Asuragen®

AmplideX®

PCR/CE *CFTR* Kit

Protocol Guide

Research Use Only. Not for use in diagnostic procedures.

REF A00076

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Purpose

The AmplideX® PCR/CE *CFTR* Kit is an *in vitro* nucleic acid amplification kit to simultaneously detect and identify a panel of mutations and variants in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The kit is a qualitative genotyping test designed for PCR on extracted genomic DNA from human whole blood performed on standard laboratory-validated thermal cyclers, followed by resolution on general laboratory-validated capillary electrophoresis platforms.

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

Limitations

- Blood samples processed into genomic DNA (gDNA) for use in this product must be collected using K_2EDTA-treated blood collection tubes and processed into gDNA within 14 days.
- Variant MAF may differ within a specific ethnicity, and population specific coverage should be determined based on known carrier rates for each specific region as needed.
- Polymorphisms that occur in primer binding regions could cause QC failure and/or false zygosity. Using gnomAD V3.1 on the GRCh38 build of the human genome as a source of Minor Allele Frequencies, the risk of polymorphism interference is calculated to be ~3.6% for D1152H and less than 0.06% for all other variants.
- INDELS located within primer binding sites could cause allele dropout. INDELs located between primer binding sites could cause a false positive or false negative. For more information, see the **Known Variant Conflicts and Limitations** section.
- Zygosity of variant 2184delA, 2184insA, and 2183AA>G may not be resolved if Mix B is excluded from testing and analysis. For more information, see the **Known Variant Conflicts and Limitations** section.
- Exon deletions may result in heterozygous mutations called as homozygous mutations. For more information, see the **Known Variant Conflicts and Limitations** section.
- Instrument configurations listed in the **Required but Not Provided** section have been verified. If desired, human cell line-derived samples with confirmed *CFTR* genotypes may be purchased from the Coriell Institute for Medical Research to confirm performance (**Table 2**).

Warnings and Precautions

- Use appropriate personal protective equipment when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910.1030, CLSI M29, or other applicable guidance when handling human samples.
- DNase contamination can cause degradation of DNA samples or PCR products. Use nuclease-free filter pipette tips and nuclease-free tubes. Clean bench surfaces before beginning work.
- PCR carryover contamination can result in false positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Prior to use, ensure that pipettes, thermal cyclers, and CE instruments are maintained, calibrated, and validated according to the manufacturer's instructions.

- **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.
- 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.
- Do not pool components from different reagent batches, lots, or other kits.
- Do not use reagents after the labeled expiration date.
- Ensure that Mix A and Mix B Primer Mixes are handled separately with care to minimize the risk of cross-contamination. Never have Mix A and Mix B Primer Mix tubes open simultaneously. Take care to keep the master mixes and plating as separate as possible. Separate Mix A and Mix B reactions on the PCR plate as much as possible. Refer to the recommended layout provided in the **PCR master mix Setup and Thermal Cycling** section.
- Do not interchange the reagent tube caps; this 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. The PCR master mix is viscous and can accumulate within the pipette tip.
- The reagents have been verified for up to 8 total uses through volumetric and freeze-thaw studies. Additional uses are not recommended.
- Use of alternative plastics and instruments other than those listed in **Required but Not Provided** section may increase risk of QC failures.
- Do not dilute samples with water stored in environments that are decontaminated with UV lights; plasticizers leached from the bottle may interfere with PCR amplification of gDNA.
- The Applied Biosystems 3730*xl* Genetic Analyzer with the 96-well capillary configuration is not recommended for fragment analysis and has not been verified for use with this assay.

Principle of the Procedure

The AmplideX® PCR/CE *CFTR* Kit is a multiplexed allele-specific PCR assay used to genotype and determine zygosity of up to 65 of the most prevalent mutations in *CFTR*. The *CFTR* mutation panel targets at least one pathogenic mutation in >99% of CF patients (Castellani et al., 2018), and represents 92.2% of variant alleles in an ethnically diverse US population (Beauchamp et al., 2019). Panel detection covers all American College of Obstetricians and Gynecologists (ACOG) recommended 23 (CF23) mutations and 11 additional variants in a single tube (CFTR Primer Mix A), representing 86% MAF. A second tube is used to detect 31 additional lower-frequency variants (CFTR Primer Mix B).

The AmplideX PCR/CE Reporter software used in conjunction with the AmplideX PCR/CE *CFTR* Analysis Module is an all-in-one data analysis and reporting tool. It combines peak detection with associated allele classification and sample level QC. For peak identification, the software tool automatically determines peak position (bp), peak height (RFU), and dye channel. The software detects peak position and dye channel of the PolyT/TG region to determine both repeat size and phase. The primer mix is automatically determined by the software within an FSA file and associates both FSAs for a single sample using file naming conventions. This association is confirmed by comparing highly polymorphic biomarkers shared between both primer mixes to ensure files are properly matched.

Background Information

Cystic Fibrosis (CF) is an autosomal recessive condition caused by mutations in the *CFTR* gene responsible for chlorine transport across the cell membrane. Mutations in the coding region result in dysfunctional ion transport (Brennan and Schrijver, 2016). This typically results in increased chloride concentration in sweat, thicker mucus linings in bronchi, impaired pancreatic exocrine function and intestinal absorption. Carrier status within the US for mutations associated with *CFTR* is ~1:30 (Caucasian) to ~1:90 (Asian), and ~1:3000 Caucasians have CF in the US (Palomaki et al., 2004).

The *CFTR* gene is one of the largest in the human genome with 27 exons across 189,000 bp that code for 1,480 amino acids. There are ~2,000 known mutations in the *CFTR* gene, however only ~300 have been reported pathogenic and each occurs at different allele frequencies in different populations (Pereira et al., 2019). The most prevalent mutation is a three nucleotide deletion resulting in a loss of phenylalanine at amino acid 508 (F508del; rs113993960; ~70% in Caucasians). A committee opinion from ACOG in 2017 reaffirmed genetic testing for a core panel of 23 mutations (CF23) on the basis of mutation frequency for women who are currently pregnant or considering pregnancy (Rink et al., 2017). ACMG (Amos et al., 2008, Gregg et al., 2021), ESHG (Dequeker et al, 2009), and ECFS (Castellani et al, 2018) have also released guidelines for testing and include the CF23 panel.

In 2019, a large whole-gene NGS study of *CFTR* MAF (herein referred to as MAF-US) was completed across an ethnically diverse US population of >115,000 individuals, including ~13,000 at risk couples and ~92,000 patients referred for routine carrier screening (Beauchamp et al., 2019). This study provides a contemporary view of *CFTR* variant frequency in the US. They identified 213 unique pathogenic variants that accounted for 3,965 pathogenic alleles observed. In addition, the authors confirmed the miss rate for CF23, indicating that 21% of observed mutant alleles would have been missed by the CF23 panel.

The AmplideX® PCR/CE *CFTR* Kit also sizes the PolyT region upstream of Exon 10, which is recommend as a reflex test when a mutant R117H allele is present (Grody et al., 2001). Further, the kit accommodates size and phasing of a dinucleotide TG-repeat directly 5' of the PolyT tract. When expanded and in phase with a 5T repeat, reduced active *CFTR* protein expression has been reported (Groman, et al., 2004).

Note: Exon numbering is based on GRCh38.p13 build of the human genome.

The AmplideX PCR/CE *CFTR* Kit determines genotypes for 61/213 variants identified in the study (Beauchamp et al., 2019), representing 92.2% coverage based on MAF. In total, the kit can resolve zygosity across 65 variants. Supported variants are listed in **Table 1** and MAF for each variant can be found in Beauchamp et al., 2019.

Table 1. *CFTR* variants detected by the AmplideX® PCR/CE *CFTR* Kit.

Legacy Variant Name	HGVS (cDNA)	rsID (build 154)	Primer Mix
1717-1G>A*	c.1585-1G>A	rs76713772	A
1898+1G>A*	c.1766+1G>A	rs121908748	A
2184delA*	c.2052del	rs121908746	A
2184insA	c.2052dup	rs121908746	A
2789+5G>A*	c.2657+5G>A	rs80224560	A
3120+1G>A*	c.2988+1G>A	rs75096551	A
3272-26A>G	c.3140-26A>G	rs76151804	A
3659delC*	c.3528del	rs78984783	A
3849+10kbC>T*	c.3718-2477C>T	rs75039782	A
621+1G>T*	c.489+1G>T	rs78756941	A
711+1G>T*	c.579+1G>T	rs77188391	A
A455E*	c.1364C>A	rs74551128	A
D1152H	c.3454G>C	rs75541969	A
F508del*	c.1521_1523del	rs113993960	A
G542X*	c.1624G>T	rs113993959	A
G551D*	c.1652G>A	rs75527207	A
G622D	c.1865G>A	rs121908759	A
G85E*	c.254G>A	rs75961395	A
I507del*	c.1519_1521del	rs121908745	A
L206W	c.617T>G	rs121908752	A
N1303K*	c.3909C>G	rs80034486	A
P5L	c.14C>T	rs193922501	A
PolyT	c.1210-12T[5_9]	rs1805177	A
PolyTG	c.1210-34TG[10_12]	rs3832534	A
R1070Q	c.3209G>A	rs78769542	A
R1162X*	c.3484C>T	rs74767530	A
R117H*	c.350G>A	rs78655421	A
R334W*	c.1000C>T	rs121909011	A
R347H	c.1040G>A	rs77932196	A
R347P*	c.1040G>C	rs77932196	A
R553X*	c.1657C>T	rs74597325	A
R560T*	c.1679G>C	rs80055610	A
S945L	c.2834C>T	rs397508442	A

Legacy Variant Name	HGVS (cDNA)	rsID (build 154)	Primer Mix
W1282X*	c.3846G>A	rs77010898	A
2183AA>G	c.2051_2052delAAinsG	rs121908799	A (WT) / B (MUT)
1078delT	c.948del	rs75528968	B
1154insTC	c.1019_1020dup	rs387906360	B
1548delG	c.1418del	rs397508205	B
1677delTA	c.1545_1546del	rs121908776	B
1811+1.6kbA>G	c.1680-886A>G	rs397508266	B
1898+5G>T	c.1766+5G>T	rs121908796	B
2789+2insA	c.2657+2_2657+3insA	rs397508414	B
3876delA	c.3744del	rs121908784	B
3905insT	c.3773dup	rs121908789	B
394delTT	c.262_263del	rs121908769	B
A559T	c.1675G>A	rs75549581	B
E60X	c.178G>T	rs77284892	B
F311del	c.935_937del, c.933_935del	rs121908768	B
G970D	c.2909G>A	rs386134230	B
I618T	c.1853T>C	rs139468767	B
M1101K	c.3302T>A	rs36210737	B
P67L	c.200C>T	rs368505753	B
Q493X	c.1477C>T	rs77101217	B
Q890X	c.2668C>T	rs79633941	B
R1066C	c.3196C>T	rs78194216	B
R1070W	c.3208C>T	rs202179988	B
R1158X	c.3472C>T	rs79850223	B
R117C	c.349C>T	rs77834169	B
R352Q	c.1055G>A	rs121908753	B
R75X	c.223C>T	rs121908749	B
S549N	c.1646G>A	rs121908755	B
V456A	c.1367T>C	rs193922500	B
V520F	c.1558G>T	rs77646904	B
Y1092X	c.3276C>A, c.3276C>G	rs121908761	B
Y122X	c.366T>A	rs79660178	B

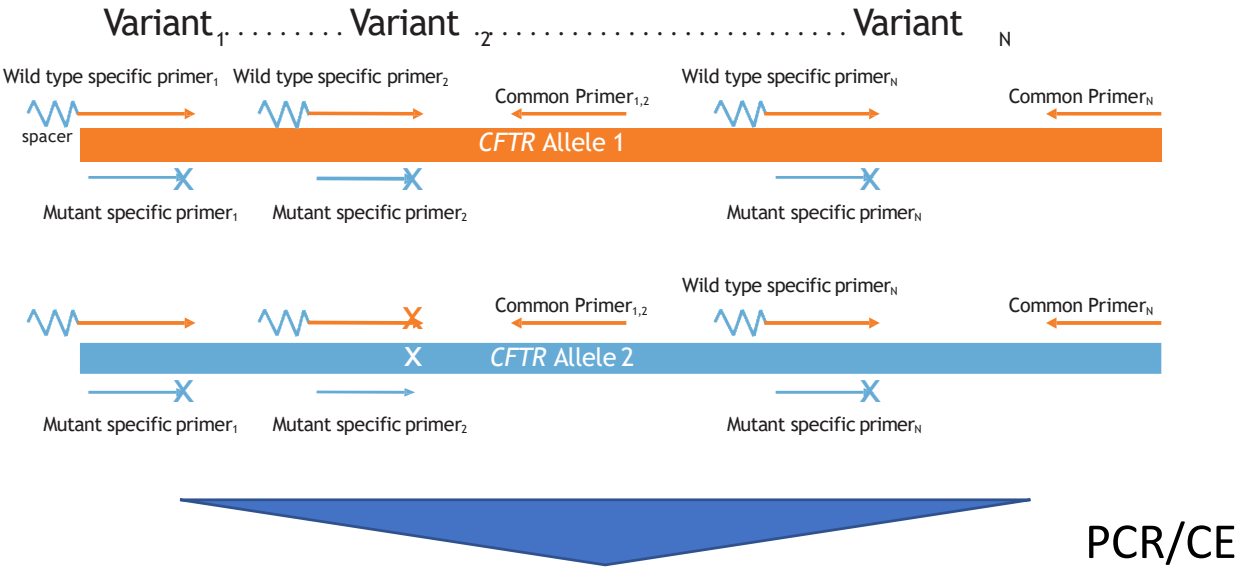
*CF23 mutations

PCR Methods

The kit includes reagents for multiplexed PCR to produce either wild type (WT) or mutant (MUT) amplicons labeled with either FAM or HEX dyes to determine zygosity across 65 variants distributed across two tubes (**Figure 1**). The kit sizes and phases the PolyT/TG region based on channel (FAM, HEX, NED) and peak size (bp), respectively.

In order to avoid sample mix up, three different short tandem repeats (STRs) have been incorporated in the NED channel across both reactions as fingerprinting biomarkers. There is approximately 1:10,000 chance that two samples will have the same fingerprint determined from published STR frequencies across US (NIST, 2017) and European (STRidER, 2019) populations.

After PCR amplification, the dye-labeled fluorescent products are resolved by CE and associated to expected variant size (bp) using the included ROX 1000 Size Ladder. Sample electropherograms include peaks in FAM, HEX, NED, and ROX channels (**Figure 1**). Every allele (MUT or WT) corresponds to a specific peak size (bp) in a specific channel. Heterozygotes are detected by both MUT and WT peaks. Homozygotes are detected by a single MUT or WT peak.



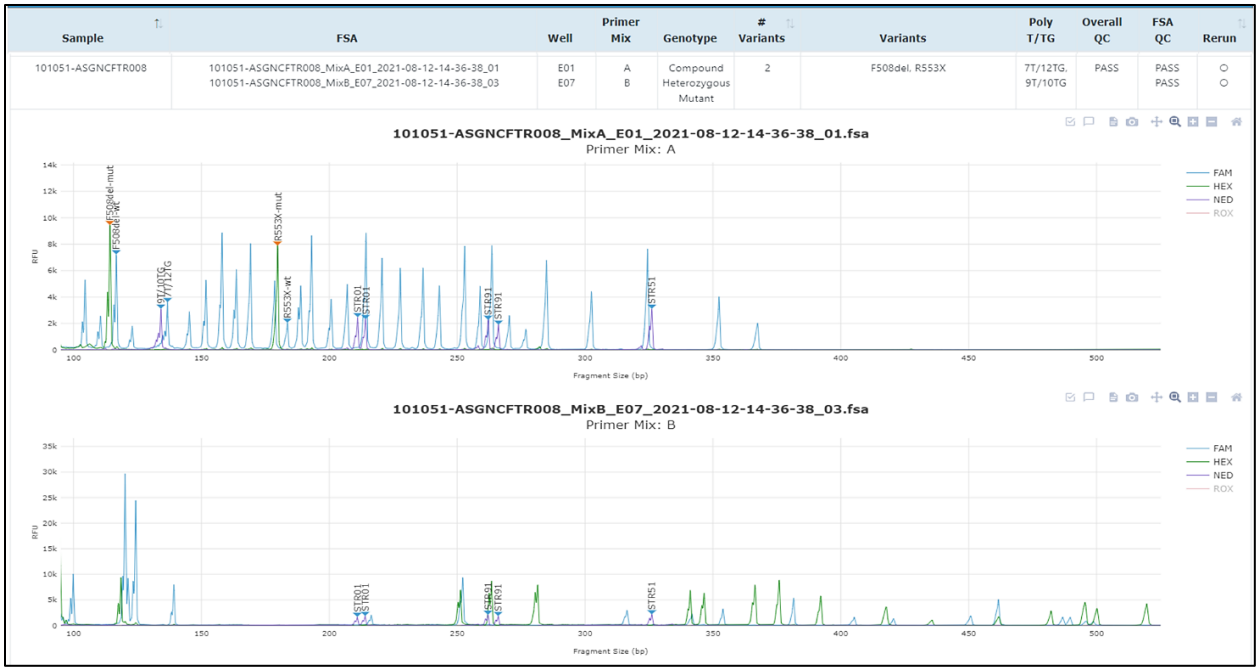


Figure 1. Assay design and example of AmplideX® PCR/CE *CFTR* Kit electropherogram. Allele-specific primers amplify wild type (WT) or mutant (MUT) allele variants with a set of common primers. Amplicons are labeled during amplification and are resolved by CE with detection in the FAM, NED, or HEX channels. The amplicon mobility shift (bp) and/or dye channel differentiates alleles for each variant. CE traces are visualized by overlapping channels in AmplideX PCR/CE Reporter with peaks binned based on expected sizes for Primer Mix A and Primer Mix B.

Workflow

The assay workflow includes PCR master mix setup (2 tubes or wells per sample), thermal cycling, CE amplicon resolution, and software analysis. For this process, add purified gDNA derived from human cell lines or whole blood to a PCR well containing a master mix of 2X PCR Mix and CFTR Primer Mix A or CFTR Primer Mix B in a final reaction volume of 10 µL. After thermal cycling is complete, add PCR products to a CE master mix comprised of Hi-Di™ Formamide and ROX 1000 Size Ladder. Following denaturation, resolve amplicons on an Applied Biosystems® Genetic Analyzer (e.g., 3130*xl*, 3500xL, 3730*xl*, or SeqStudio). See **Figure 2** for a schematic of the workflow and turn-around time.

After resolving the PCR products by CE, the resulting electropherograms must be processed by the AmplideX PCR/CE *CFTR* Analysis Module. Results are generated for zygosity and size/phase of PolyT/TG.

Figure 2. The workflow is streamlined from sample-to-answer and can be performed in less than 5 hours with ~60 minutes of total hands-on time for 12 samples. CE instrument time was documented for a single injection of 24 reactions using an Applied Biosystems 3500xL Genetic Analyzer (24 capillary).

Use of Batch Run Controls

Use of a negative control (i.e., NTC) is optional. If an NTC is desired, the Diluent included in the kit should be used in place of sample DNA.

If desired, human cell line-derived samples with confirmed *CFTR* genotypes may be purchased from the Coriell Institute for Medical Research to confirm performance (**Table 2**). These samples are not required for proper functionality of the assay.

Table 2. Human cell line-derived samples with confirmed *CFTR* positive-variant alleles. Wild type (WT) is listed in the Allele 2 column if no additional mutant alleles were identified. When the same allele is listed in the Allele 1 and Allele 2 columns, this signifies a homozygous mutation.

DNA ID	Expected *CFTR* Allele 1	Expected *CFTR* Allele 2	PolyT/TG
CD00003*	1078delT	WT	7T/11TG, 7T/11TG
CD00008*	2184delA	WT	7T/11TG, 9T/10TG
CD00009*	394delTT	WT	7T/11TG, 9T/10TG
NA00999*	621+1G>T	1154insTC	7T/10TG, 9T/10TG
NA01531*	F508del	F508del	9T/10TG, 9T/10TG
NA07464*	R553X	WT	7T/11TG, 7T/12TG
NA07732*	F508del	E60X	7T/11TG, 9T/10TG
NA08338*	G551D	WT	7T/10TG, 9T/11TG
NA11277*	I507del	WT	7T/10TG, 7T/10TG
NA11278*	F508del	Q493X	7T/10TG, 9T/10TG
NA11283*	F508del	A455E	9T/10TG, 9T/10TG
NA11284*	F508del	R560T	7T/10TG, 9T/10TG
NA11496*	G542X	G542X	9T/10TG, 9T/10TG
NA11723*	W1282X	WT	5T/12TG, 7T/10TG
NA11860*	3849+10kbC>T	3849+10kbC>T	7T/10TG, 7T/10TG
NA12926*	Q493X	WT	7T/10TG, 7T/10TG

DNA ID	Expected *CFTR* Allele 1	Expected *CFTR* Allele 2	PolyT/TG
NA12960*	R334W	WT	7T/11TG, 7T/12TG
NA12961*	V520F	WT	7T/10TG, 7T/11TG
NA13591*	F508del	R117H	5T/12TG, 9T/10TG
NA18801*	3272-26A>G	WT	7T/10TG, 7T/11TG
NA18802*	Y122X	R1158X (not listed in Coriell Remarks, confirmed by Sanger Sequencing)	7T/10TG, 7T/11TG
NA18803*	F508del	2183AA>G	7T/10TG, 9T/10TG
NA20741*	3876delA	WT	7T/11TG, 9T/10TG
NA20745*	S549N	WT	7T/10TG, 9T/10TG
NA20836*	3905insT	WT	7T/11TG, 9T/10TG
NA20837*	S549N	WT	7T/10TG, 9T/10TG
NA20924*	R75X	WT	7T/11TG, 9T/10TG
CD00007	1898+1G>A	WT	7T/11TG, 7T/11TG
GM04345	F508del	W1282X	7T/10TG, 9T/10TG
NA00897	F508del	R347P	7T/11TG, 9T/10TG
NA07441	3120+1G>A	621+1G>T	7T/11TG, 9T/10TG
NA07857	M1101K	M1101K	7T/10TG, 7T/11TG
NA11275	F508del	3659delC	7T/11TG, 9T/10TG
NA11280	621+1G>T	711+1G>T	7T/11TG, 9T/10TG
NA11859	2789+5G>A	2789+5G>A	7T/10TG, 7T/10TG
NA12444	1717-1G>A	WT	7T/10TG, 7T/11TG
NA12585	R1162X	WT	7T/10TG, 7T/10TG
NA12785	G551D	R347P	7T/10TG, 7T/11TG
NA13423	G85E	D1152H	7T/11TG, 7T/11TG
NA18800	F508del	1898+1G>A	7T/11TG, 9T/10TG
NA20737	R347H	WT	7T/11TG, 9T/10TG
NA21069	3876delA	WT	7T/11TG, 9T/10TG
NA21080	394delTT	WT	7T/10TG, 7T/11TG
NA21847	F508del	WT	7T/11TG, 9T/10TG

*Coriell cell lines tested in design verification studies

Reagents Supplied with the Kit

*Item #	Description	Cap Color	Storage Temp
A00075*	2X PCR Mix	Yellow	-15 to -30 °C
A00072*	CFTR Primer Mix A	Green	-15 to -30 °C
A00073*	CFTR Primer Mix B	Blue	-15 to -30 °C
145188*	ROX 1000 Size Ladder	Red	-15 to -30 °C
145388	Diluent	Clear	-15 to -30 °C

*One kit component tube included in Kit A00076; two kit component tubes included in Kit A00077

Required but Not Provided

Item numbers, where provided, may vary by region. Please contact Asuragen® Technical Support 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 QIAamp® DNA Blood Mini Kit (QIAGEN®) or similar kit. See additional notes in the **Pre-Analytical Steps** section.
- General laboratory equipment and workspace to perform PCR and CE procedures
- Centrifuge capable of spinning 96-well plates
- Micro-centrifuge or benchtop centrifuge capable of spinning reagent tubes: Eppendorf® 5810 or equivalent
- Vortex mixer
- Pipettes 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 thermal cycler: Applied Biosystems® ProFlex™ 96-well Thermal Cycler, Applied Biosystems Veriti™ 96-well Thermal Cycler, Applied Biosystems GeneAmp™ PCR System 9700 (gold block), Bio-Rad C1000 Touch™ Thermal Cycler (96-well Fast Reaction Module)
  Note: For best results, thermal cyclers should have a ramp rate of 2.6-5.0°C/sec
- 96-well PCR semi-skirted plates for use with the Applied Biosystems Veriti 96-well Thermal Cycler, Applied Biosystems ProFlex 96-well Thermal Cycler, Applied Biosystems GeneAmp PCR System 9700 (gold block), and Applied Biosystems Genetic Analyzers: Phenix Research Products, P/N MPS-3580, or equivalent
- MicroAmp™ Optical Film Compression Pad for use with Applied Biosystems thermal cyclers: Applied Biosystems P/N 4312639, or equivalent
- Plastic Seals (Applied Biosystems P/N AB0558, or equivalent) or Adhesive Foil Seals (Beckman Coulter P/N 538619, or equivalent) for use with Applied Biosystems thermal cyclers
- 96-well PCR fully-skirted plates for use with the Bio-Rad C1000 Touch Thermal Cycler: Bio-Rad, P/N HSP9631 with Microseal ‘B’ MSB-1001 or ‘C’ MSC-1001 plate seal film, Pierceable Foil Heat Seal #181-4040
- Applied Biosystems Genetic Analyzers running POP-7™ polymer (3130, 3500 or 3730 series) or POP-1™ polymer (SeqStudio™)
    - SeqStudio:
        - Cathode Buffer: Applied Biosystems P/N A33401
        - Cartridge: Applied Biosystems P/N A33671 or A41331 (V2)

    - 3130 Series:
        - POP-7 Polymer: Applied Biosystems P/N 4363785, or equivalent
        - Running Buffer: Applied Biosystems P/N 402824
    - 3500 Series:
        - POP-7 Polymer: Applied Biosystems P/N 4393708, or equivalent
        - Anode Buffer: Applied Biosystems P/N 4393925, or equivalent
        - Cathode Buffer: Applied Biosystems P/N 4408258, or equivalent
    - 3730 Legacy Series:
        - POP-7 Polymer: Applied Biosystems P/N 4363935, or equivalent
        - Running Buffer (10X): Applied Biosystems P/N 4335613
- Hi-Di™ Formamide: Applied Biosystems P/N 4311320, or equivalent
- DS-30 Matrix Standard Kit (Dye Set D): Applied Biosystems P/N 4345827

Number of Reactions

- The provided reagents are sufficient for up to 50 reactions (A00076) each of Mix A and Mix B or 100 reactions (A00077) each of Mix A and Mix B, including master mix overage.
- The master mixes can be prepared for the appropriate number of samples with a recommended total number of at least 5 reactions per run. 15% overage for PCR and 10% for CE master mix is recommended.

Reagent Stability

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

Storage & Handling

- Store frozen reagents in a non-frost-free freezer protected from light at -15 to -30°C.
- The reagents have been validated for up to 8 total uses through freeze-thaw studies. Additional uses are not recommended.
- Completely thaw reagents at room temperature before use (at least 30 minutes). Vortex all reagents after thawing. Once thawed, individual kit components are stable for up to 45 minutes per thawing event at room temperature.
- Ensure that CFTR Primer Mix A and CFTR Primer Mix B are handled with care to minimize the risk of cross-contamination. Do not interchange the Primer Mix tube caps as described in **Warnings and Precautions.**
- 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 to 25°C). Once assembled, master mixes are stable for up to one hour at room temperature prior to aliquoting onto the sample plate.

Procedural Steps

Pre-Analytical Steps

Human genomic (gDNA) samples extracted from K_2EDTA-treated whole human blood (fresh or stored at 2 to 8°C for up to 14 days) and collected via laboratory-validated procedures may be isolated using various extraction and purification methods (precipitation, silica column, or functionalized magnetic beads).

It is essential to use a validated isolation method to ensure that DNA is consistently of high quality and purity. Commercially available silica column, precipitation, and magnetic bead purification technologies have all been tested and are generally compatible, as are many automated DNA purification methods. For questions about specific isolation methods, contact **support@asuragen.com**.

DNA amount and purity should be quantified using a spectrophotometer (e.g., Thermo Scientific® NanoDrop™).

Sample requirements are as follows:

- 20 to 60 ng DNA input
- 2 µL of DNA per PCR reaction

Sample purity for optimal performance are as follows:

- $A_{260}/A_{280} = 1.8 \pm 0.3$
- $A_{260}/A_{230} \geq 1.25$ (recommended)

PCR products and ladder standards are observed in 4 dye channels. Before beginning CE, ensure the calibrated instrument detects FAM™, HEX™, NED™ and ROX™ using the DS-30 Matrix Standard Kit (Dye Set D, Applied Biosystems®) according to the manufacturer's instructions. For further details on setting up the genetic analyzer instrument, refer to the **Capillary Electrophoresis Using a Genetic Analyzer** section.

Overview of the AmplideX PCR/CE *CFTR* Kit Protocol

The test protocol involves four key sets of procedures:

1. PCR master mix setup and thermal cycling
2. Capillary electrophoresis using a genetic analyzer
3. Sample Naming
4. Fragment analysis (PCR/CE)

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 (A00076) each of Mix A and Mix B or 100 reactions (A00077) each of Mix A and Mix B, performed in up to 8 independent batches, including 15% overage for PCR and 10% overage for CE injection master mix formulations.

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.

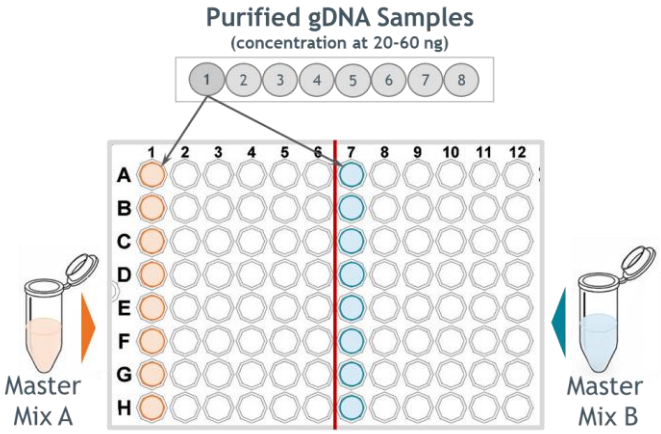
PCR Master Mix Setup and Thermal Cycling

1. Thaw the 2X PCR Mix, both tubes of Primer Mix, and Diluent (if NTC is to be included) at room temperature until each component is a uniform liquid (up to 30 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: Ensure that CFTR Primer Mix A and CFTR Primer Mix B are handled separately with care to minimize the risk of cross-contamination.
2. Assemble the reagents in the order listed according to the PCR master mix in the following table. Volumes indicated are for a single reaction and do not include the recommended overage. We recommend creating a master mix with 15% overage for each reagent (volume/reaction * number of reactions * 1.15 for each reagent, rounded to nearest 0.1 µL). Prepare sufficient volume of PCR master mix for each sample to be tested. Inclusion of a reaction with diluent in place of DNA as an NTC is optional.
   Note: The 2X PCR Mix is viscous; therefore, retract pipette piston slowly to acquire the desired solution. The PCR master mix is stable for up to an hour at room temperature prior to adding template, however it is recommended to add sample immediately after mixing and aliquoting.

Reagent	PCR Setup Mix A Volume/reaction (µL)	PCR Setup Mix B Volume/reaction (µL)
2X PCR Mix	5.0	5.0
CFTR Primer Mix A	3.0	-
CFTR Primer Mix B	-	3.0
Sample* or NTC*	2.0*	2.0*
Total Volume	**10.0**	**10.0**

   *Do not add to bulk master mix

3. Thoroughly vortex the PCR master mix for approximately 5 seconds at maximum speed and briefly centrifuge prior to aliquoting to the PCR plate.
   Note: The master mix must be vortexed prior to dispensing to ensure adequate mixing of all reagents.
4. Dispense 8.0 µL of the PCR master mix A or master mix B to each well. Use a repeater pipette if available.
   Note: Both Mix A and Mix B support the same cycling protocol. Samples tested with Mix A and Mix B should be located on the same plate in an order that is documented to prevent sample mix up. See recommended PCR plate layout to minimize risk of cross-contamination.



5. Add 2.0 µL of the appropriate DNA sample to each well of Mix A and Mix B (see **Pre-Analytical Steps** for sample input requirements). 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 x g).
   Note: Ensure all bubbles are removed from the bottom of the plate wells.
9. Transfer the sealed PCR plate(s) to a preprogrammed thermal cycler, and run the following cycling protocol setting the reaction volume to 10 µL.
   Note: Use default ramp rates for the Applied Biosystems® Veriti, Applied Biosystems 9700 (gold block), and Bio-Rad C1000 thermal cyclers. For the Applied Biosystems ProFlex, set ramp rate to 5.0°C/s. Thermal cyclers with maximum ramp rates < 2.6 °C/s are not recommended. For thermal cyclers not listed here, use the Veriti/ProFlex conditions and contact Asuragen® Technical Support for assistance.

Stage	Temperature	Time	Veriti (3.9°C/s), ProFlex (5.0°C/s)*	9700 gold block (2.6°C/s)	C1000 (3.3°C/s)
Initial Denaturing	95°C	5 minutes	1 hold	1 hold	1 hold
Allele Specific	95°C	15 seconds	2 cycles	2 cycles	2 cycles
	60°C	4 minutes			
Expansion	95°C	15 seconds	20 cycles	20 cycles	17 cycles
	72°C	4 minutes			
Tagging	95°C	30 seconds	7 cycles	6 cycles	7 cycles
	60°C	30 seconds			
	72°C	4 minutes			
Conditioning	72°C	10 minutes	1 hold	1 hold	1 hold
End Storage	4°C	Hold	1 hold	1 hold	1 hold

*Veriti Emulation mode is also compatible

10. Transfer PCR products for CE analysis or store at -15 to -30°C until analyzed.
    Note: Once the PCR cycling is complete, PCR products are stable for up to 48 hours at 2 to 8°C. When stored in a freezer, PCR products are stable at -15 to -30°C for at least 1 month. Storage of amplicons at ambient temperature is not recommended.

Capillary Electrophoresis Using a Genetic Analyzer

1. Thaw the Hi-Di™ Formamide and ROX 1000 Size Ladder at room temperature until each component is a uniform liquid.
2. Thoroughly vortex (max speed for 5 to 10 seconds) and briefly centrifuge to collect the contents.
3. Prepare a CE master mix solution by adding components in the order listed according to the **CE master mix** table. Volumes indicated are for a single reaction and do not include the recommended overage. Creating a master mix with 10% overage for each reagent (volume/reaction * number of reactions * 1.10 for each reagent, rounded to nearest 0.1 µL) is recommended.

CE master mix

Reagent	Per Reaction (µL) (SeqStudio, 3500 series)	Per Reaction (µL) (3130, 3730 series)
Hi-Di Formamide	11.0	8.0
ROX 1000 Size Ladder	2.0	1.0
PCR Product*	2.0*	1.0*
Total Volume	**15.0**	**10.0**

*Do not add to bulk master mix

4. Mix all added reagents (vortex at max speed for 5 seconds), and briefly centrifuge to collect the contents.

5. Aliquot 13.0 µL (if using a SeqStudio™ or 3500 series Genetic Analyzer) or 9.0 µL (if using a 3130 or 3730 series Genetic Analyzer) of CE master mix to each well of a new CE analysis plate compatible with the genetic analyzer.
   Note: Match layout of the CE plate to the injection configuration of the genetic analyzer in appropriate groups of 4, 8, 16, 24, or 48 capillaries depending upon CE model (e.g., A1–H3, A4–H6, A10–H12 for 3500xL). If running less than the number of samples for any injection group, fill empty wells subject to injection with 15.0 µL (SeqStudio or 3500 series) or 10.0 µL (3130 or 3730 series) of Hi-Di™ Formamide.
6. Prior to transfer, gently vortex and then centrifuge the plate containing PCR products to collect the contents at bottom of wells and prevent contamination when opening (1 minute at 1600 x g).
7. Transfer the PCR products to the CE plate (2.0 µL if using the SeqStudio or 3500 series, or 1.0 µL if using the 3130 or 3730 series), pipetting up and down at least 3 times to mix.
   Note: A multi-channel pipette is recommended for this transfer, especially for multiple columns of PCR products.
8. Seal the plate with an adhesive film seal ensuring that all the wells and the plate edges are properly sealed.
9. Gently vortex the plate.
10. Centrifuge the plate to collect the contents at bottom of wells (1 minute at 1600 x g).
11. Denature the CE plate on a thermal cycler for 2 minutes at 95°C followed by a 4°C hold for at least 2 minutes. Centrifuge the plate to collect the contents at bottom of wells (1 minute at 1600 x g), ensuring that no bubbles are present within plate wells. After the denaturation step, store the plate on ice and protect from light.
    Note: The amplicons must be denatured prior to CE analysis and may be run up to 48 hours after denaturing; instrument deck time stability has been verified up to 48 hours.
12. Prepare the genetic analyzer for data acquisition according to the manufacturer instructions. Import sample template and run module files can be provided by Asuragen® Technical Services if needed for each instrument. Final injection and run conditions must be validated by the end user and may differ between instruments.
    Note: Per the manufacturer instructions, instrument buffers and polymer must be at room temperature before installing on the instrument.
    a. The following considerations apply:
        i. Calibrate the instrument for the detection of FAM™, HEX™, NED™ and ROX™ fluorescent dyes using the DS-30 Matrix Standard Kit.
        ii. Adjust the injection conditions and run time according to the particular instrument configuration. The following table lists recommended starting values.
        iii. For the 3130 and 3730 series Genetic Analyzer, the 10X running buffer must be diluted to 1X with high quality purified water per manufacturer recommendations prior to installing on the instrument. For best results, dilute the running buffer immediately prior to use. Once installed on the instrument, diluted 1X running buffer is stable for up to 24 hours. Diluted 1X running buffer may be stored for up to 7 days at either room temperature or 2 to 8 °C.
            Note: 1X running buffer must be at room temperature prior to installation on the instrument.
        iv. Genetic analyzer performance can be affected by fluctuations in room temperature according to the manufacturer. Ensure that the room temperature is compliant with manufacturer recommendations before and during use. For best results, we recommend a room temperature of 18 (20 °C for 3130 series) to 30 °C with a maximum change of less than 2 °C per 24 hours.

The following instrument default run modules can be used as templates:

- 3130, 3130*xl*: FragmentAnalysis36_POP7
- 3500, 3500xL: FragmentAnalysis50_POP7, FragmentAnalysis50_POP7xl, FragmentAnalysis36_POP7, FragmentAnalysis36_POP7xl
- 3730, 3730*xl*: GeneMapper50_POP7, GeneMapper36_POP7
- SeqStudio™: FragAnalysis

Adjust the injection conditions in default run module templates as follows:

Instrument	Capillary Length	Injection	Pre-Run	Run	Oven Temperature
3130†, 3130*xl*†	36 cm	3.5 kV, 35 s	15 kV*, 900 s	15 kV*, 1500 s	60 °C*
3500, 3500xL	50 cm	2.5 kV, 20 s	15 kV*, 900 s	19.5 kV*, 2100 s	60 °C*
3500, 3500xL	36 cm	2.5 kV, 20 s	15 kV*, 900 s	15 kV*, 1500 s	60 °C*
3730, 3730*xl*††	50 cm	2.5 kV, 10 s	15 kV*, 900 s	15 kV*, 2900 s	63 °C*
3730, 3730*xl*††	36 cm	2.5 kV, 10 s	15 kV*, 900 s	15 kV*, 1500 s	63 °C
SeqStudio	28 cm	6.0 kV, 2 s	13 kV*, 180 s*	6.0 kV, 3000 s	60 °C*

* Indicates settings that are identical to default instrument settings for the given capillary length and polymer type
† Ambient temperatures ≤20°C may impact performance
†† Performance on Applied Biosystems® 3730 Refresh series has not been verified. Only 3730 instruments with 48-capillary configurations should be used; 96-capillary instruments are not recommended for fragment analysis

Note: SeqStudio users may experience a high number of QC flags related to the size standard identified by the instrument after a run using default settings. These instrument size standard flags are only visible on the SeqStudio instrument interface, and do not impact downstream analysis or indicate an issue with the run. For size standard quality evaluation, refer to the QC Information section of 00003130, AmplideX® PCR/CE *CFTR* Analysis Module Software User Guide. For more information, contact Asuragen® Technical Support**.**

13. Name samples according to the following format with filename fields separated by a common delimiter. For more information, refer to the **Sample Naming** section.

Sample Naming

FSA file names contain one or more informational components, called *fields* or *attributes*, herein referred to as *attributes*. These attributes may appear in any order. Attributes are separated by a non-alphanumeric delimiter.

Permissible delimiters are restricted to (see note below):

- Underscore "_"
- Hyphen "-"

Pre-defined attributes include, but are not limited to:

- <Sample Name>
- <Well Position>
- <Date>
- <Instrument ID>

Examples of User-defined attributes are:

- <Operator Name>
- <Lot ID>

Note: Only underscore "_" or hyphen "-" are recognized as delimiters by the AmplideX® PCR/CE *CFTR* Analysis Module. Use of any other delimiters available within Applied Biosystem® Data Collection Software will result in analysis failure.

When programming a file naming convention for the AmplideX PCR/CE *CFTR* Analysis Module, any number of attributes may be included, in any order, however the following rules must be observed:

1. <Sample Name> must be in the filename.
2. Additional attributes, if any, must be separated by either underscore "_" or hyphen "-".
3. The characters \ / : * ? ' " < > | are not permitted.
4. When both CFTR Primer Mix A and CFTR Primer Mix B are used:
    1. <Sample Name> position must be the same for all file names in the batch.
    2. <Sample Name> spelling must match exactly in both file names.

Note: When hyphen "-" is selected as the delimiter within the AmplideX PCR/CE *CFTR* Analysis Module, attributes that contain hyphen "-" as a non-delimiting character (e.g. <Date> in the format yy-mm-dd) are treated as multiple attributes. In the analysis module's settings, adjust the position of <Sample Name> accordingly if such an attribute precedes <Sample Name>.

Optionally, **MixA** and **MixB** may be included in the file name in any position, including the delimiter, when both CFTR Primer Mix A and CFTR Primer Mix B reactions are performed. This may be done by either:

1. Appending directly to the <Sample Name> attribute, separated by the chosen delimiter (e.g. **<Sample Name>(delimiter)MixA** and **<Sample Name>(delimiter)MixB)**, when manually entering sample names into the instrument's Data Collection Software, or
2. Including a <User Defined> attribute in the file naming convention, if an XML file is used to import sample information into the Data Collection Software.

By default, the AmplideX PCR/CE *CFTR* Analysis Module uses underscore "_" as delimiter and Position 1 for <Sample Name>. These default settings may be changed in the Admin tab of the AmplideX PCR/CE *CFTR* Analysis Module (see 00003130, AmplideX PCR/CE *CFTR* Analysis Module Software User Guide).

Table 3. All examples use underscore "_" as the delimiter between attributes, however hyphen "-" can also be used. Attributes in **bold** are required by the software; all other attributes are optional, including MixA and MixB. Red indicates examples and reasons for incorrect naming.

Position of Sample Name field	Sample Name Example	FSA File Naming Convention	Sample Name Format	Reason for Sample Match/Mismatch
1	Sample-1_MixA	**<Sample Name>**_<Well>_<Date>.fsa	CORRECT	Sample Name in position 1 matches for both fsa files.
	Sample-1_MixB	**<Sample Name>**_<Well>_<Date>.fsa		
1	Sample1_MixA	**<Sample Name>**_<Well>_<Date>.fsa	CORRECT	Sample Name in position 1 matches for both fsa files.
	Sample1_MixB	**<Sample Name>**_<Well>_<Date>.fsa		
1	A01_Sample-1_MixA	<Well>_**<Sample Name>**_<Date>.fsa	INCORRECT	The position does not match the location of the Sample Name. Mix A processes independently and results in a Label QC (LA) flag for the Mix B sample. See the next example for resolution.
	B01_Sample-1_MixB	<Well>_**<Sample Name>**_<Date>.fsa		
2	A01_Sample-1_MixA	<Well>_**<Sample Name>**_<Date>.fsa	CORRECT	Sample Name in position 2 matches for both fsa files.
	B01_Sample-1_MixB	<Well>_**<Sample Name>**_<Date>.fsa		
1	Sample1_Rep1_MixA Sample1_Rep1_MixB	**<Sample Name>**_<Well>_<Date>.fsa	INCORRECT	In this example, four fsa files would be processed with the same Sample Name in the 1st position leading to failed analysis. See the next example for resolution.
	Sample1_Rep2_MixA Sample1_Rep2_MixB	**<Sample Name>**_<Well>_<Date>.fsa		
1	Sample1-Rep1_MixA Sample1-Rep1_MixB	**<Sample Name>**_<Well>_<Date>.fsa	CORRECT	Sample Name at position 1 matches exactly and uniquely for each replicate prior to the underscore "_" delimiter.
	Sample1-Rep2_MixA Sample1-Rep2_MixB			

Fragment analysis (PCR/CE)

The AmplideX® PCR/CE *CFTR* Kit produces electropherogram data that is converted into sample-specific peak patterns that are automatically interpreted to determine variant zygosity or repeat size using an analysis module within AmplideX Reporter. Please refer to document 00003130, AmplideX PCR/CE *CFTR* Analysis Module Software User Guide for complete instructions. Anticipated peak sizes for all WT and MUT peaks are listed alphabetically by variant in **Table 4**.

Table 4. Anticipated peak sizes in base pairs (bp) for wild type (WT), mutant (MUT), STR, or PolyT peaks. Size ranges generally applicable across all CE models. Peak sizes may show slight size increase (0.5 – 1 bp) on Applied Biosystems® SeqStudio™ instruments.

Marker Name	Allele	Primer Mix	Channel	Peak Min	Peak Max
1717-1G>A	WT	A	FAM	102.0	106.0
1717-1G>A	MUT	A	HEX	99.5	103.5
1898+1G>A	WT	A	FAM	234.5	238.5
1898+1G>A	MUT	A	HEX	231.5	235.5
2183AA>G/2184insA/2184delA	WT	A	FAM	366.0	370.0
2184delA	MUT	A	HEX	356.0	360.0
2184insA	MUT	A	HEX	365.5	369.5
2789+5G>A	WT	A	FAM	212.1	216.1
2789+5G>A	MUT	A	HEX	209.0	213.0
3120+1G>A	WT	A	FAM	218.5	222.5
3120+1G>A	MUT	A	HEX	215.3	219.3
3272-26A>G	WT	A	FAM	261.3	265.3
3272-26A>G	MUT	A	HEX	258.5	262.5
3659delC	WT	A	FAM	225.5	229.5
3659delC	MUT	A	HEX	221.7	225.7
3849+10kbC>T	WT	A	FAM	148.5	152.5
3849+10kbC>T	MUT	A	HEX	146.5	150.5
621+1G>T	WT	A	FAM	167.2	171.2
621+1G>T	MUT	A	HEX	164.4	168.4
711+1G>T	WT	A	FAM	198.5	202.5
711+1G>T	MUT	A	HEX	195.6	199.6
A455E	WT	A	FAM	161.4	165.4
A455E	MUT	A	HEX	158.6	162.6
D1152H	WT	A	FAM	322.0	326.0
D1152H	MUT	A	HEX	319.5	323.5
F508del	WT	A	FAM	114.0	118.0
F508del	MUT	A	HEX	112.1	116.1
G542X	WT	A	FAM	142.0	146.0
G542X	MUT	A	HEX	139.9	143.9
G551D	WT	A	FAM	175.9	179.9
G551D	MUT	A	HEX	173.0	177.0
G622D	WT	A	FAM	186.0	190.0
G622D	MUT	A	HEX	183.1	187.1
G85E	WT	A	FAM	155.6	159.6
G85E	MUT	A	HEX	153.0	157.0
I507del	WT	A	FAM	106.9	111.9
I507del	MUT	A	HEX	105.2	109.2
L206W	WT	A	FAM	274.5	278.5
L206W	MUT	A	HEX	271.0	275.0

Marker Name	Allele	Primer Mix	Channel	Peak Min	Peak Max
N1303K	WT	A	FAM	250.4	254.4
N1303K	MUT	A	HEX	248.0	252.0
P5L	WT	A	FAM	282.1	286.4
P5L	MUT	A	HEX	279.0	283.0
R1070Q	WT	A	FAM	350.0	354.0
R1070Q	MUT	A	HEX	348.0	352.0
R1162X	WT	A	FAM	267.4	272.0
R1162X	MUT	A	HEX	264.5	268.5
R117H	WT	A	FAM	299.4	304.0
R117H	MUT	A	HEX	297.5	301.5
R334W	WT	A	FAM	120.5	124.5
R334W	MUT	A	HEX	118.0	122.0
R347H/R347P	WT	A	FAM	240.8	244.8
R347H/R347P	MUT	A	HEX	238.0	242.0
R553X	WT	A	FAM	181.2	185.2
R553X	MUT	A	HEX	178.2	182.2
R560T	WT	A	FAM	204.8	208.8
R560T	MUT	A	HEX	201.6	205.6
S945L	WT	A	FAM	256.6	260.6
S945L	MUT	A	HEX	253.5	257.5
W1282X	WT	A	FAM	190.9	194.9
W1282X	MUT	A	HEX	188.0	192.0
5T	PolyT	A	HEX	126.5	140.0
7T	PolyT	A	FAM	126.4	140.0
9T	PolyT	A	NED	123.5	138.5
STR01	Biomarker	A/B	NED	184.5	220.5
STR51	Biomarker	A/B	NED	301.0	377.0
STR91	Biomarker	A/B	NED	244.5	300.5
1078delT	WT	B	FAM	337.2	343.5
1078delT	MUT	B	FAM	330.1	336.2
1154insTC	WT	B	FAM	400.0	406.5
1154insTC	MUT	B	FAM	407.4	413.2
1548delG	WT	B	FAM	352.0	356.0
1548delG	MUT	B	FAM	358.0	362.0
1677delTA*	WT	B	FAM	485.5	491.0
1677delTA*	MUT	B	FAM	477.1	486.5
1811+1.6kbA>G	WT	B	HEX	338.9	343.2
1811+1.6kbA>G	MUT	B	HEX	333.7	338.7
1898+5G>T	WT	B	FAM	136.5	140.9
1898+5G>T	MUT	B	FAM	132.6	136.6
2183AA>G	MUT	B	HEX	310.8	314.8
2789+2insA	WT	B	HEX	261.0	265.0

Marker Name	Allele	Primer Mix	Channel	Peak Min	Peak Max
2789+2insA	MUT	B	HEX	265.8	269.8
3876delA	WT	B	HEX	480.1	484.0
3876delA	MUT	B	HEX	475.0	479.0
3905insT	WT	B	HEX	497.0	501.0
3905insT	MUT	B	HEX	502.4	506.4
394delTT	WT	B	HEX	493.0	496.9
394delTT	MUT	B	HEX	488.0	492.0
A559T	WT	B	FAM	214.0	218.0
A559T	MUT	B	FAM	220.0	224.0
E60X	WT	B	HEX	415.0	419.0
E60X	MUT	B	HEX	411.0	414.9
F311del	WT	B	FAM	313.7	317.7
F311del	MUT	B	FAM	308.9	312.9
G970D	WT	B	HEX	116.3	120.3
G970D	MUT	B	HEX	111.6	115.6
I618T	WT	B	HEX	517.3	521.8
I618T	MUT	B	HEX	510.8	516.0
M1101K	WT	B	HEX	248.0	252.0
M1101K	MUT	B	HEX	253.1	257.1
P67L	WT	B	HEX	432.3	436.7
P67L	MUT	B	HEX	427.0	431.4
Q493X	WT	B	FAM	417.0	422.9
Q493X	MUT	B	FAM	423.0	427.9
Q890X	WT	B	FAM	457.7	463.6
Q890X	MUT	B	FAM	464.1	470.0
R1066C	WT	B	HEX	364.0	368.0
R1066C	MUT	B	HEX	358.7	362.7
R1070W	WT	B	HEX	343.8	347.8
R1070W	MUT	B	HEX	349.0	353.0
R1158X	WT	B	FAM	378.6	383.4
R1158X	MUT	B	FAM	382.7	387.9
R117C	WT	B	HEX	373.5	377.5
R117C	MUT	B	HEX	378.4	382.4
R352Q	WT	B	FAM	447.0	453.0
R352Q	MUT	B	FAM	437.1	443.0
R75X	WT	B	HEX	459.0	463.0
R75X	MUT	B	HEX	454.0	458.0
S549N	WT	B	FAM	249.0	253.6
S549N	MUT	B	FAM	245.0	249.0
V456A	WT	B	FAM	121.1	125.1
V456A	MUT	B	FAM	127.5	131.5
V520F	WT	B	FAM	491.3	501.0

Marker Name	Allele	Primer Mix	Channel	Peak Min	Peak Max
V520F	MUT	B	FAM	501.0	510.0
Y1092X	WT	B	HEX	278.2	282.2
Y1092X	MUT	B	HEX	283.0	287.0
Y122X	WT	B	HEX	389.5	393.5
Y122X	MUT	B	HEX	394.0	398.0

*Expected peak size can vary depending on the status of F508del and I507del

NOTE: PolyTG size is calculated from the measured size of the detected fragment based on amplicon design, PolyT status, and a dye specific mobility correction factor.

Known Variant Conflicts and Limitations

- Where present, the following alleles will not be detected when they occur in phase (i.e. on the same chromosome) with the following variants:
    - R117C not detected when in phase with Y122X mutant
    - R117C not detected when in phase with R117H mutant
    - R117H not detected when in phase with R117C mutant
    - R1070W not detected when in phase with R1066C mutant
    - 394delTT not detected when in phase with 405+3A>C mutant (not detected by kit)
    - G551D not detected when in phase with A559T mutant
- Where present, the following alleles will be reported as Not Detected (ND) in the CSV file:
    - R1070W may be reported as ND in the presence of homozygous R1066C-mut
    - 394delTT may be reported as ND in the presence of homozygous 405+3A>C-mut (not detected by kit)
    - G551D may be reported as ND in the presence of homozygous A559T-mut
    - I507del may be reported as ND in the presence of homozygous F508del-mut
    - F508del may be reported as ND in the presence of homozygous I507del-mut. This case would erroneously trigger a DI QC failure though the assay is acting as expected
- Where present, the following alleles will be reported as Other in the CSV file:
    - If 2183/84 wt and 2184delA mut peaks are found within the FSA signal: 2184delA is marked HET, 2184insA is marked Other, 2183AA>G is marked Other
    - If 2183/84 wt and 2183AA>G mut peaks are found within the FSA signal: 2184delA is marked Other, 2184insA is marked Other, 2183AA>G is marked HET
    - If only the 2184delA mut peak is found within the FSA signal: 2184delA is marked MUT, 2184insA is marked Other, 2183AA>G is marked Other
    - If 2184delA mut and 2184insA mut peaks are found within the FSA signal: 2184delA is marked HET, 2184insA is marked HET, 2183AA>G is marked Other
    - If 2184delA and 2183AA>G mut peaks are found within the FSA signal: 2184delA is marked HET, 2184insA is marked Other, 2183AA>G is marked HET
    - If 2184insA mut and 2183AA>G mut peaks are found within the FSA signal: 2184delA is marked Other, 2184insA is marked HET, 2183AA>G is marked HET
    - If only the 2183AA>G mut peak is found within the FSA signal: 2184delA is marked Other, 2184insA is marked Other, 2183AA>G is marked MUT

- Where present, the following alleles will be reported as “Multiple” genotype in the UI, PDF, and CSV file:
    - If the F508del-mut and I507del-mut peaks are found within the FSA signal: I507del-wt and F508del-wt peaks may drop out and I507del and F508del may be reported as homozygous mutant, sample genotype is marked “Multiple”
- Where present, the following allele wild type peak may be shifted:
    - If F508del-mut or I507del-mut peaks are found within FSA signal for Mix A: expected Mix B variants 1677delTA and V520F peaks will be shifted
- Where present as heterozygous mutant in conjunction with an exon deletion, the following alleles may be reported as homozygous mutant:
    - E60X, P67L, R75X, 394delTT, and G85E may be reported as homozygous mutant if sample contains an Exon 3 deletion
    - M1101K, R1066C, R1070Q, R1070W, Y1092X, and 3272-26A>G may be reported as homozygous mutant if sample contains an Exon 20 deletion

Quality Control Procedures

No Template Control (NTC)

Use of a negative control (i.e., NTC) is optional. If an NTC is desired, the kit Diluent should be used in place of sample DNA. An NTC is run as a normal sample and is displayed in the list of samples tested. A properly functioning NTC exhibits a flat CE electropherogram and produces an “LS” FSA QC failure (Low Signal) and “FAIL” Overall QC in Results view of the software. In the exported reports, the NTC will display FAIL in the Overall QC column of the CSV report and will display FAIL in the Overall QC Status section of the PDF report for the relevant sample.

Figure 3. Example of valid NTC CE trace. Top is Primer Mix A, bottom is Primer Mix B.

Cell Line Samples

If desired, human cell line-derived samples with confirmed *CFTR* genotypes may be purchased from the Coriell Institute for Medical Research to confirm performance (**Table 2**). These samples are not required for proper functionality of the assay. See **Figure 4** for expected results from a cell line sample NA04345.

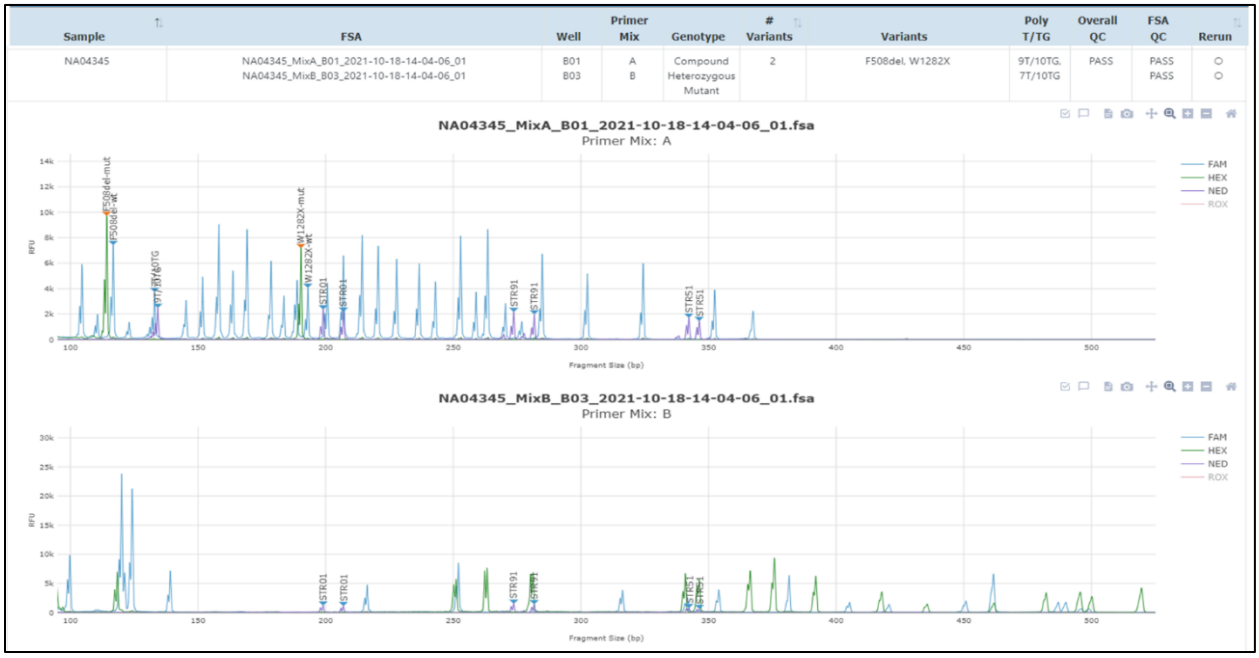


Figure 4. Coriell cell line sample NA04345. Top is Primer Mix A, bottom is Primer Mix B.

Samples

To ensure quality results, the AmplideX® PCR/CE Reporter with the *CFTR* Analysis Module provides automated QC checks for each FSA file associated with a sample. QC failures are summarized in **Table 5**.

Table 5. QC failure mode summaries. For more details, refer to 00003130, AmplideX® PCR/CE *CFTR* Analysis Module Software User Guide. Refer to the Troubleshooting section for resolution suggestions.

QC	Level	Description	QC Type & Cause	Sample Level Reporting	Variant Level Reporting
ROX QC (LD)	FSA File	The ROX QC flags samples that have aberrant sizing of the ladder and/or mobility correction factors that cannot be accurately determined. These prevent accurate peak sizing and variant association and are typically encountered when unexpected peak artifacts present in the ROX channel are similar in size and shape to expected ROX peaks, or when the ROX signal is too low.	Fail: Ladder is abnormal or missing	No genotype, number, or list of mutant variants	No variant zygosity in CSV; No peak annotations in UI, PDF, or CSV
Signal Magnitude QC (LS)	FSA	The Signal Magnitude QC is designed to flag samples with insufficient signal for multiple variants (low signal).	Fail: Low or undetected peak RFU associated with multiple variants	No genotype, number, or list of mutant variants	No variant zygosity in CSV; No peak annotations in UI, PDF, or CSV
Saturation QC (SA)	FSA	The Saturation QC will identify saturated peaks or excessive cross talk between channels which can cause incorrect variant zygosity determination.	Fail: PCR over-amplification or sample purity issues	No genotype, number, or list of mutant variants	No variant zygosity in CSV
Contamin-ation QC (CT)	FSA	The Contamination QC will flag samples when two or more human DNA templates are observed in the signal.	Fail: Multiple samples in a Primer Mix, sample	No genotype, number, or list of mutant variants	No variant zygosity in CSV
Label QC (LA)	Paired FSAs	The Label QC will flag a sample if two Primer Mix A sample traces are associated to a single Sample Name.	Fail: Samples named incorrectly	No genotype, number, or list of mutant variants	No variant zygosity in CSV
		The Label QC will flag a sample if a single Primer Mix B sample trace is associated to a Sample Name.	Fail: Unsupported primer mix	No genotype, number, or list of mutant variants	All variant zygosity and peak annotations
Tube Mismatch (TM)	Paired FSAs	The Tube Mismatch QC will flag samples with different patterns in the highly polymorphic STR biomarkers between the two primer mixes.	Fail: Samples from two different donors attributed to the same sample name; file naming or sample setup issue	No genotype	All variant zygosity and peak annotations
Data Integrity (DI)	Paired FSAs	The Data Integrity QC will flag samples when a variant is dropped (e.g. no WT or MUT peak is detected for a variant). The AmplideX PCR/CE *CFTR* Analysis module indicates peaks missing from the analysis.	Fail: Low signal for one or more variants	No genotype	All variant zygosity and peak annotations

Troubleshooting

Observation	Potential Cause	Action
Dropout of WT peak(s) observed	The purity of samples is insufficient (e.g. $A_{260/230} \leq 1.25$, $A_{260/280} \leq 1.5$ or $A_{260/280} > 2.1$)	• Re-purify the nucleic acid and repeat PCR. A kit similar to Zymo DNA Clean & Concentrator® Kit is recommended for desalting and removal of protein contaminates.
	Input quantity is below the recommended range for the assay.	• Increase input quantity (see **Pre-Analytical Steps** for supported range) or re-purify the nucleic acid and repeat PCR.
	Cycling conditions are not optimal for thermal cycler in use. Each amplicon within a Multiplex Allele-Specific PCR reaction does not have the same amplification efficiency; therefore, certain variants are more sensitive to cycling conditions.	• Review supported thermal cyclers in **Required but Not Provided** section. For thermal cyclers not listed use the Veriti/ProFlex conditions and contact Asuragen® Technical Support for assistance.
Low peak heights across all HEX labeled assay peaks and/or FAM labeled assay peaks for all samples included in a run.	Incorrect thermal cycling, incorrect instrument settings, incompatible instrumentation, incorrect CE formulation, incompatible reagent storage conditions, or handling	• Ensure compatible instrumentation and appropriate thermal cycler settings, CE instrument settings, and CE formulations as described in the Procedural Steps section. • Ensure that reagents are stored and handled as described in **Storage & Handling** and **Warnings and Precautions** sections**.** • Verify that Mix A and Mix B Primer Mixes have a light pink color. If the liquid is colorless, it may indicate a storage or contamination issue. Contact support@asuragen.com for more information.
	Saturating primer dimer peaks in the range of 85-90 bp due to cross contamination of Mix A and Mix B Primer Mixes	• Ensure that reagents are stored and handled as described in **Storage & Handling** and **Warnings and Precautions**. • Review the laboratory's contamination control procedures.
Spectral calibration issue of CE instrument or extra peaks detected (75-95 bp) in CE traces of	Carry over from AmplideX® PCR/CE *CFTR* Kit may be impacting spectral calibration of the CE instrument or other assays.	• Perform 4X injections of DI water or Hi-Di™ only to flush out carry-over contaminants. • Repeat spectral calibration.

Observation	Potential Cause	Action
other assays post AmplideX® PCR/CE *CFTR* Kit CE run.		

Sample fails Signal Magnitude QC criteria (LS in FSA QC column)	DNA input is below mass input range (<20 ng), DNA sample impurity, no DNA input due to pipetting error, aged capillary, or improper instrument calibration.	• Ensure the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D).
		• Capillary age can affect signal height; check the capillary age and number of injections to confirm it is within manufacturer specifications.
		• Ensure that reagents are stored and handled as described in **Storage & Handling** and **Warnings and Precautions** sections.
		• Pay special attention to pipetting technique, particularly when adding the DNA sample.
		• **If only one of the reactions (either Mix A or Mix B) for a sample failed LS QC**
		  ○ Repeat CE plate injection for failed reactions of the affected samples if ≤48 hours have passed since preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples).
		  ○ After CE injection repeat of the affected reactions, pair the FSAs with the previously passed corresponding Mix A or Mix B FSA file for paired-analysis in software.
		• **If both Mix A and Mix B reactions for a sample failed LS QC**
		  ○ Repeat CE plate injection for both Mix A and Mix B reactions of the affected samples if ≤48 hours have passed since preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples).
		  ○ After CE injection, perform paired-analysis in software.
		• Check DNA sample concentration and purity to ensure it is within assay range;

Observation	Potential Cause	Action
		note that some contaminants can affect quantitation of DNA concentration. • Check the raw CE data of the ROX ladder as well as FAM, HEX and NED channels for issues (e.g. saturating primer dimer in the range of 85-90 bp). • If root cause is due to low DNA input or contamination of reagents, repeat protocol starting at the **PCR master mix Setup and Thermal Cycling** section with all affected reactions using DNA input as per recommended range (20-60 ng). After PCR and CE injection repeat of the affected reactions, pair the FSAs with the previously passed corresponding Mix A or Mix B FSA file for paired-analysis in software. If the sample was initially within the assay range, increase to higher input within the recommended range. • Repeat DNA isolation and review procedure and reagents for potential sources of contamination or degradation of nucleic acids during purification workflow.
Sample fails Saturation level QC criteria (SA in FSA QC column)	DNA input is above mass input range, an incompatible thermal cycler or CE model was used, incorrect thermal cycling or CE protocol used, improper instrument calibration, incorrect instrument settings	• Ensure compatible instrumentation and appropriate thermal cycler settings, CE instrument settings, and CE formulations were used as described in **Procedural Steps**. • Check the genetic analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Check the raw CE data of the ROX ladder as well as FAM, HEX, NED channels for issues (e.g., extra peaks). • **If only one of the reactions (either Mix A or Mix B) for a sample failed SA QC** ○ Repeat CE plate injection for failed reactions of the affected samples if ≤48 hours have passed since preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples). ○ After CE injection repeat of the affected reactions, pair the FSAs with the previously passed

Observation	Potential Cause	Action

		corresponding Mix A or Mix B FSA file for paired-analysis in software.
		• **If both Mix A and Mix B reactions for a sample failed SA QC**
		  ○ Repeat CE plate injection for both Mix A and Mix B reactions of the affected samples if ≤48 hours have passed since preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples).
		  ○ After CE injection, perform paired-analysis in software.
		• Check the sample concentration to ensure it is within the recommended assay range (20-60 ng); note that some contaminants can affect quantitation of DNA concentration.
		• Dilute the sample with Diluent included in the kit; if the sample was initially within the assay range, dilute to 10 ng/µL such that the DNA mass input targets 20 ng.
		• Repeat protocol starting at the **PCR master mix Setup and Thermal Cycling** section with all affected reactions. After PCR and CE injection repeat of the affected reactions, pair the FSAs with the previously passed corresponding Mix A or Mix B FSA file for paired-analysis in software.

Sample fails Data Integrity QC criteria (DI in Overall QC column)	DNA input is below mass input range (<20 ng), DNA sample impurity, an incompatible thermal cycler or CE model was used, incorrect thermal cycling or CE protocol used, incorrect instrument settings, improper instrument calibration.	• Ensure compatible instrumentation and appropriate thermal cycler settings, CE instrument settings, and CE formulations were used as described in **Procedural Steps**. • Check the Genetic Analyzer is calibrated with the appropriate Matrix Standard (DS-30, Dye Set D). • Capillary age can affect signal height; check the capillary age and number of injections to confirm it is within manufacturer specifications. • **If only one of the reactions (either Mix A or Mix B) for a sample failed DI QC** ○ Repeat CE plate injection for failed reactions of the affected samples if ≤48 hours have passed since

Observation	Potential Cause	Action
		preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples). ○ After CE injection repeat of the affected reactions, pair the FSAs with the previously passed corresponding Mix A or Mix B FSA file for paired-analysis in software. • **If both Mix A and Mix B reactions for a sample failed DI QC** ○ Repeat CE plate injection for both Mix A and Mix B reactions of the affected samples if ≤48 hours have passed since preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples). ○ After CE injection, perform paired-analysis in software. • Check DNA sample concentration and purity to ensure it is within assay range; note that some contaminants can affect quantitation of DNA concentration. • If root cause is not identified above and sample concentration is sufficient, repeat protocol starting at the **PCR master mix Setup and Thermal Cycling** section with all affected samples. Pay special attention to pipetting technique, particularly when adding the DNA sample. • Repeat DNA isolation and review procedure and reagents for potential sources of contamination or degradation of nucleic acids during purification workflow.
	INDELs located within or between primer binding sites that are not covered by the AmplideX® PCR/CE *CFTR* Kit may shift the amplicon peak outside of the expected size bin causing allele dropout. For more information, see the	• Check the CE trace for extra peaks outside the peak size bin of the dropped variant. In this case, DI QC failure will not be resolved by repeating CE or PCR.

Observation	Potential Cause	Action
	Known Variant Conflicts and Limitations section.	
Sample fails ROX QC criteria (LD in FSA QC column)	Issue with ROX ladder sizing or peak intensity.	• Capillary age can affect consistent migration; check the capillary age and number of injections to confirm it is within manufacturer specifications. • **If only one of the reactions (either Mix A or Mix B) for a sample failed ROX QC** o Repeat CE plate injection for failed reactions of the affected samples if ≤48 hours have passed since preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples). o After CE injection repeat of the affected reactions, pair the FSAs with the previously passed corresponding Mix A or Mix B FSA file for paired-analysis in software. • **If both Mix A and Mix B reactions for a sample failed ROX QC** o Repeat CE plate injection for both Mix A and Mix B reactions of the affected samples if ≤48 hours have passed since preparation (otherwise, repeat protocol starting at the **Capillary Electrophoresis Using a Genetic Analyzer** section; i.e. reformulate CE plate using previously amplified PCR products for all affected samples). o After CE injection, perform paired-analysis in software. • If failure persists, check raw data for issues indicating loss of resolution, such as declining peak heights in the ROX ladder, or inconsistent migration of ROX ladder peaks between samples. If issues are observed, inject Hi-Di™ only for 5 to 10 injections to clean system. • If repeating CE does not resolve the issue, repeat protocol starting at the **PCR master mix Setup and Thermal Cycling** section (both Mix A and Mix B) with all affected samples. Ensure DNA concentration of the affected samples are within the recommended range.

Observation	Potential Cause	Action
Sample fails Contamination QC criteria (CT in FSA QC column)	Contamination of samples **NOTE**: The AmplideX® PCR/CE *CFTR* Analysis Module allows for extra alleles to be detected within an STR region as to not be overly sensitive but limited to 2 alleles above what is biologically feasible. The CT QC will be triggered by 3 alleles present in ≥ 2 STR regions, or by ≥ 4 alleles in one STR region.	• Check STR biomarker peaks in the NED channel for issues indicating sample contamination, specifically presence of more than 2 peaks per STR group (STR01, STR51, STR91). • Review procedure for potential sources of contamination of sample during purification workflow or assay set up. • Repeat protocol starting at the **PCR master mix Setup and Thermal Cycling** section with all affected reactions. After PCR and CE injection repeat of the affected reactions, pair the FSAs with the previously passed corresponding Mix A or Mix B FSA file for paired-analysis in software. Pay special attention to pipetting technique, particularly when adding the DNA sample. • If repeating PCR does not resolve the issue, repeat DNA isolation. Pay special attention to contamination of nucleic acids during purification workflow.
Sample fails Label QC criteria (LA in Overall QC column)	FSAs have an incompatible combination of kit mixtures (i.e., A and A, B and B) due to mislabeling or an unsupported primer mix (i.e. B only)	• Review file names for both Mix A and Mix B reactions for each sample is as per sample naming format provided in **Capillary Electrophoresis Using a Genetic Analyzer** section. Ensure file names of both reactions for a sample are associated with the correct Primer Mix and rerun analysis. • If failure was due to not testing Mix A for a sample, perform protocol starting at the **PCR master mix Setup and Thermal Cycling** section for Mix A reaction with all affected samples. Pay special attention to file naming. After CE injection of the Mix A reactions, pair the FSAs with the previously passed corresponding Mix B FSA file for paired-analysis in software.
	Issue with sizing or signal of Mix B target peaks in the range of 400-530 bp.	• Review the CE trace of affected samples to confirm anticipated peak sizes in **Table 4.** Anticipated peak sizes in base pairs (bp) for wild type (WT), mutant (MUT), STR, or PolyT peaks. Size ranges generally applicable across all CE models. Peak sizes may show slight size increase (0.5 – 1 bp) on Applied Biosystems® SeqStudio™ instruments. If failure was due to allele dropouts or

Observation	Potential Cause	Action
		incorrect sizing of alleles in the range of 400-530 bp, perform protocol starting at the **PCR master mix Setup and Thermal Cycling** section with all affected samples.
Sample fails Tube Mismatch criteria (TM in Overall QC column)	Biomarker STR patterns do not match between Mix A and Mix B.	• Review procedure for potential sources of sample mix up. • Review file names for both Mix A and Mix B reactions ensuring each sample is as per sample naming format provided in **Capillary Electrophoresis Using a Genetic Analyzer** section. Ensure Mix A and corresponding Mix B reaction are associated with the correct sample. Rerun analysis after fixing errors. • If Mix A and corresponding Mix B reaction cannot be identified, then reformulate CE reactions for the affected samples. • If failure persists, repeat protocol starting at the **PCR master mix Setup and Thermal Cycling** section with all affected samples (both Mix A and Mix B). Pay special attention to file naming.

Analytical Assessment

Method Comparison

In order to assess performance of the AmplideX® PCR/CE *CFTR* Kit, we tested 84 unique DNA samples consisting of human whole blood (n=51), cell lines (n=31), or synthetic DNA (n=2). Collectively, the panel included samples positive for all 63 variants detected by the assay, and contained PolyT repeat sizes of 5, 7, and 9 and PolyTG repeat sizes of 9, 10, 11, and 12. DNA samples were isolated using silica column (n=10), functionalized magnetic bead (n=31), or precipitation-based purification methods (n=41). To determine reference results for all variants detected by the assay, DNA samples were tested using either Sanger sequencing or a PCR/probe hybridization-based method.

All 84 samples were tested in singleton using the AmplideX PCR/CE *CFTR* Kit and analyzed using the following Applied Biosystems® Genetic Analyzers: 3500xL (36 cm and 50 cm capillary lengths), SeqStudio™ (28 cm capillary length), 3130*xl* (36 cm capillary length), and 3730*xl* (with 48-capillary configuration, 36 cm and 50 cm capillary lengths). In total, 6 unique CE instrument configurations were tested. QC failures were excluded from analysis.

Out of 489 valid sample measurements and 30,626 variant calls generated by the study, the variant-level statistics were all >99% in agreement with reference results for each CE instrument configuration (**Table 6**). Additionally, 475/489 (97.1%) sample measurements were correctly genotyped (**Table 7**). Among the incorrectly genotyped samples, 11/14 (78.6%) were due to incorrect variant zygosity (heterozygous vs. homozygous) rather than incorrect presence/absence of a variant, explaining the consistently high

performance across variant-level metrics (**Table 6**). Furthermore, 6/14 misses (42.9%) were from a single sample tested across all six CE configurations, indicating a sample-specific issue.

Taken together, these data demonstrate that the AmplideX PCR/CE *CFTR* Kit is highly concordant with reference methods across the full range of *CFTR* variants detected by this assay using multiple sample purification methods on all supported CE instrument configurations.

Table 6. Variant-level metrics for the method comparison study of the AmplideX® PCR/CE *CFTR* Kit. For each metric, the number of expected variant calls per instrument configuration is listed. Each sample measurement generates 62 unique variant calls. All variants detected by the assay were included in the panel. PolyT/TG agreement was evaluated separately (99.4% agreement across all measurements). PPV=positive predictive value, PPA=positive percent agreement, NPA=negative percent agreement, OPA=overall percent agreement (OPA), PA_z=zygosity agreement.

Measure	N (per config)	3130 36 cm	3500 36 cm	3500 50 cm	3730 36 cm	3730 50 cm	SeqStudio 28 cm
PPV	113	100%	100%	100%	100%	99.1%	100%
PPA	113	99.1%	100%	100%	100%	99.1%	100%
NPA	4492	100%	100%	100%	100%	100%	100%
OPA	5105	100%	100%	100%	100%	100%	100%
PA_z	5105	99.9%	100%	100%	100%	99.9%	100%

Table 7. Sample-level agreement for the method comparison study of the AmplideX PCR/CE *CFTR* Kit. Number of variants detected are compared to expected number of variants from reference method results. Sample genotypes reported by the kit are interpreted as, Wild type: 0 variants. Heterozygous: 1 variant. Homozygous, Compound Heterozygous, or Multiple: ≥2 variants.

		Expected Number of Variants			
		0	**1**	**≥2**	**Sum**
Measured Number of Variants	**0**	59	1	0	**60**
	1	1	274	1	**276**
	≥2	0	11	142	**153**
	Sum	**60**	**286**	**143**	**489**

Within-lab Precision

To assess the precision of the AmplideX® PCR/CE *CFTR* Kit, 11 unique samples consisting of DNA isolated from human cell lines (n=5) or whole blood (n=6) with eight unique variants (2 indels, 6 SNVs) were tested using 20 ng DNA input per reaction. Samples were isolated using precipitation, column, or automated magnetic bead methods.

Testing was performed in duplicate across 12 batch runs using two operators, one reagent lot, and two Applied Biosystems® Veriti™ 96-well thermal cyclers, generating 24 measurements per sample, or 264 sample measurements in total. The study was analyzed on two Applied Biosystems® 3500xL Genetic Analyzers.

Out of 264 valid sample measurements and 16,344 variant calls generated by the study, the variant-level statistics were all 100% in agreement with reference results for each operator and overall (**Table 8**). Additionally, all 264 sample measurements were correctly genotyped (**Table 9**).

Taken together, these data demonstrate that the AmplideX PCR/CE *CFTR* Kit has sufficient precision to unambiguously resolve variant status, zygosity, and sample genotype across multiple operators, runs, CE instruments, and thermal cyclers.

Table 8. Variant-level metrics for the within-lab precision study of the AmplideX PCR/CE *CFTR* Kit. For each metric, the number of variant calls included in the calculation per operator is listed. Each sample measurement generates 64 unique variant calls. 10 unique positive variants were included in the panel, including F508del, R117H, D1152H, R1158X, Y122X, V456A, 2789+2insA, and G551D. PolyT/TG agreement was evaluated separately (100% agreement). PPV=positive predictive value, PPA=positive percent agreement, NPA=negative percent agreement, OPA=overall percent agreement (OPA), PA_z=zygosity agreement.

Measure	N (per operator)	Op1	Op2	Overall
PPV	144	100%	100%	100%
PPA	144	100%	100%	100%
NPA	8,028	100%	100%	100%
OPA	8,172	100%	100%	100%
PA_Z	8,172	100%	100%	100%

Table 9. Sample-level agreement for the within-lab precision study of the AmplideX® PCR/CE *CFTR* Kit. Number of variants detected are compared to expected number of variants from reference method results. Sample genotypes reported by the kit are interpreted as, Wild type: 0 variants. Heterozygous: 1 variant. Homozygous, Compound Heterozygous, or Multiple: ≥2 variants.

		Expected Number of Variants			
		0	**1**	**≥2**	**Sum**
Measured Number of Variants	**0**	48	0	0	**48**
	1	0	120	0	**120**
	≥2	0	0	96	**96**
	Sum	**48**	**120**	**96**	**264**

DNA Input

In order to assess the DNA input range of the AmplideX PCR/CE *CFTR* Kit, eight unique samples consisting of DNA isolated from human cell lines (n=2) or whole blood (n=6) with six unique variants (1 indel, 5 SNVs) were tested in duplicate with five different DNA input amounts including 15, 20, 40, 60, or 70 ng of DNA per PCR using one reagent lot, generating 80 sample measurements. Samples were tested on one Applied Biosystems® 3500xL Genetic Analyzer.

Out of 80 valid sample measurements and 4,950 variant calls generated by the study, the variant-level statistics were all 100% in agreement with reference results for DNA inputs from 15 ng to 70 ng of DNA per reaction (**Table 10**). Additionally, all 80 sample measurements were correctly genotyped. These results support the assay DNA input range of 20 to 60 ng.

Table 10. Variant-level metrics for the DNA input study of the AmplideX PCR/CE *CFTR* Kit. All measurements with inputs from 15 ng to 70 ng were included in calculations. For each metric, the number of variant calls included in the calculation is listed. Each sample measurement generates 62 unique variant calls. 7 unique positive variants were included in the panel, including F508del, R117H, G542X, E60X, 621+1G>T, and W1282X. PolyT/TG agreement was evaluated separately (100% agreement). PPV=positive predictive value, PPA=positive percent agreement, NPA=negative percent agreement, OPA=overall percent agreement (OPA), PA_Z=zygosity agreement, PA_G=sample-level genotype agreement.

Measure	N	Overall
PPV	100	100%
PPA	100	100%
NPA	4,850	100%
OPA	4,950	100%
PA_Z	4,950	100%
PA_G	80	100%

Analytical Sensitivity

In order to assess the analytical sensitivity of the AmplideX® PCR/CE *CFTR* Kit, eight unique samples consisting of DNA isolated from human cell lines (n=7) or whole blood (n=1) were selected to include eleven low peak height alleles based on previous results. Samples were tested at a DNA input of 20 ng, the lowest supported concentration of the assay, with four replicates per sample. Samples were tested using an Applied Biosystems Veriti™ 96-well Thermal Cycler analyzed on an Applied Biosystems 3500xL Genetic Analyzer, and using an Applied Biosystems GeneAmp™ PCR System 9700 (gold block) analyzed on an Applied Biosystems 3130*xl* Genetic Analyzer. In total, 384 measurements were generated across 11 peaks of interest (I507del-MUT, R1158X-MUT, R75X-MUT, E60X-MUT, Q493X-MUT, V456A-MUT, Q493X-WT, R334W-WT, L206W-WT, 1677delTA-WT, and V520F-WT; MUT =mutant peak, WT =wild type peak).

Out of 384 measurements of peaks of interest generated by the study, all were 100% in agreement with expected results (**Table 11**). Mean peak heights were higher on the 3500 Genetic Analyzer compared to the 3130 Genetic Analyzer as expected, given the differences in RFU dynamic range between the platforms (30,000 RFU saturation limit for 3500, 8,000 RFU saturation limit for 3130). This trend is apparent when comparing peak height distributions between the platforms across the 11 peaks of interest in this study (**Figure 5**).

These results indicate that the AmplideX PCR/CE *CFTR* Kit has sufficient sensitivity to detect the most challenging variant peaks included in the assay at the lowest supported input level.

Table 11. Percent agreement across 11 peaks of interest for the sensitivity study of the AmplideX PCR/CE *CFTR* Kit. WT=wild type peak, MUT=mutant peak.

Variant-Peak	N	N correct	Percent Agreement	Mean RFU (3130)	Mean RFU (3500)
1677delTA-WT	64	64	100%	2602	2879
E60X-MUT	16	16	100%	534	946
I507del-MUT	8	8	100%	394	2110
L206W-WT	64	64	100%	281	2157
Q493X-MUT	16	16	100%	1471	2088
Q493X-WT	64	64	100%	792	1436
R75X-MUT	8	8	100%	680	1351
R334W-WT	64	64	100%	661	2391
R1158X-MUT	8	8	100%	842	3836
V456A-MUT	8	8	100%	1517	4310
V520F-WT	64	64	100%	610	1307
All	**384**	**384**	**100%**	NA	NA

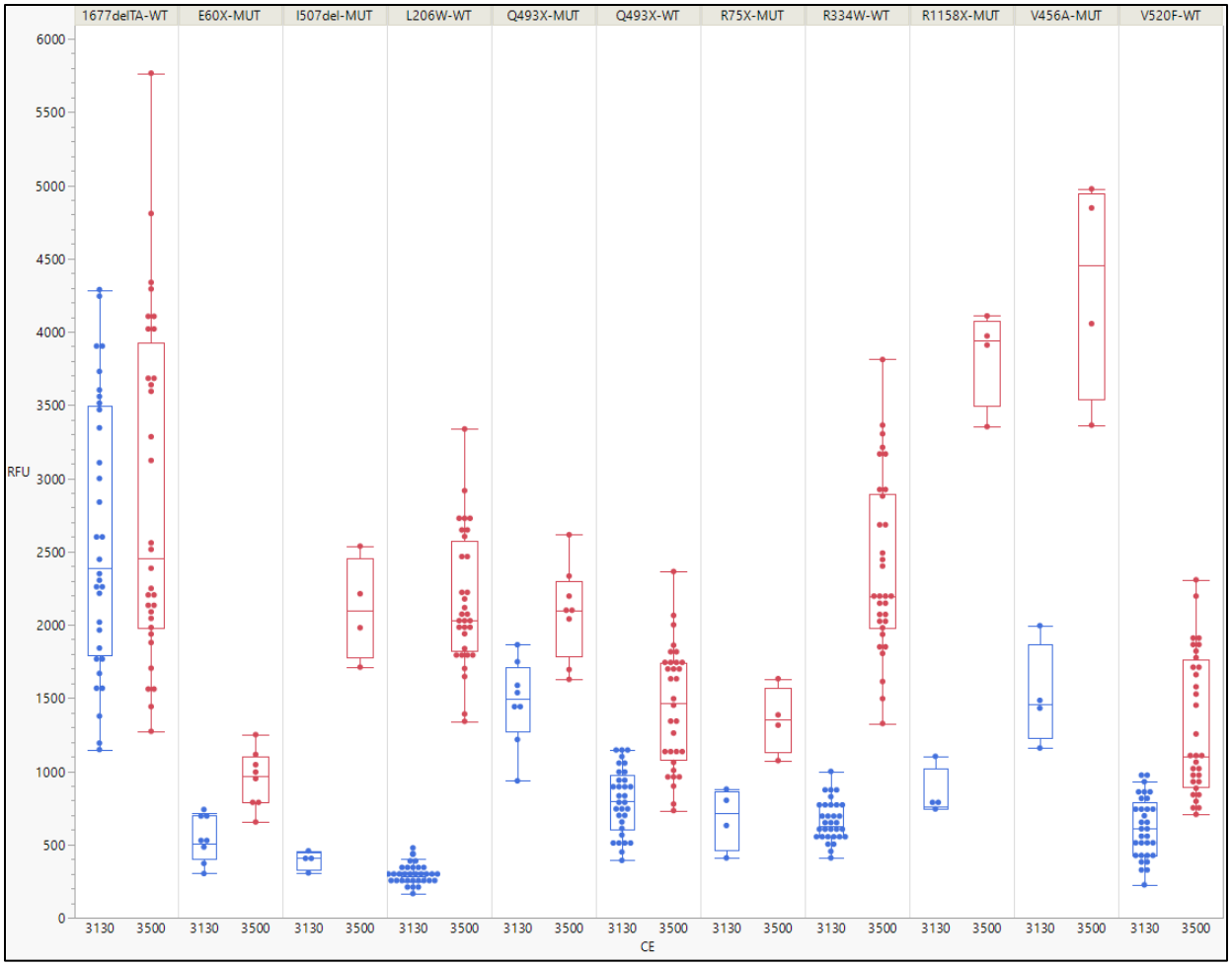


Figure 5. Peak heights across 11 peaks of interest for the sensitivity study of the AmplideX® PCR/CE *CFTR* Kit. Variant and peak of interest (WT or MUT) are indicated at top of figure. WT=wild type peak, MUT=mutant peak. CE instrument (3130 or 3500) are labeled at bottom of figure. Blue=3130, Red=3500.

Analytical Specificity

For the analytical specificity study, a subset of data generated on the Applied Biosystems Veriti™ 96-well Thermal Cycler and analyzed on an Applied Biosystems 3500xL Genetic Analyzer with 50 cm capillary length from the within-lab precision study, DNA input study, thermal cycler equivalency study, and method comparison study were combined for analysis.

To assess analytical specificity (exclusivity) for all variants detected by the assay, percent agreement with Sanger sequencing was determined with all homozygous wild type variant-level zygosity measurements (n=963). Across all variants, 100% of zygosity measurements were in agreement with Sanger sequencing for negative homozygous variant measurements (**Table 12**).

To assess analytical specificity (inclusivity) for all variants detected by the assay, percent agreement with Sanger sequencing was determined with all homozygous mutant variant-level zygosity measurements (n=113). Across all variants, 100% of zygosity measurements were in agreement with Sanger sequencing for positive homozygous variant measurements (**Table 12**).

Taken together, these data demonstrate that the AmplideX® PCR/CE *CFTR* Kit has sufficient specificity to determine variant status for all variants detected by the assay.

Table 12. Variant-level zygosity agreement for the specificity study of the AmplideX PCR/CE *CFTR* Kit. Detected variant zygosity is compared to expected zygosity of variants from reference method results. WT=wild type, MUT=homozygous positive.

		Expected Variant Status		
		WT	**MUT**	**Sum**
Measured Variant Status	**WT**	963	0	**963**
	MUT	0	113	**113**
	Sum	**963**	**113**	**1076**

Disclaimers

- This product is intended for Research Use Only. Not intended for use in diagnostic procedures.
- This product may not be resold, modified for resale, or used to manufacture commercial products without the written approval of Asuragen.
- Asuragen® and AmplideX® are trademarks of Asuragen®, Inc.
- All instrumentation must be maintained and operated according to manufacturer's instructions.
- TO THE EXTENT PERMITTED BY APPLICABLE LAW, IN NO EVENT SHALL ASURAGEN BE LIABLE IN ANY WAY (WHETHER IN CONTRACT, TORT (INCLUDING NEGLIGENCE), STRICT LIABILITY OR OTHERWISE) FOR ANY CLAIM ARISING IN CONNECTION WITH OR FROM THE USE OF THIS PRODUCT.

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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
	Manufacturer

Asuragen, Inc.
2150 Woodward St.
Suite 100
Austin, TX 78744
USA
+1.512.681.5200
+1.877.777.1874

https://asuragen.com/myasuragen

Customer Experience (Technical Support): 1.877.777.1874
Email: **support@asuragen.com**