Verification of a Targeted PCR/CE *CFTR* Assay and Companion Software that Addresses 93% Mutation Prevalence Across Diverse Ethnic Groups

Connor Parker, Kevin Kelnar, Elliot Hallmark, Sarah Edelmon, Shobha Gokul, Pranesh Rao, John N Milligan and Bradley Hall

Asuragen, a Bio-Techne brand, Austin, TX

Summary

- Current targeted *CFTR* variant panels fail to bridge the gap of genetic diversity across the US demographic, and often require cumbersome workflows and/or specialized instrumentation.
- In response, we developed the AmplideX® PCR/CE *CFTR* Kit which interrogates the most prevalent pathogenic variants for multiple ancestries.
- The Kit combines reagents and companion AmplideX PCR/CE Reporter software to generate genotypes within 5 hours with only 40 minutes of hands-on-time.
- The Kit design was verified across multiple operators, thermal cyclers, CE instruments, and DNA inputs, resulting in robust, accurate, and precise performance.

Introduction

Cystic Fibrosis (CF) is an autosomal recessive condition caused by mutations in the CF transmembrane conductance regulator gene (*CFTR*) resulting in dysfunctional ion transport across the cell membrane.[1] This typically results in increased chloride concentration in sweat, thicker mucus linings in bronchi, and impaired pancreatic exocrine function and intestinal absorption.

Here we describe the analytical performance of the AmplideX PCR/CE *CFTR* Kit and Reporter software. The AmplideX PCR/CE *CFTR* Kit is a multiplexed allele-specific PCR assay used to genotype and determine zygosity of up to 67 of the most prevalent mutations in *CFTR*. The *CFTR* mutation panel targets at least one pathogenic mutation in >99% of CF patients[2], and represents 93% of variant alleles in an ethnically diverse US population.[3] Panel detection covers all American College of Medical Genetics and Genomics (ACMG) minimally recommended 23 (CF23) mutations and 11 additional variants in a single tube (*CFTR* Primer Mix A), representing 86% MAF. *CFTR* Primer Mix A can also size and phase the PolyT and PolyTG regions upstream of Exon 10. A second tube is used to detect 33 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 peak identification, visualization, and variant reporting tool which combines peak detection with associated allele and sample level classification and QC across two tubes.

Materials and Methods

Multiple study-specific sample panels consisting, in total, of 56 residual clinical DNA samples from whole blood, 36 cell line DNA samples (Coriell Institute for Medical Research), and 2 gBlock pool mixes were used to evaluate the AmplideX PCR/CE *CFTR* Kit performance for accuracy, single-site precision and DNA input range among other studies. Variant status was determined using multiple genotype comparator methods including Sanger sequencing, Luminex xTAG CF60v2 (IVD), and SALSA MLPA Probemix P091-D2 *CFTR* assay (version D2-03; 11May2020, CE-IVD).

Samples were amplified using the AmplideX PCR/CE *CFTR* Kit on the Applied Biosystems™ (ABI) Veriti, ABI 9700, ABI ProFlex, and Bio-Rad C1000 thermal cyclers and PCR products were resolved by capillary electrophoresis (CE) on the ABI 3130*xl*, 3730*xl*, 3500xL, and SeqStudio™ Genetic Analyzers. FSA files were analyzed using the AmplideX PCR/CE Reporter software with AmplideX PCR/CE *CFTR* Analysis Module. Variant-level accuracy metrics, positive predictive value (PPV), positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) were calculated by comparison of assay result to reference variant status, with positive defined as variant present, heterozygous mutant (HET) or homozygous mutant (MUT), and negative defined as variant absent, homozygous wild-type (WT). Variant-level zygosity percent agreement with reference variant zygosity was also calculated along with sample-level genotype percent agreement.

Figure 1. Total-Assay-Time for the AmplideX PCR/CE *CFTR* Kit. Workflow is streamlined allowing for sample-to-result in <5 hours with <1 hour of hands-on time. Times based on average time to assay 12 samples across 2 operators.

*Research Use Only – Not for Use in Diagnostic Procedures
Presented at AMP 2021

Results

Sample	FSA	Well	Primer Mix	Genotype	# Variants	Variants	Poly T/TG	Overall QC	FSA QC	Rerun
103111-ASGNCFTR015	103111-ASGNCFTR015_MixA_C02_2021-08-14-10-15-47_01 103111-ASGNCFTR015_MixB_C08_2021-08-14-10-15-47_03	C02 C08	A B	Compound Heterozygous Mutant	2	W1282X, 1677delTA	7T/10TG, 7T/11TG	PASS	PASS PASS	○ ○

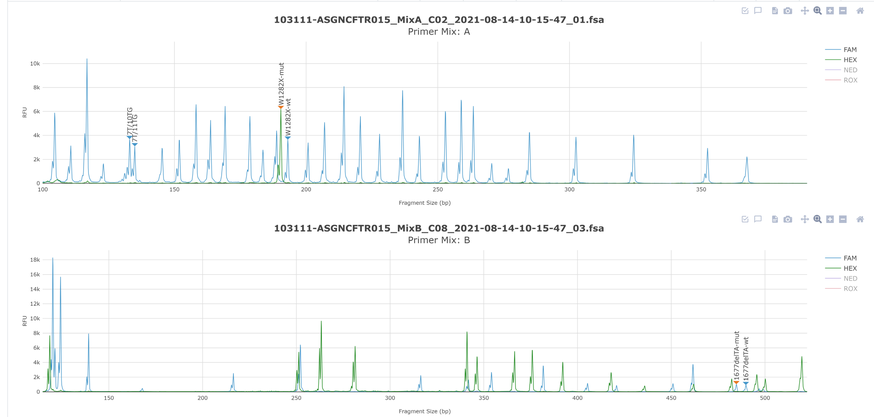


Figure 2. AmplideX PCR/CE Reporter Electropherogram View. The AmplideX PCR/CE Reporter interface with the *CFTR* module displays both electropherograms from two-tube assay and labels allele peaks corresponding to identified variants along with PolyT/TG peaks. Wild-type and variant allele peaks are segregated by dye-color and/or fragment mobility. Sample genotype, variants detected, and QC results are displayed in table format. Results are easily exported as PDF and CSV files.

Variant-level Accuracy Analytical Performance					
CE	PPV (N)	PPA (N)	NPA (N)	OPA (N)	Zygosity Agreement (N)
3130	100.0% (114)	99.1% (115)	100.0% (5151)	>99.9% (5266)	99.9% (5266)
3500	100.0% (231)	99.6% (232)	100.0% (10305)	>99.9% (10537)	>99.9% (10534)
3730	99.6% (232)	99.6% (232)	>99.9% (10304)	>99.9% (10536)	99.9% (10534)
SeqStudio	100.0% (116)	100.0% (116)	100.0% (5153)	100.0% (5269)	>99.9% (5269)
Combined	99.9% (693)	99.6% (695)	>99.9% (30913)	>99.9% (31608)	99.9% (31603)

Table 1. PPA, NPA, PPV, and Zygosity Agreement Were >99% on All CE Instrument Platforms in Accuracy Study. The study utilized 51 clinical DNA samples, 31 cell line DNA samples, and 2 gBlock pool mixes that together represented all kit-supported variants. Whole blood DNA was isolated with three different DNA extraction methods: precipitation, column, and magnetic-bead. Assays were performed by one operator using an ABI Veriti thermal cycler and six CE configurations (model and capillary length): ABI 3130*xl* (36 cm), 3500xL (36 and 50 cm), 3730*xl* legacy (36 and 50 cm), and SeqStudio (28 cm) CE Genetic Analyzers.

	Sample Genotype Agreement		Genotype Comparator Methods			
	Variants Identified / Sample Genotype		0 Homozygous WT	1 Heterozygous MUT	≥ 2 Homozygous MUT/ Compound HET/ Multiple	
AmplideX PCR/CE *CFTR* Kit	0	Homozygous WT	53	2	0	
	1	Heterozygous MUT	1	268	1	
	≥ 2	Homozygous MUT/ Compound HET/ Multiple	0	10*	154	Total
	Sample Genotype Agreement		98.1% (53/54)	95.7% (268/280)	99.4% (154/155)	97.1% (475/489)

Table 2. Sample Genotype Agreement with Genotype Comparator Methods of All Whole Blood and Cell Line Samples Assayed Across CE Instrument Platforms in Accuracy Study. AmplideX Reporter sample level genotypes for all accuracy study runs (Table 1), except gBlock pools and samples that did not pass overall QC, are included in table. The majority (10/14) of sample genotype disagreements were due to HET samples being genotyped as having more than one variant present.

*Note that 6/10 of these instances (i.e., same sample across 6 CE configurations) were called homozygous MUT when the heterozygous call was expected due to a SNP in the primer binding site that, when present, only affected D1152H zygosity.

3A

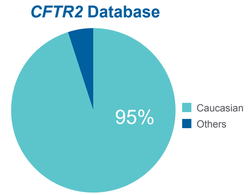
Single-Site Precision			
Operator	PPA (N)	NPA (N)	Zygosity Agreement (N)
Operator 1	100.0% (168)	100.0% (8268)	100.0% (8436)
Operator 2	100.0% (168)	100.0% (8268)	100.0% (8436)
Combined	100.0% (336)	100.0% (16872)	100.0% (16872)

3B

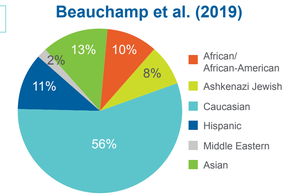
Thermal Cycler Equivalency		
Thermal Cycler	PPA (N)	NPA (N)
9700	99.0% (104)	>99.9% (6032)
C1000	100.0% (52)	>99.9% (3016)
ProFlex	100.0% (52)	100.0% (3016)
Veriti	100.0% (52)	100.0% (3016)

Table 3. PPA and NPA were 100% Across Both Operators in the Single-site Precision Study, and ≥99.0% for All Thermal Cyclers in Thermal Cycler Equivalency Study. A) Single-site precision study utilized 6 clinical DNA samples and 5 cell line DNA samples assayed in duplicate at 20 ng input by two operators on six different days (N of 24 for each sample). Assays performed using multiple ABI Veriti thermal cycler units and multiple ABI 3500xL Genetic Analyzers. **B)** Thermal cycler equivalency study utilized 6 clinical DNA samples and 6 cell line DNA samples assayed at 20 and 60 ng input, in duplicate, per PCR on four thermal cyclers and analyzed on an ABI 3500xL or 3130*xl* Genetic Analyzer. DNA input study (not shown) utilized 6 clinical DNA samples and 2 cell line DNA samples assayed in duplicate across DNA input range of 15 to 70 ng/reaction on an ABI Veriti thermal cycler and analyzed on an ABI 3500xL Genetic Analyzer. PPA and NPA were 100% for the supported DNA input range of 20 to 60 ng.

3A



3B



3C

Commercial Kit Manufacturer/Panel	Full Product Name	# of Variants Detected	% Coverage, CFTR2[4]	% Coverage, US[3]	% Coverage Difference, US[a]
Asuragen	AmplideX PCR/CE *CFTR* Kit	67	92.1%	93.0%	-
Luminex 97	xTAG Cystic Fibrosis (*CFTR*) 97 kit (Custom)	97	92.2.%	88.2%	4.8%
Illumina	MiSeqDx Cystic Fibrosis 139 Variant Assay	139	94.3%	87.7%	5.3%
Agena	iPLEXPro *CFTR* Panel	74	91.5%	86.9%	6.1%
Elucigene	CF-EU2v1	50	90.5%	86.2%	6.8%
Luminex 60	xTAG Cystic Fibrosis (*CFTR*) 60 kit v2	60	91.2%	86.1%	6.9%
Luminex 39	xTAG Cystic Fibrosis (*CFTR*) 39 kit v2	39	89.1%	80.7%	12.3%
GenMark	eSensor CF Genotyping Test	23	86.8%	78.9%	14.1%

Figure 3. AmplideX PCR/CE *CFTR* Kit Design Informed By Large NGS Studies Enabling Better Coverage, Even Compared To Large NGS Panels. A) The >89K patients in *CFTR2* database are heavily biased toward those of European or Ashkenazi Jewish descent.[4] **B)** Ethnic diversity of 115K subjects in US study[3] more accurately represents 2020 US Census demographic. **C)** Percent coverage of pathogenic and likely pathogenic variant alleles detected by commercial kits based on *CFTR*2 database[4] and US population frequencies. Sorted highest to lowest by US population coverage.

[a]Difference in percent coverage of *CFTR* variant alleles from Asuragen assay design[3]

Conclusions

- The AmplideX PCR/CE *CFTR* Kit includes reagents and assay-specific software to reliably detect 67 variants covering 93% of variant alleles in the ethnically diverse US population.
- AmplideX PCR/CE Reporter software automates quality control checks, peak detection and allele classification, and provides a detailed sample genotype report.
- The assay accurately (PPA and OPA >99%) detects variants across four CE instrument platforms, and four thermal cycler models across a DNA input range of 20 to 60 ng.
- Zygosity agreement with reference methods was >99%, and overall sample level genotype provided by the AmplideX PCR/CE Reporter software was >97%.

References

1. Brennan, M.-L. and Schrijver, I. (2016) Cystic Fibrosis A Review of Associated Phenotypes, Use of Molecular Diagnostic Approaches, Genetic Characteristics, Progress, and Dilemmas. The Journal of Molecular Diagnostics, 18, 3–14.
2. Castellani, C., Duff, A. J. A., Bell, S. C., Heijerman, H. G. M., Munck, A., Ratjen, F., et al. (2018). ECFS best practice guidelines: the 2018 revision. Journal of Cystic Fibrosis: Official Journal of the European Cystic Fibrosis Society, 17(2), 153–178.
3. Beauchamp, K.A., Johansen Taber, K.A., Grauman, P.V., Spurka, L., Lim-Harashima, J., Svenson, A., Goldberg, J.D. and Muzzey, D. (2019) Sequencing as a first-line methodology for cystic fibrosis carrier screening. Genet Med, 37, 773–8.
4. The Clinical and Functional Translation of *CFTR* (*CFTR*2); available at http://*CFTR*2.org.

Asuragen a bio-techne brand