Rapid, Equitable Molecular Confirmation of Pathogenic Variants in the *CFTR* Gene for Cystic Fibrosis Testing with Dried Blood Spots

Poster #9

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Summary

- Accurate and equitable molecular testing of the *CFTR* gene is critical as a second-tier confirmation for positive newborn screening results, especially in ethnic minority populations where many assay panels have lower coverage which can lead to missed variant detection and suboptimal outcomes.
- The AmplideX PCR/CE *CFTR* Kit* includes reagents and automated analysis software to reliably detect 65 variants covering 92% of variant alleles in the ethnically diverse US population by using latest peer reviewed data from a diverse cohort[1].
- The kit design was verified on dried blood spot (DBS) samples across multiple isolation methods, operators, thermal cyclers, and genetic analyzers, resulting in accurate and precise assay performance.
- The AmplideX PCR/CE *CFTR* Kit* can determine zygosity and genotype on DNA extracted from DBS in less than 5 hours, enabling rapid, accurate, and equitable characterization of *CFTR*.

Introduction

Cystic Fibrosis (CF) is a progressive hereditary disease and a core condition in the Recommended Uniform Screening Panel (RUSP).[2] CF is caused by pathogenic variants in the *CFTR* gene. Over 2,000 CFTR variants have been identified but most are benign or have unknown significance, and variant frequencies differ significantly between ancestries. There is a critical need for rapid and equitable second-tier confirmation of molecular changes in *CFTR* for positive newborn screening results. Lack of representative coverage in variant panels that can be run on accessible testing platforms can lead to missed detection and delayed confirmation in affected individuals. Fragment analysis on capillary electrophoresis (CE) instruments is a reliable and proven testing methodology that is already employed in a myriad of genetic applications.

Here, we evaluate the performance of the AmplideX PCR/CE *CFTR* Kit on DBS samples. The kit is comprised of a PCR/Capillary Electrophoresis assay that interrogates 65 pathogenic (P) or likely pathogenic (LP) *CFTR* variants. The assay design covers 92% P/LP variant prevalence in the US[1], provides consistently high coverage across ancestries, and includes at least one P/LP variant in >99% of CF patients[3]. The accompanying AmplideX PCR/CE Reporter software is an all-in-one visualization and variant reporting tool that combines peak detection with allele association and sample level classification and QC across two reaction tubes.

Materials & Methods

Study-specific sample panels consisting of 96 unique DBS samples were used to evaluate the AmplideX PCR/CE *CFTR* Kit performance for precision and accuracy, including studies to assess bias of sample matrix and instrument configurations. Fifty-three of the DBS samples were obtained from the Texas Department of State Health Services[4]. For the matrix study, results from DBS samples were compared to results from sample-matched whole blood. Reference results were determined using the TruSight Cystic Fibrosis assay (Illumina), xTAG CF60v2 assay (Luminex), or Sanger sequencing.

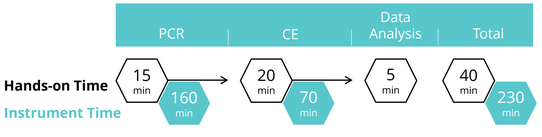

Figure 1. Total Assay Time for the AmplideX PCR/CE *CFTR* Kit. The workflow is streamlined for sample-to-result in <5 hours with <1 hour of total hands-on time. Times are based on average time to assay 12 samples across 2 operators.

Samples were isolated with three common methods: Quantabio Extracta DBS, Qiagen Generation DNA purification and elution solutions, and Qiagen QIAamp® DNA micro columns. Samples were tested using 2 µL of extraction eluate per PCR as input following the kit protocol.

DBS samples were amplified using the AmplideX PCR/CE *CFTR* Kit on the Applied Biosystems (ABI) Veriti, ABI 9700 (gold block), ABI ProFlex, ABI SimpliAmp, ABI VeritiPro, and Bio-Rad C1000 thermal cyclers. PCR products were resolved by capillary electrophoresis (CE) on the ABI 3500xL (50 cm), 3730*xl* (36 and 50 cm), SeqStudio (28 cm), and SeqStudio Flex (36 and 50 cm) Genetic Analyzers. Resultant .fsa files were analyzed using the AmplideX PCR/CE Reporter software with the AmplideX PCR/CE *CFTR* Analysis Module. Variant-level accuracy, zygosity, and PolyT/TG agreement metrics for positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) were calculated compared to reference results defined above.

Results

Table 1. Within-Lab Precision in DBS. Two operators utilized 3 unique lots of the AmplideX PCR/CE *CFTR* Kit over 12 days with two replicates of each sample per run to assess between-operator, lot-to-lot, day-to-day, and run-to-run variability. The sample panel included 12 DBS samples, and all PCRs were amplified on the ABI Veriti and analyzed on the ABI 3500xL (50 cm) Genetic Analyzer, generating 552 total measurements. OPA, NPA, and PPA for individual kit lots, run day, or operator was >99.34%, and PolyT/TG agreement was 100.00%.

Kit Lot	OPA (N)	NPA (N)	PPA (N)	PolyT/TG Agreement (N)
1	99.97% (5952)	99.98% (5800)	99.34% (152)	100.00% (192)
2	99.85% (5952)	99.84% (5800)	100.00% (152)	100.00% (192)
3	99.71% (5952)	99.71% (5800)	100.00% (152)	100.00% (192)
Overall	**99.84% (17856)**	**99.84% (17400)**	**99.78% (456)**	**100.00% (576)**

Table 2. Matrix Equivalency between Dried Blood Spots (DBS) and Whole Blood. Forty-one unique matched whole blood and DBS sample pairs were extracted using a silica column method to generate 41 extractions per sample type and tested in duplicate. PCRs were amplified on the ABI Veriti and analyzed on an ABI 3500xL Genetic Analyzer. Across 2,479 variant calls, agreement was perfect (100%) between matrix types. WT = wildtype, HET = heterozygous mutant, MUT = homozygous mutant.

Whole Blood

DBS	WT	HET	MUT
WT	2416	0	0
HET	0	57	0
MUT	0	0	6

Table 3. Comparison of AmplideX PCR/CE *CFTR* Kit and reference assay results. The study utilized 54 unique DBS samples, 53 of which were residual neonatal samples obtained from the Texas DSHS[4]. Available samples were divided into three groups and extracted using three extraction methods. All PCRs were amplified on the ABI Veriti and analyzed on six CE configurations (see Methods). **3A:** Sample-level genotype agreement was 100% across all six CE Genetic Analyzer configurations, combined. Numbers of variants corresponds with genotype as follows: WT = wildtype; HET = heterozygous mutant; MUT = homozygous mutant, compound heterozygous mutant, or multiple (3 or more variants). **3B:** OPA, NPA, PPA, PPV, and variant zygosity was 100% for all six CE Genetic Analyzers, individually (Table 3B). Similarly, all sample and variant-level statistics were 100% for all three extraction methods tested (see Methods).

3A.

Reference Genotype

Assay Result	WT	HET	MUT
WT	83	0	0
HET	0	175	0
MUT	0	0	56

3B.

Genetic Analyzer (capillary length)	OPA (N)	NPA (N)	PPA (N)	PPV (N)	Variant Zygosity (N)
ABI 3500xL (50 cm)	100.00% (3343)	100.00% (3297)	100.00% (46)	100.00% (46)	100.00% (3343)
ABI 3730*x*l (36 cm)	100.00% (3902)	100.00% (3844)	100.00% (58)	100.00% (58)	100.00% (3902)
ABI 3730*x*l (50 cm)	100.00% (3343)	100.00% (3297)	100.00% (46)	100.00% (46)	100.00% (3343)
ABI SeqStudio (28 cm)	100.00% (3281)	100.00% (3235)	100.00% (46)	100.00% (46)	100.00% (3281)
ABI SeqStudio Flex (36 cm)	100.00% (3343)	100.00% (3297)	100.00% (46)	100.00% (46)	100.00% (3343)
ABI SeqStudio Flex (50 cm)	100.00% (3343)	100.00% (3297)	100.00% (46)	100.00% (46)	100.00% (3343)

Table 4. OPA, PolyT/TG, and Zygosity Agreement Across Multiple Thermal Cyclers. The thermal cycler equivalency study utilized 9 DBS samples with genotypes representing wild type (n=1), heterozygous (n=4), and compound heterozygous mutant (n=4), covering 9 unique SNVs/INDELs. DBS samples on six thermal cyclers (ramp rate range 2.6 to 5.0 °C/s) and analyzed on an ABI 3500xL Genetic Analyzer. OPA and zygosity agreement were >99.82% and PolyT/TG agreement was 100.00% (420/420).

Thermal Cycler	OPA (N)	PolyT/TG Agreement (N)	Zygosity Agreement (N)
ABI 9700 (gold)	99.82% (1674)	100.00% (54)	99.82% (1674)
ABI ProFlex	100.00% (1674)	100.00% (54)	100.00% (1674)
ABI SimpliAmp	99.87% (3162)	100.00% (102)	99.87% (3162)
ABI VeritiPro	100.00% (3162)	100.00% (102)	100.00% (3162)
ABI Veriti	100.00% (1674)	100.00% (54)	100.00% (1674)
Bio-Rad C1000	99.94% (1674)	100.00% (54)	99.94% (1674)

Conclusion

- We demonstrated strong performance of the AmplideX PCR/CE *CFTR* Kit on DBS samples across three of the most common DBS extraction methods and a range of relevant sample genotypes.
- The assay accurately detects variants across four CE Genetic Analyzer platforms and six thermal cycler models, including an agreement to reference method of >99.82% for OPA, NPA, PPA, PPV, variant zygosity, and PolyT/TG.
- Following the same workflow of <5 hours from DNA sample-to-answer, matrix comparison of two sample types across 41 unique samples demonstrated 100% agreement between whole blood and DBS.
- This workflow enables rapid, accurate, and equitable characterization of variants in the *CFTR* gene from DBS that could help address delayed confirmation times in historically underrepresented populations.

References

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