A Multiplex PCR/CE *CFTR* Prototype Assay and Software Targets Mutations Including Poly-T/TG and Addresses >90% Allele Frequency in US Population

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Summary

- Mutations in the *CFTR* gene on Chromosome 7 are responsible for Cystic Fibrosis (CF), which affects 1 in 3000 births.
- Reliable detection of CF mutations informs disease diagnosis, therapy decisions, carrier screening and pre-natal testing.
- We developed a simple and rapid two-tube PCR/CE assay to genotype 63 *CFTR* variants, including all 23 ACMG/ACOG-recommended, to address >92% mutant prevalence in an ethnically diverse US demographic and CFTR2.org.

Introduction

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

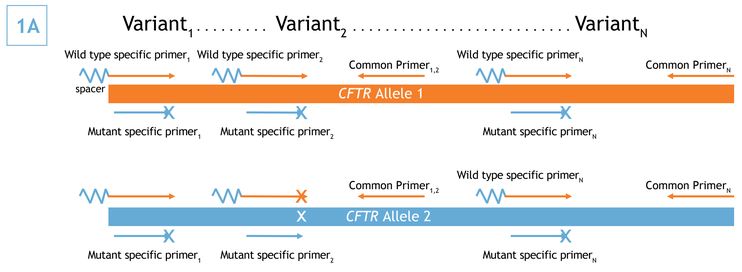
ACMG and ACOG recommend a panel of 23 mutations (CF23) on the basis of known mutation frequency[2,3]. Recent published clinical data by Beauchamp et al. (2019)[4], in combination with databases such as CFTR2.org[5], have identified pathogenic variants beyond CF23 that are relatively common, particularly for the US demographic where CF23 accounts for <80% of prevalence and could be missing observed variants in >25% of CF carriers.

Here we describe a prototype two-tube PCR/CE assay that detects 63 pathogenic variants. The assay can size/phase the Poly-T/TG (IVS8) region often associated with R117H, and detect the large exonic deletion CFTR2,3dele. This prototype panel addresses 92.3% of the US demographic, and includes eight relatively prevalent *CFTR* variants not detected by other on-market targeted mutation kits.

Materials and Methods

Cell-line genomic DNA samples with known *CFTR* mutations (n=58) or normal (n=22) were obtained from Coriell Cell Repository (CCR) or Asuragen. Genomic DNA was isolated from whole blood (WB) samples of 294 presumed healthy donors. Genomic DNA was amplified using AmplideX® PCR chemistry, and resolved by capillary electrophoresis (CE) on a 3500xL genetic analyzer (Thermo Fisher Scientific). Amplicon sizes were determined by mobility shift compared to a ROX ladder. Wild type and mutant allele identification was determined by both automated peak calling algorithms under development and GeneMapper panels based on expected sizes. Sanger sequencing was used as a comparator method to confirm cell line (n=80), WB carriers (n=9) and WB wild type (n=10).

1A



1B

Figure 1. Design and Workflow of a Prototype Two-tube, Multiplexed *CFTR* PCR/CE Assay. A) Allele-specific primers amplify wild-type (WT) or mutant (MUT) allele variants with a set of common primers. Amplicons are tagged with FAM, NED, or HEX during amplification. The amplicon mobility shift (bp) and/or dye channel differentiates alleles for each variant. **B)** The streamlined workflow can be performed in <5 hours from DNA to genotype, and requires <1 hr of hands on time.

*This product is under development. Future availability and performance cannot be ensured.

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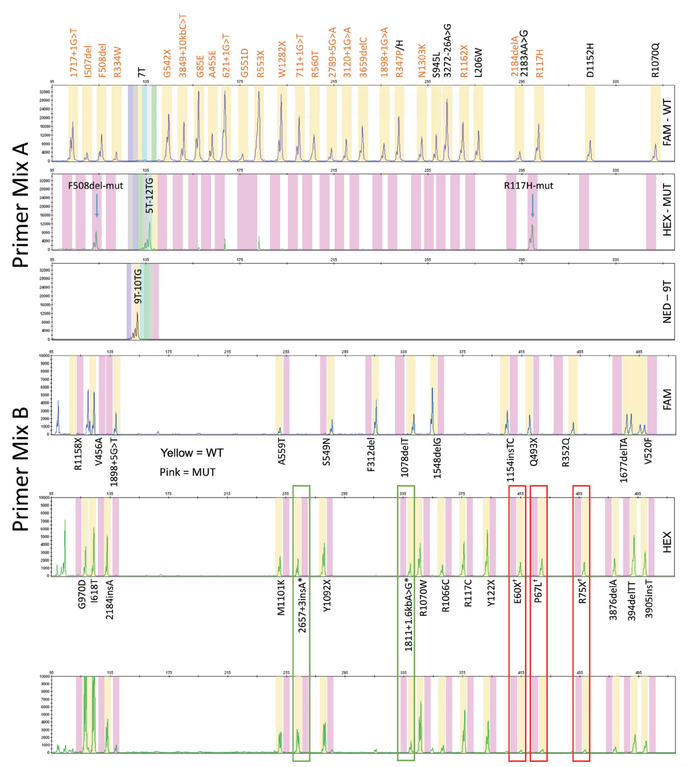
Results

2A



2B

Figure 2. Variant Zygosity and IVS8 Size/Phase is Accurately Resolved for Sample. CE traces are visual by GeneMapper 5.0. Peaks are binned based on expected sizes. The poly-T IVS8 allele is sized by dye channel: 5T (HEX), 7T (FAM) and 9T (NED). Peak size (bp) determines the poly-TG size and phases both tracts. **A)** CE traces for both Primer Mix A and B are shown split across dye channels for a compound heterozygote cell line (NA13591). Mutant peaks for F508del and R117H in Primer Mix A HEX channel and all WT variants across both mixes are observed. F508del is confirmed in Mix B based on split peaks for 1677delTA and V520F. The IVS8 genotype is 5T-12TG/9T-10TG. All samples with F508del were also observed to have at least one 9T allele for IVS8. **B)** Comparing peak height ratios of two WT alleles outside of Exon 3 (* green boxes) to three WT alleles in Exon 3 († red boxes) identifies the large exon deletion found in NA18668. Allele common names are listed above (Mix A) or below (Mix B) each peak. CF23 allele names in orange, additional alleles in **black**.

3A

IVS8 Size Distribution and Agreement	10 TG	11 TG	12 TG	Totals
5T	-	2	2	4/4 (100%)
7T	42	69	10	121/121 (100%)
9T	31	4	-	35/35 (100%)
	73/73 (100%)	75/75 (100%)	12/12 (100%)	160/160 (100%)

3B

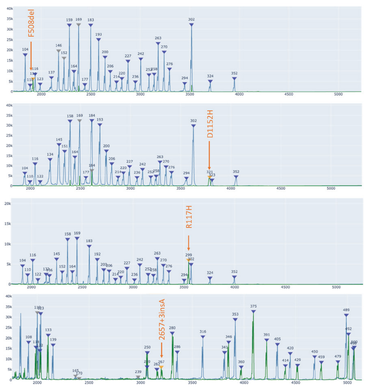
Agreement with Comparator Assay for Observed Allele Peak			Sanger Sequencing: homozygous wt/wt	Sanger Sequencing: homozygous mut/mut	Sanger Sequencing: heterozygous wt/mut	Variant Agreement
CFTR PCR/CE Assay	homozygous	wt/wt	4785**	-	-	4785/4785 (100%)
		mut/mut	-	5 4 var. - Mix A 1 var. - Mix B	-	5/5 (100%)
	heterozygous	wt/mut	-	-	64 28 var. - Mix A 15 var. - Mix B	64/64 (100%)

**Observed allele dropout for 1811+1.6kbA>G in 26/80 cell lines was caused by a SNP in the allele specific primer binding region of both alleles. The SNP was confirmed by sequencing in 26/26 (100%) of the samples and identified as non-pathogenic in dbSNP. Primer redesign fixed the issue. Two sets of variants (R347H/R347P and 2184delA/2183AA>G) are detected by the same allele specific primers and cannot be differentiated.

Figure 3. The Prototype Assay Agreed with Comparator Method for Allele Zygosity and IVS8 Sizing/Phasing. Cell lines (n=80) were sequenced to identify truth for each of the 63 variants covered by the assay. Zygosity across 5,040 variants covering 10,080 alleles were determined by the comparator method. **A)** Poly-T/TG sizing and phasing agreed with comparator for each cell line and with published results for IVS8 where available (n=43)[6]. **B)** The assay agreed with comparator for both WT and MUT alleles in all cell lines. Split peaks were observed in 16/16 (100%) F508del or I507del mutant allele samples. For variants where a mutant allele was not available in cell line material†, 63 synthetic mutant gBlocks were used to determine primer performance, and agreed with the designed sequence.

†[1548delG, 1677delTA, 1811+1.6kbA>G, 1898+5G>T, 2184insA, 2657+3insA, A559T, F311del, G970D, I618T, L206W, P67L, R1066C, R117C, R352Q, S945L, Y1092X]: No Coriell cell line DNA available for these variants.

4A



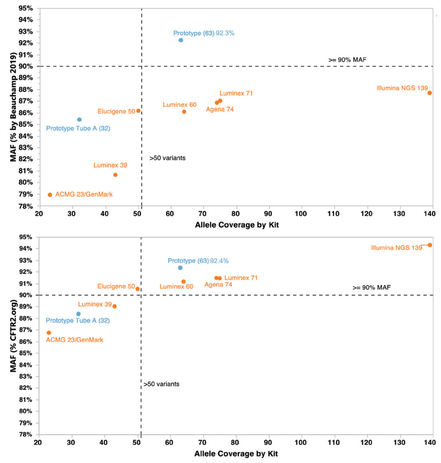
4B

4C

	Expected	Actual
Primer Mix A	1/35 * 85.4% = 2.44%, 7-8	8/294 (2.72%)
Primer Mix B	1/35 * 6.83% = 0.20%, 0-1	1/294 (0.34%)

Figure 4. Analysis of 294 Presumed Normal WB Donors Identified 9 Mutant Alleles. Prototype automated peak calling and classification software was developed to determine genotypes automatically. Across 6 experiments, 13907/14274 (97.4%) variants were called by the automated software concordantly with manual analysis. Sequencing was performed on a subset of samples (all nine with mutant allele and 10 WT) confirmed both manual and automated analysis. **A)** Of the nine, six had an F508del mutant allele which is the most common *CFTR* mutation. All the F508del carriers also had a 9T allele, consistent with published studies[7]. **B)** Of the remaining carriers, mutant alleles were detected for R117H, D1152H, and 2657+3insA. The last two would have been missed in a CF23-only test and 2657+3insA would have been missed by other on-market targeted mutation kits. The donor with a heterozygous R117H mutant allele did not have a 5T. **C)** Observed carrier frequency across samples matched expectations for the 63-variant set and was similar to the published carrier rate observed in the US population (~3% or one in 35)[8].

5A



5B

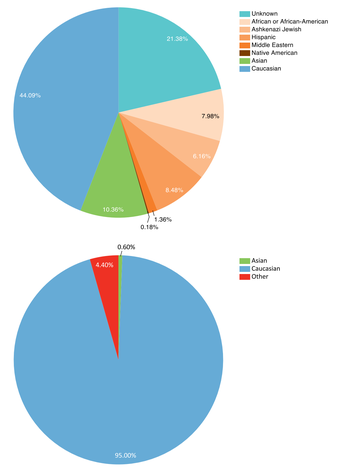


Figure 5. The Prototype 63-plex Assay Covers 92.3% of Pathogenic Alleles as Reported by Beauchamp et al.[4] Using Whole Gene NGS Analysis of *CFTR* Mutant Allele Frequency for an Ethnically Diverse US Population of >115K Individuals That Includes Mutations of Higher Frequency Than Many of Those in the CF23 Panel. A) Allele frequency of various on-market *CFTR* kits (orange) measured by Beauchamp et al. (top) or CFTR2.org (bottom) compared with the prototype (blue). **B)** Reported ethnic representation of each data set.

Conclusions

- We developed and evaluated a 63-variant PCR/CE *CFTR* assay that achieved 100% genotype agreement to reference results across 80 cell lines and 10,080 wild-type or mutant allele calls.
- Prototype automated software results were 97.4% concordant with manually called peaks and incorporated multiple QC checks, matched-tube assignment, and automated peak calling/classification.
- Genotyping was accomplished from a two-tube, PCR and CE injection using a workflow that readily slots within an 8-hour shift, and with the flexibility to support high-throughput screening or lower-volume diagnostic applications.
- The current panel content covers 92.3% of the pathogenic variants reported in an ethnically diverse US population including all mutations recommended by ACMG/ACOG guidelines. This coverage exceeds on-market *CFTR* assay kits with a simpler workflow.

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