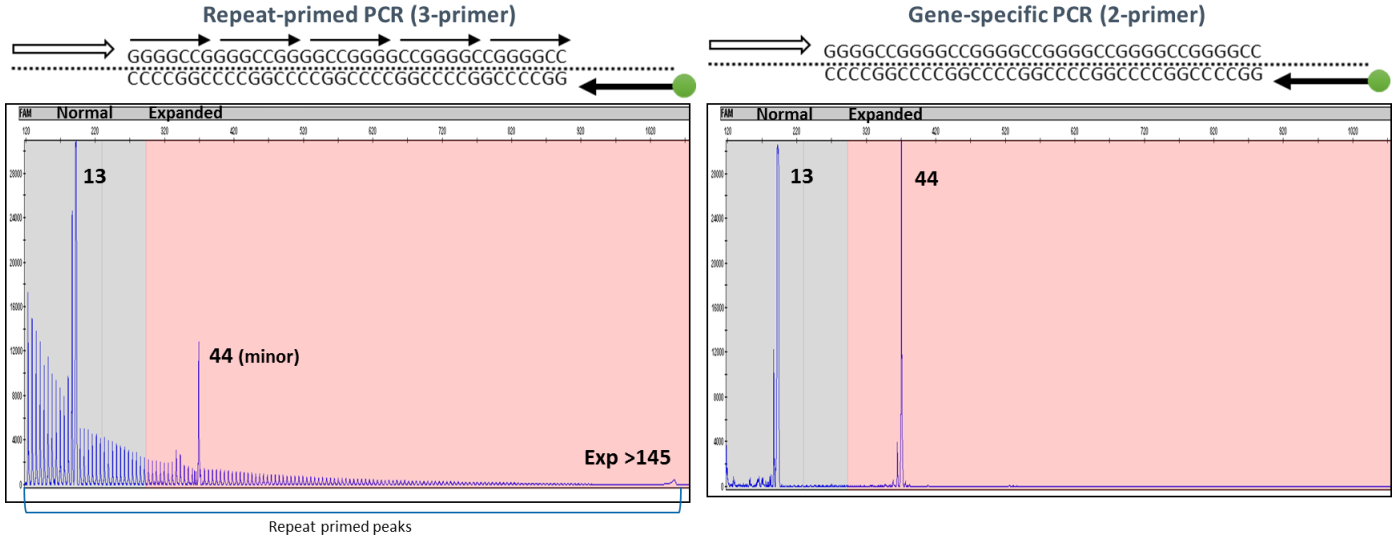

Figure 1. *C9orf72* PCR Methodologies highlighting the features of the 2- and 3-primer PCR systems.

The G_4C_2 RP-PCR repeat profile can telegraph the presence of longer alleles in the amplification, irrespective of whether such alleles are detected as full-length products. Consequently, the risk of PCR dropout of the longer allele is reduced (see Figure 1 above). The full-length gene-specific product peaks may be converted from size in base pairs to the number of G_4C_2 repeats using predefined conversion factors. In addition, the repeat peak profile can provide very accurate $(G_4C_2)_n$ repeat quantification by directly counting the number of G_4C_2 repeat primed amplicon peaks up to ~145 G_4C_2.

Workflow

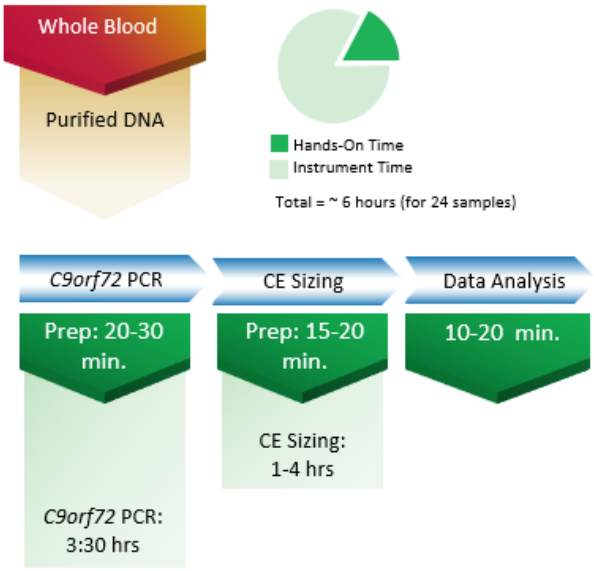

Figure 2. Overview of the AmplideX® PCR/CE *C9orf72* Workflow, showing key steps and time estimates for each step (thermal cycling for gene-specific PCR is ~4 hours). The CE analysis protocol requires approximately a 1 hour run time for each set of 16 to 96 samples per injection depending on the model used.

The workflow for the test includes PCR master mix setup, thermal cycling, and analysis using CE. Genomic DNA is added to a PCR reaction well containing a master mix composed of GC-Rich Amp Buffer (P/N 145425), GC-Rich Polymerase Mix (P/N 145153), and the *C9orf72* Gene-Specific Primer Mix for gene-specific PCR (P/N 145432). The *C9orf72* Repeat Primer (P/N 145433) is also added to the master mix to enable G_4C_2 RP-PCR. After ~4 hrs of thermal cycling, unpurified PCR products are directly mixed with Hi-Di™ Formamide and Asuragen's ROX 1000 Size Ladder (P/N 145427). Following denaturation, the products the amplicons are sized on any Applied Biosystems Genetic Analyzer running POP-7 polymer. A schematic of the workflow is shown in Figure 2.

After resolving PCR products on CE, the resulting electropherograms are analyzed to identify full-length gene-specific product peaks. Repeat peaks are detected within the linear range of the instrument (~900 bp) up to approximately 145 G_4C_2 repeats. These peaks are converted from size in base pairs to the number of G_4C_2 repeats using correction factors derived for that instrument configuration. Beyond 145 G_4C_2 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 [10]. Thus, *C9orf72* PCR products exceeding 145 G_4C_2 are identified categorically as >145 G_4C_2. In addition to sizing information, qualitative trace features that can differentiate homozygous alleles from those with one large expanded allele, as well as sequence variability such as insertions or deletions near the repeat region, may be derived from the G_4C_2 RP-PCR profile.

Use of Controls

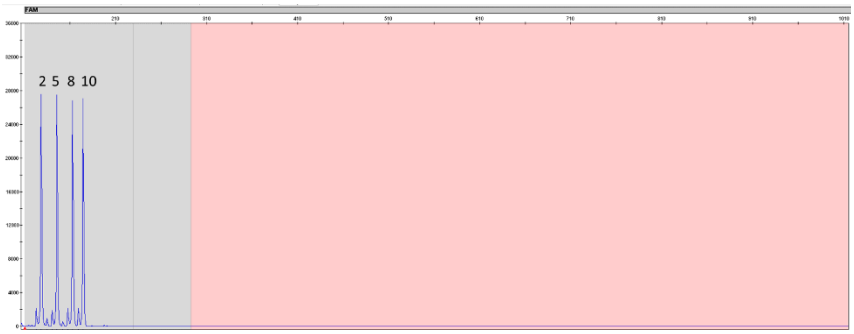

Figure 3. Electropherogram of the provided *C9orf72* PCR Control (P/N 145426). A pooled cell-line control showing the G_4C_2 RP-PCR results resolved on CE, which produce 4 gene-specific peaks corresponding to 2, 5, 8 and 10 repeats.

Positive and negative controls are recommended in every run. The Diluent provided within the reagent set (P/N 145157) may be used as a negative no-template control. A PCR Control (P/N 145426) comprised of an admixture of 4 normal *C9orf72* alleles (2, 5, 8 and 10 G_4C_2 repeats; Figure 3) is also provided and can be used as a general positive PCR control and as a calibrator for size (in bp) to G_4C_2 repeat unit conversion (see Fragment Size Analysis section). Genomic DNA extracted from well-characterized cell lines with expanded *C9orf72* alleles may be used for positive controls. Cell lines or corresponding purified genomic DNA can be obtained from various repositories such as the Coriell Cell Repository (CCR) [11]. Representative examples of CE traces of AmplideX PCR/CE *C9orf72* products from these materials are shown in the Data Interpretation section below.

Reagents

Reagents Provided with the Kit

Table 1. AmplideX® PCR/CE *C9orf72* Kit Components (RUO) (P/N 49581)

Item #	Item Description	Volume	Storage Temp
145432	*C9orf72* Gene Specific Primer Mix	28 µL	-15 to -30°C
145433	*C9orf72* Repeat Primer	28 µL	-15 to -30°C
145425	GC-Rich Amp Buffer	650 µL	-15 to -30°C
145153	GC-Rich Polymerase Mix	5 µL	-15 to -30°C
145427	ROX 1000 Size Ladder	110 µL	-15 to -30°C
145157	Diluent	1.0 mL	-15 to -30°C
145426	*C9orf72* PCR Control	12 µL	-15 to -30°C

Handling and Storage

- Store the reagents in a non-frost-free freezer in the dark at -15 to -30 °C.
- Allow reagents (except GC-Rich Polymerase Mix) to completely thaw at room temperature before use (approximately 10 minutes). Vortex all reagents (except GC-Rich Polymerase Mix) 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 (approximate range of 18-25 °C).

Number of Reactions

- The provided reagents are sufficient for up to 50 reactions of gene-specific PCR or G_4C_2 RP-PCR, and 50 CE analyses.
- The reagents have been verified for use up to five freeze-thaw cycles. Additional cycles are not recommended.
- Master mixes can be prepared for the appropriate number of samples with a recommended total number of at least 10 reactions per run.

Reagent Stability

- The product will maintain performance through the expiration date printed on the label when stored under the specified conditions.

Reagents Required but not Provided

- Reagents for DNA isolation are not included. DNA can be extracted via common, laboratory-validated sample preparation methodologies that ensure high quality, intact DNA.

Capillary Electrophoresis Materials Required but not Provided

- ABI Genetic Analyzers running POP-7 polymer (e.g. 3130, 3730 or 3500 series).
- POP-7 Polymer: Applied Biosystems, #4363785 or equivalent
- Hi-Di™ Formamide: Applied Biosystems, #4311320 or equivalent
- Dye set calibrators for FAM and ROX, DS-30 or DS-31 dye set: Applied Biosystems #4345827, #4345829, or equivalent

Consumables & Equipment Required but not Provided

- General laboratory equipment and workspace to perform PCR

- Thermal cycler: ABI 9700 or ABI Veriti. Additional thermocyclers may require user validation
- Centrifuge capable of spinning 96-well plates
- Vortex
- Micro-centrifuge
- Pipettes: Units with an accuracy range between 0.2-2 µL, 1-10 µL, 2-20 µL, 20-200 µL and 100-1000 µL
- Multi-channel pipette unit capable of pipetting 1-10 µL
- 96-Well PCR Plates: AB Gene #AB-0900 or equivalent
- PCR Plate Seals: AB Gene #AB-0558, Phenix LMT-0028 or equivalent
- PCR Compression Pad: Applied Biosystems # 4312639 or equivalent

Positive Controls Recommended but not Provided

- CCR, NINDS ALS genomic DNA samples: ND06769, ND10518, ND10966 and ND12028 (See Figure 10 below) or other commercially-available genomic DNA or cell-line DNA standards.

Warnings and Precautions

- Use proper personal protective equipment. Wear appropriate protective eyeglasses, protective gloves, and protective clothing when working with these materials. Use nuclease-free lab ware (e.g., pipettes, pipettes tips, reaction vials).
- **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.
- Follow Universal Precautions when handling human samples.
- Substances that may interfere with the PCR of DNA 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 filter pipette tips and nuclease-free tubes.
- 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 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.
- 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.
- Prior to use, ensure that the Genetic Analyzer is calibrated according to the manufacturer's instructions.

Caution: The toxicological properties of the PCR reagents have not been fully investigated. Avoid contact with skin and mucous membranes. Do not ingest. Safety Data Sheets are available upon request.

Pre-Analytical Steps

Genomic DNA extracted via common sample preparation methodologies from whole blood collected in EDTA is compatible with the AmplideX® PCR/CE *C9orf72* Kit (RUO). It is recommended that the purified genomic DNA be evaluated for concentration (e.g. via OD260) and purity (OD260/280 and OD260/230 ≥ 1.7) and to store DNA samples below -15°C. Input 20 – 80 ng into each reaction (2 µL of DNA at 10 – 40 ng/µL).

AmplideX® PCR/CE *C9orf72* Kit (RUO) Protocol

The test protocol involves three key sets of procedures:

1. PCR master mix setup and thermal cycling
2. Capillary electrophoresis
3. Fragment sizing analysis

The instructions below are written for the preparation and analysis of gene-specific PCR or G_4C_2 RP-PCR products. There is only one difference between the two protocols: Gene-specific PCR is performed without the *C9orf72* Repeat Primer (P/N 145433) 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 provided reagents are sufficient for up to 50 reactions performed in up to 5 independent batches, also including 10% overage for master mix preparation. The minimum number of reactions per batch is 10 and no more than 5 freeze-thaw cycles are supported. Examples of recommended overage for a given batch size are provided in Table 2.

Table 2. Examples of PCR Master Mix Setup

Sample Batch Size	Recommended 10% Overage
10	+1
25	+2.5
50	+5

The workflow should proceed in a uni-directional 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 risk of amplicon contamination.

PCR Master Mix Setup and Thermal Cycling

1. Thaw all reagents except the GC-Rich Polymerase Mix for approximately 10 minutes at room temperature. Place GC-Rich Polymerase Mix on ice. Briefly vortex all tubes (3-5x pulse vortexing) except the Polymerase Mix.

   Note: GC-Rich Polymerase Mix should be stored on ice at all times. The GC-Rich Amp Buffer may be occluded or have observable precipitation when cold. After completely thawing the tube, vortex to ensure mixing.

2. Add the appropriate components to a 1.5 mL microfuge tube in the exact order specified in Table 3.

Table 3. PCR Master Mix Setup

Component	2-Primer Gene-specific PCR	3-Primer RP-PCR
GC-Rich Amp Buffer (P/N 145425)	11.45 µL	11.45 µL
C9orf72 Gene Specific Primer Mix (P/N 145432)	0.50 µL	0.50 µL
C9orf72 Repeat Primer (P/N 145433)	0 µL	0.50 µL
Diluent (P/N 145157)	1.00 µL	0.50 µL
GC-Rich Polymerase Mix (P/N 145153)	0.05 µL	0.05 µL
DNA Sample or PCR Control (P/N 145426)	2.00 µL	2.00 µL
Total Volume per Reaction	**15.00 µL**	**15.00 µL**

Note: The GC-Rich Amp buffer is viscous; retract piston slowly to acquire solution.

Important! Excess GC-Rich Polymerase Mix may inhibit the reaction. Ensure that there are no additional droplets on the pipette tip prior to dispensing to the master mix.

3. Thoroughly vortex master mix (3-5 times pulse vortexing) prior to aliquoting to PCR plate or strip-tubes.

   Critical! The master mix must be vortexed prior to dispensing to ensure adequate mixing of all reagents.

4. Dispense 13.0 µL master mix to each well or tube. Use a repeater pipette if available. Switch pipette tip at the start of every column of the plate if using a standard pipettor.

5. Add 2.0 µL of the appropriate DNA sample to each well. Pipette up/down at least twice to ensure adequate mixing.

6. Seal the plate with an adhesive film seal; ensure that all of the wells and plate edges are well sealed.

7. Gently vortex the plate.

8. Centrifuge the plate to remove bubbles (1 min at 1600 rcf).

   Important! Ensure all bubbles are removed from the wells.

9. Transfer the sealed PCR plate to a preprogrammed supported thermal cycler and run the following cycling protocol:

Gene-specific PCR and G_4C_2 RP-PCR	
Description	**Duration**
1 hold	98°C for 5 min
37 Cycles	97°C for 35 sec
	62°C for 35 sec
	72°C for 3 min
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. PCR product stability at -15 to -30 °C has been verified for up to 10 days.

Capillary Electrophoresis using POP-7

1) Thaw the formamide and ROX 1000 Size Ladder (P/N 145427) at room temperature. Thoroughly vortex (15 seconds) and spin tubes before use.

2) Prepare a master mix solution by adding components in the order listed:

Hi-Di™ Formamide	11 µL
ROX 1000 Size Ladder	2 µL
Total Volume per well	**13 µL**

3) Mix all added reagents (by pulse vortexing 3-5 times), and spin down briefly to collect.

4) Aliquot 13.0 µL of Formamide/ROX solution to each well of a new CE analysis plate.

   Important! Samples must be matched to the injection configuration of the Genetic Analyzer (e.g. A1-H2, A3-H4…A11-H12) in appropriate groups of 8, 16 or 24 capillaries. If running less than the number of samples for any injection group, fill empty wells subject to injection with 15 µL of Hi-Di™ Formamide.

5) Transfer 2 µL of PCR products to the CE plate, pipetting up and down 2 to 3 times to mix. A multi-channel pipette is recommended for transfer.

6) Seal the plate, vortex, centrifuge to remove bubbles and transfer to a thermal cycler.

7) Denature for 2 min at 95°C followed by 4°C until ready for injection on the CE instrument. Alternatively, the products may be stored on ice and protected from light after the denaturation step.

   Critical! The samples must be denatured prior to CE analysis.

Note: Samples may be run up to 24 hours after denaturation.

8) Prepare Genetic Analyzer for data acquisition according to manufacturer's directions. Final injection and run conditions must be validated by the end user and may differ between instruments. The following considerations apply:

- The instrument must be calibrated for the detection of both FAM and ROX fluorescent dyes.
- Use the factory-installed Fragment Analysis Protocol for POP-7 polymer and capillary length for your instrument as a base protocol.
- Adjust the injection conditions and run time according to the particular instrument configuration and capillary length. Recommended starting values are listed in Table 4.

Table 4. 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
3130, 3130xL	36 cm	2.5 kV, 20 s	2400 s
3730, 3730xL	36 cm	2.0 kV, 10 s	2400 s
3500, 3500xL	50 cm	2.5 kV, 20 s	2400 s

9) After the run, the data may be analyzed for amplicon size and conversion to $(G_4C_2)_n$ repeat length.

Fragment Sizing Analysis

Fragment sizing analysis of gene-specific or G_4C_2 RP-PCR data involves a series of steps 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 Data Analysis section. The terms used for analysis refer to GeneMapper 4.0/4.1/5.0 features. An overview of the fragment sizing analysis workflow is shown in Figure 4.

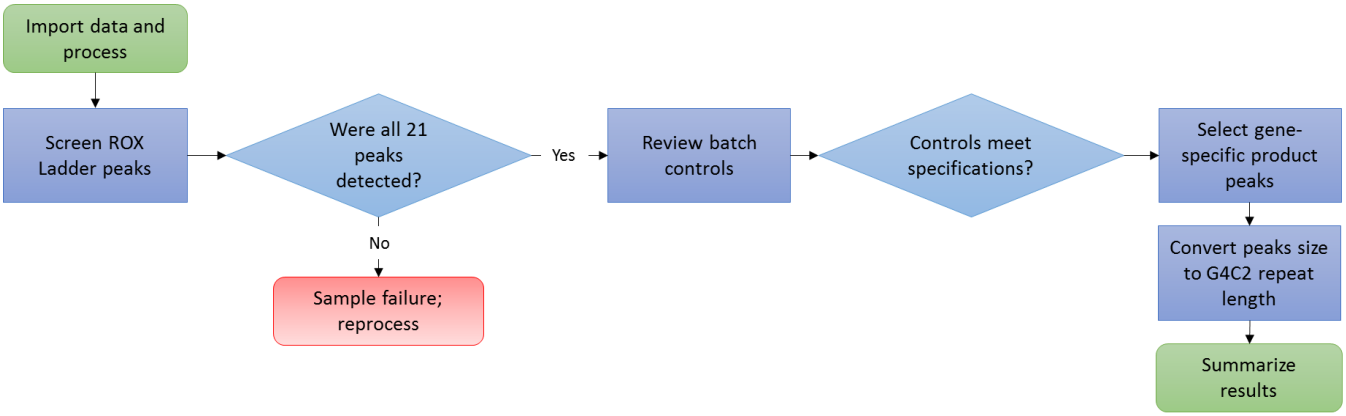

Figure 4. An overview of the fragment sizing analysis workflow including sample file processing, scoring the ladder peaks, qualifying the batch run, selecting gene-specific peaks, and summarizing results.

1. Import data and process
   a. Import the *.fsa files into GeneMapper®.
   b. Process files according to the methods, panels and size standard settings established for *C9orf72* PCR product analysis.
2. Qualify the run
   a. Screen ROX ladder peaks.

b. 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.

Critical! Ladders must be properly called befor proceeding with analysis.

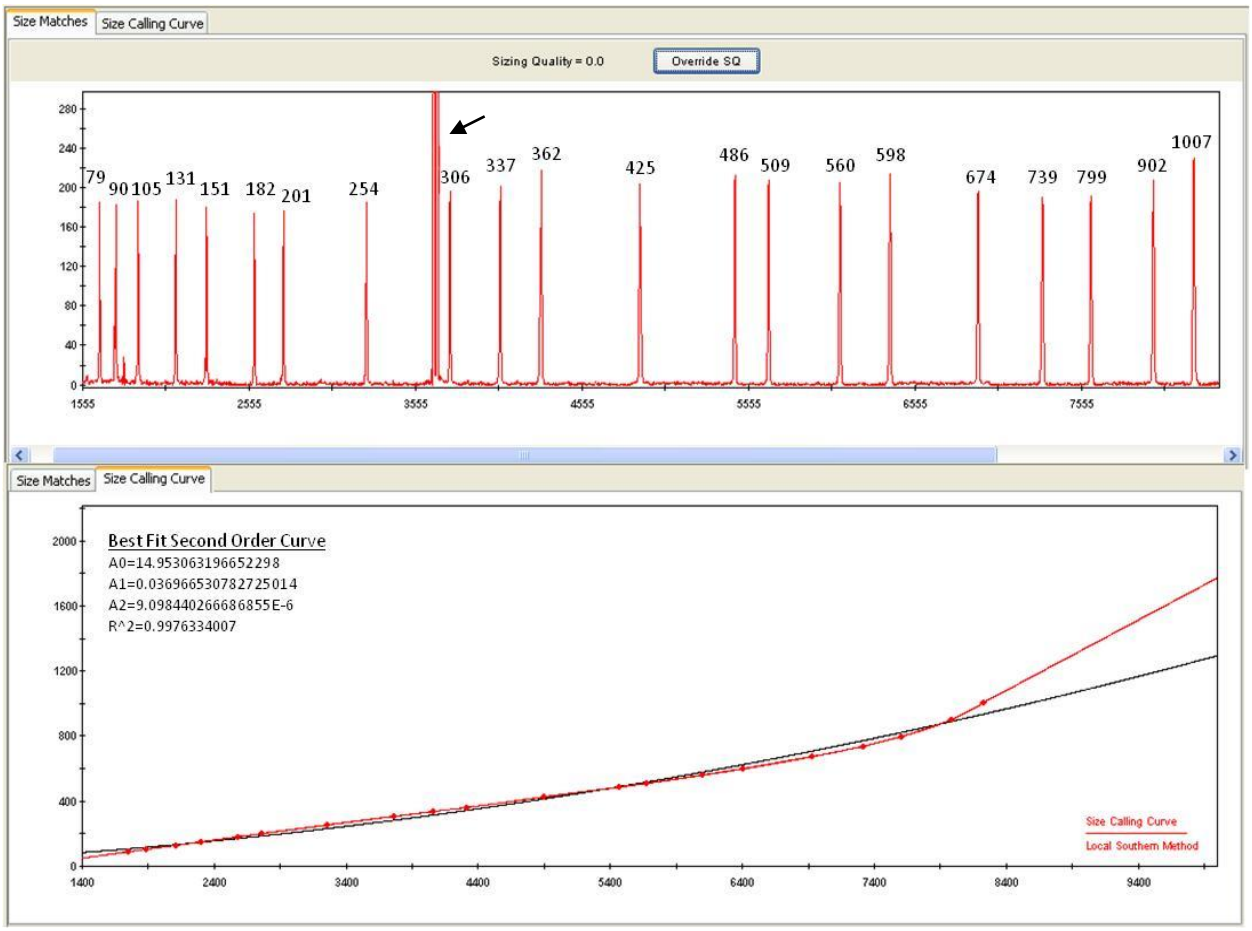

Figure 5. ROX 1000 Size Matches and Size Calling Curve. The Size Match view highlights the 21 peaks of the ladder and includes an example spectral pull-up peak (arrow) from the FAM-labeled target peak that should be ignored in the ROX channel.

Note: 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.

c. Review batch controls

I. Ensure that a negative control included in the batch run meets specifications.

II. Ensure that positive controls meet specifications. See Examples of Controls in the Data Interpretation section.

3. Select gene-specific target peaks

a. The electropherogram traces are reviewed for peak selection criteria. For analysis of G_4C_2 RP-PCR products, the multiplicity of G_4C_2 RP peaks is deselected in order to simplify tracking, export, and conversion of the full-length gene-specific PCR product peak to $(G_4C_2)_n$ repeat length. An example of this process is highlighted below in Figure 6 for a representative (13, 44 (minor), Exp) sample and described in the next section.

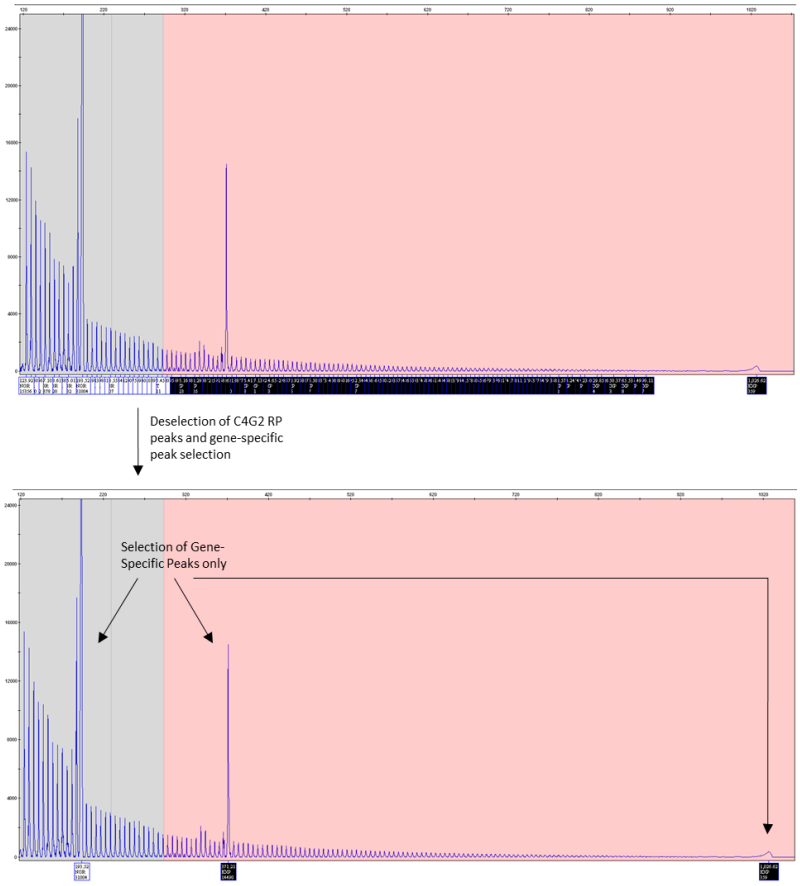

Figure 6. Example electropherogram with default analysis settings (top) and only gene-specific full-length peaks and high molecular weight expanded (>145 repeats) peak pile-up selected from the G_4C_2 RP-PCR electropherogram (bottom).

NOTE: If processing the results of a gene-specific PCR, only the gene-specific peaks will be present and only these peaks need to be selected. Deselection will not be required.

b. Deselect all peaks, then select gene-specific full-length product peaks (Figure 6). By default, peaks exceeding an instrument-specific cutoff are automatically selected. Minor or low intensity peaks may be manually selected using a user defined cutoff of either 3X above baseline signal height, or according to the guidelines in Table 5.

Table 5. Manufacturer default signal intensity cutoffs and low peak ranges for different CE instrument configurations

Instrument	Cutoff (rfu)	Low Peak Range (rfu)
3130, 3130xL	50	10-49
3730, 3730xL	150	50-174
3500, 3500xL	150	50-174

c. After general deselecting all peaks, identify gene-specific full-length product peaks using the guidelines illustrated in Figure 7.

I. Identify signal start site – First RP peak at ~121-125bp.

II. Select the highest peaks in the profile – Gene-specific peaks are typically present at significantly higher signal levels than the background RP profile signal.

d. Once all gene-specific peaks are selected, identify a possible expanded RP profile.

I. Detect the presence of an expanded RP profile which extends past the largest gene-specific allele.

II. Identify and select high molecular weight pile-up peak at the >900 bp range if present.

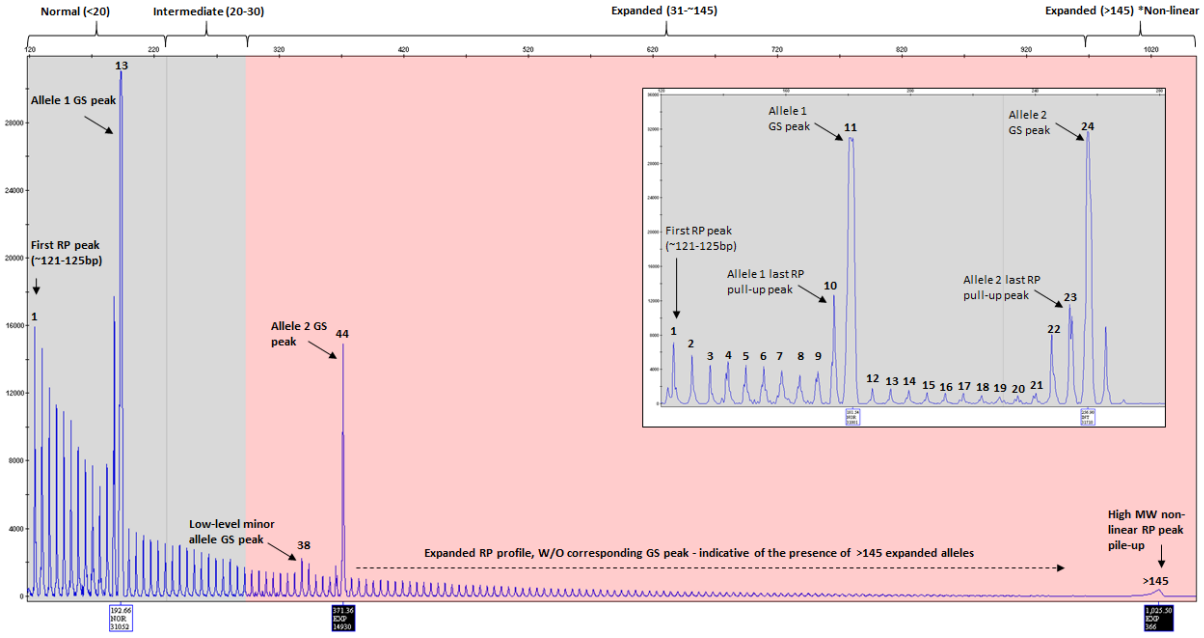

Figure 7. Peak selection guidelines based on size range and electropherogram features. A (13, 44 (minor), Exp) sample with multiple peaks at various levels (minor and major) is presented. **Inset:** A (11, 24) sample, denoting the RP peak counting scheme and unique RP and GS features to support manual size analysis.

4. Convert peak size to $(G_4C_2)_n$ repeat length – Direct repeat peak counting method

The Repeat Profile generated from the degenerate priming of all amplifiable repeat units is tightly correlated to the gene-specific peak size. Direct counting of RP peaks (inset in Figure 7) can serve as an accurate method for sizing using the following steps:

I. Identify signal start site – First RP peak at ~121-125 bp.

II. Select the highest peaks in the profile – Gene-specific peaks are present at significantly higher signal levels than background RP profile signal.

III. Count all ~6 bp apart (RP) peaks from the signal start site (peak 1 selected in step i.) to the selected gene-specific peak (selected in step ii.). The repeat size of the gene-specific peak is identical to its position within the count.

5. Convert peak size to $(G_4C_2)_n$ repeat length – Calibration Curve method

After CE, the size of the target amplicon can generally be derived from comparison to a co-injected size standard, e.g. the supplied ROX 1000 Size Ladder (P/N 145427). However, GC-rich PCR products of repeat regions, such as the *C9orf72* $(G_4C_2)_n$ element, have a faster migration than the generic DNA composition of the size standard [12, 13]. In the absence of the incorporation of an appropriate correction factor, this higher migration rate may result in underreporting of repeat length. To this end, the AmplideX® PCR/CE *C9orf72* Kit (RUO) includes a PCR Control sample (P/N 145426) which generates 4 peaks of known repeat lengths (Figure 8) that establishes a *linear calibration curve* and correction factors for conversion of size in base pairs to the number of $(G_4C_2)_n$ repeats for each allele. The size of each peak may be converted to repeat length according to the Equation below:

$$(\mathrm{G_4C_2})_n = \frac{Peak_n - C_0}{m_0}$$

Where: $Peak_n$ is the size in base pairs of a given gene-specific product peak; C_0 is the a Size Correction Factor (i.e. Intercept (b)); and m_0 is the Mobility Correction Factor (i.e. Slope (a)) extracted from the *linear calibration curve* ($y = ax + b$); See example in Figure 8). The Size Correction Factor represents the common region of DNA included in the primers but omits the $(G_4C_2)_n$ repeats. The Mobility Correction Factor accounts for the apparent change in mobility of the GC-rich repeat DNA. Correction factors may vary slightly depending on the specific run conditions and different instrument configuration used. Proposed correction factors for supported configurations are listed in Table 6.

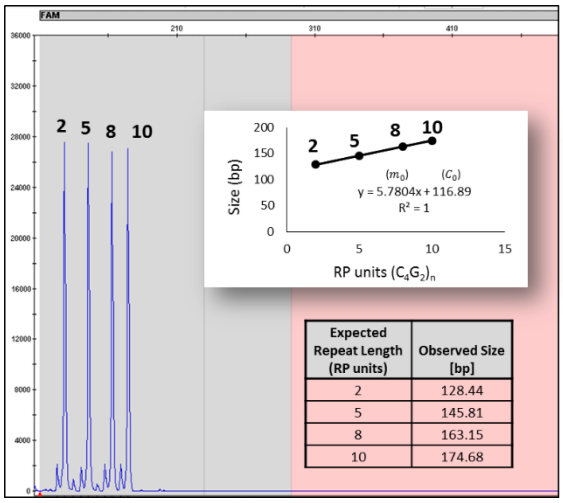

Figure 8. *C9orf72* PCR Control-derived *linear calibration curve.* Derivation of C_0 and m_0 for a representative configuration.

Table 6. Size and mobility correction factors for standard instrument configurations

Configuration		C_0	m_0
3130, 3130xL	36 cm	115.6	5.7
3730, 3730xL	36 cm	118.7	5.8
3500, 3500xL,	50 cm	117.7	5.8

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 described above and in Appendix A.

To use the linear calibration curve method follow these steps:

i. Identify and select all 4 gene-specific peaks present in the PCR control profile.

ii. Use Excel/JMP/other spreadsheet application to generate a linear curve fit, using the *Expected* RP count (i.e. 2, 5, 8 and 10) for X values and the *Observed* sizes (in bp) as the Y values.

iii. Use the linear calibration curve's ($y = ax + b$) slope ($a = m_0$) and intercept ($b = C_0$) in the following equation: $(\mathrm{G_4C_2})_n = \frac{Peak_n - C_0}{m_0}$

iv. Solve the equation for any selected gene-specific peak in a sample ($Peak_n$) to provide the corresponding repeat count ($(\mathrm{G_4C_2})_n$).

v. Subtract 1 repeat count for gene-specific peaks sized between 100 and 145.

NOTE: Minimally, only one of the methods (Section 4 and 5 above) is required for $(G_4C_2)_n$ size conversion. Nontheless, using both methods provides secondary size confirmation and may reveal sequence anomalies such as indels in the $(G_4C_2)_n$ flanking regions.

Data Interpretation

Alleles are reported as whole-integer repeats associated with a specific genotype category: normal, intermediate or expanded. The analytical reportable range is 1-145 repeats; above 145 repeats all alleles are identified as >145 G_4C_2. In

samples with multiple alleles, the indication of the longest allele is the reported one. Lower-level minor alleles may be noted as well.

Avaiable *C9orf72* gDNA reference materials

Well-characterized individual cell-line DNA samples from the CCR can be used as controls. Representative electropherograms obtained with select NINDS ALS reference materials from CCR are presented below (Figure 9; *C9orf72* allele sizes denoted):

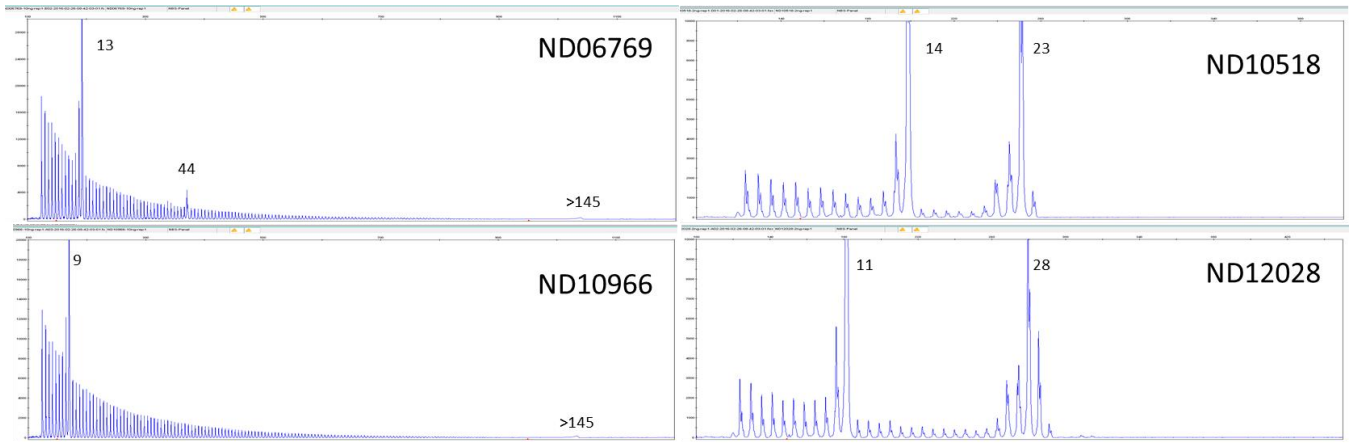

Figure 9. Electropherograms of the representative gDNA samples available from CCR.

Resolution of Zygosity

The G_4C_2 RP-PCR profile provides a unique signature to resolve homozygous normal from heterozygous samples with expanded alleles. An example profile for homozygous (7,7) and heterozygous (7,Exp) samples are shown in Figure 10. PCR products from homozygous alleles reveal a G_4C_2 RP peak profile up to the full-length product peak, and a baseline level signal for the remainder of the electropherogram range. Conversely, heterozygous alleles have a characteristic "decay" pattern of G_4C_2 RP products that exceed the normal range of $(G_4C_2)_n$ repeat lengths along with detection of both the normal and expanded alleles. Moreover, RP products will be generated even if the full-length product peak is not detected.

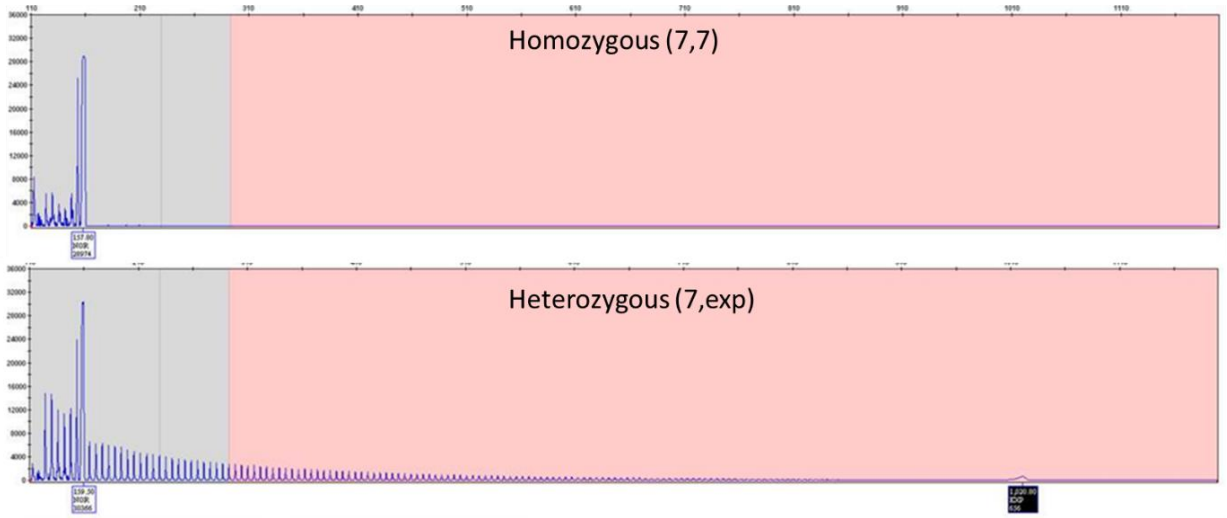

Figure 10. G_4C_2 Repeat Primed *C9ORF72* PCR reagents provide an unmistakable signature that resolve zygosity. Each $(G_4C_2)_n$ RP amplicon peak is separated by 6 bp, or 1 repeat unit.

General Disclaimers

1. The AmplideX® PCR/CE *C9orf72* Kit (RUO) reagents are produced in the USA and are for Research Use Only. Not for Use in Diagnostic Procedures.
2. All instrumentation must be maintained and operated according to manufacturer's instructions.
3. This Asuragen product may not be resold, modified for resale, or used to manufacture commercial products without prior written approval of Asuragen.

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Effective Date: 2016-06