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

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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 low coverage which can lead to missed variant detection and suboptimal outcomes.
- The Ampliplex PCR/CE CFTR Kit includes reagents and automated analytical software to reliably detect 65 variants covering 92% of variant alleles in the ethnically diverse US population using just a few pe beads reviewed data from a diverse cohort.
- The kit design was verified on dried blood spot (DBS) samples across multiple isolation methods, operators, thermal cyclers, and genotypic analyzers, resulting in accurate and precise assay performance.
- The Ampliplex 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 on the Recommended Uniform Screening Panel (RUSP). CF is caused by pathogenic variants in the CFTR gene. Over 2,000 CFTR variants have been identified but most are rare and 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 Ampliplex 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 overcomes low genetic variation in DBS samples provided consistently high coverage across ancestries, and includes at least one P/LP variant in 99% of CF patients.

The accompanying Ampliplex 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 64 unique DBS samples were used to evaluate the Ampliplex PCR/CE CFTR Kit performance for precision and accuracy, including studies to assess bias of sample matrix and instrument configurations. Fifty-three of the 64 samples were obtained from the Texas Department of State Health Services. 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 ) and XTAG CFgene assay ( Luminex ), or Sanger sequencing.

Table 1. Within-Lab Precision in DBS. Two operators utilized 3 unique lots of the Ampliplex 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 Genetic Analyzer, generating 552 total measurements. OPA, NPA, and PPA for individual kits lots, run, day, or operator was >99.34% and PolyT agreement was 100.00%.

Table 2. Matrix Equivalence Between Dried Blood Spots (DBS) and Whole Blood. For single unique matched whole blood and DBS sample pairs were extracted using a silica column method to generate a 41-extraction for each 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 both sample types. WT = wildtype, HET = heterozygous mutant, MUT = homozygous mutant.

Table 3. Comparison of AmplixPlex PCR/CFTR Kit and reference assay results. The study utilized 54 unique DBS samples, 53 of which were residual neonatal blood spots obtained from the Texas Department of State Health Services. 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 genotypes 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).

Table 4. OPA, Poly/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 SMV/HDEL. DBS samples on six thermal cycler ramp rate 2.6 to 5.0 °C and analyzed on an ABI 3500xL. Genotypic OPA and zygosity agreement were >99.82% and Poly/TG agreement was 100.00%.

Conclusion
- We demonstrated strong performance of the Ampliplex 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, variant zygosity, and Poly/TG.
- Following the same workflow of >5 hours from DNA sample-to-answer, this method compares favorably to 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
2. European Cystic Fibrosis Society. 17(2), 153
4. Texas Department of State Health Services. https://www.dshs.texas.gov/cftr

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Figure 1. Total Assay Time for the Ampliplex PCR/CE CFTR Kit. The workflow is streamlined for 15 in 5 hours to 1 hour of total hands-on time. Times are based on average time to assay 12 samples across 2 operators.