A Multiplex PCR/CE *CFTR* Assay Resolves Zygosity of the 23 ACMG/ACOG-recommended *CFTR* Variants and Sizes poly-T and TG Repeats in a Single Tube

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Summary

- Mutations in the *CFTR* gene on Chromosome 7 are responsible for Cystic Fibrosis (CF), which affects 1 in 3300 births.
- Reliable detection of CF mutations informs disease diagnosis, therapy decisions, carrier screening and pre-natal testing.
- We developed a PCR/CE assay that accurately genotypes all 23 ACMG/ACOG-recommended variants plus 7 additional high-prevalence variants and IVS8 (poly-T/TG) using a simple and rapid one-tube workflow.

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[2] recommends a panel of 23 mutations (CF23) on the basis of mutation frequency in CF patients in the US. Poly-T sizing, which affects splicing of exon 10 primarily for 5T alleles, is a modifier of R117H[3] and recommended by ACMG as a reflex test. Further, expansion of a dinucleotide TG-repeat directly 5' of the poly-T tract in phase with a 5T repeat causes reduced active CFTR protein expression[4] and should be accommodated within an assay design. Recently, Beauchamp et al.[5] published NGS results 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.

Here we describe a prototype assay for the recommended CF23 mutations plus 7 additional high-prevalence variant alleles that sizes/phases the poly-T/TG tract in a single-tube PCR/CE assay.

Materials and Methods

Cell-line genomic DNA samples with known *CFTR* mutations were obtained from Coriell Cell Repository (CCR; n=62) or Asuragen (n=11). Genomic DNA was isolated from whole blood (WB) samples of 124 presumed healthy donors. Genomic DNA was amplified using AmplideX® PCR chemistry, and resolved by capillary electrophoresis (CE) on a 3500x*L* genetic analyzer (Thermo Fisher Scientific) within eight hours from DNA to genotype. Alleles and zygosity were differentiated by mobility shift compared to a ROX ladder. Variants of the poly-T tract were further distinguished by color channel (NED, FAM or HEX). Sanger sequencing was used as a comparator method to confirm cell lines (n=73), WB carriers (n=4) and WB wild type (n=5).

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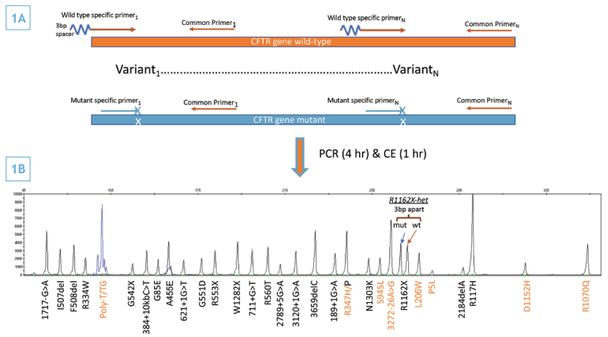

Figure 1. Design of a Prototype Single-tube, Multiplexed *CFTR* PCR/CE Assay. A) Allele-specific primers amplify wild-type (WT) or mutant (MUT) allele variants with a common primer labelled with a NED fluorophore. A three-nucleotide (nt) spacer on the WT primer differentiates the allele by size. The poly-T IVS8 allele was sized by dye channel and the poly-TG track was sized by mobility shift. **B)** A representative CE trace for the 32-plex assay. Allele common names are listed below each peak. A heterozygous allele is observed by the presence of both the MUT and the WT peaks (i.e. R1162X). CF23 allele names are given in **black**, additional alleles are shown in **orange**.

Results

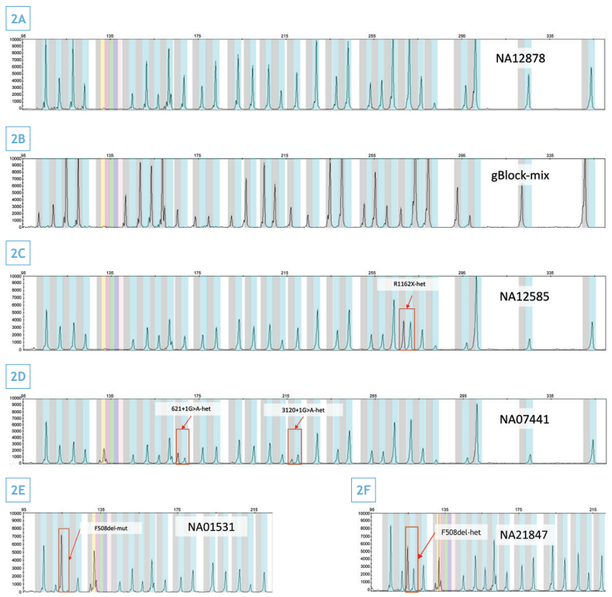

Figure 2. Zygosity is Accurately Resolved for Each Mutant *CFTR* Allele. CE traces were visualized by GeneMapper 5.0. Peaks were binned based on expected sizes. Amplicons from WT alleles were observed in teal bins and MUT in grey except for IVS8. **A)** NA12878 is homozygous WT for all alleles. This cell line does not have a 9T allele (in NED ch.). **B)** A mix of synthetic gBlock containing MUT alleles is MUT for all alleles. **C)** NA12585 is WT for all alleles except R1162X [ARG1162TER] where both WT and MUT alleles are observed, where the mutant allele peak is offset from its corresponding WT peak by 3bp. This would be common for a CF carrier. **D)** NA07441 is a compound heterozygote where both WT and MUT alleles are observed for 3120+1G>A and 621+1G>T variants. This cell line has a 9T allele phased with 10TG (yellow bin). **E)** NA01531 is a homozygous mutant for F508del while **F)** NA21847 is a carrier for F508del with a single affected allele. All samples with F508del were also observed to have at least one 9T allele for IVS8. Both D and E would be typical of a CF affected patient while C and F would reflect a CF carrier.

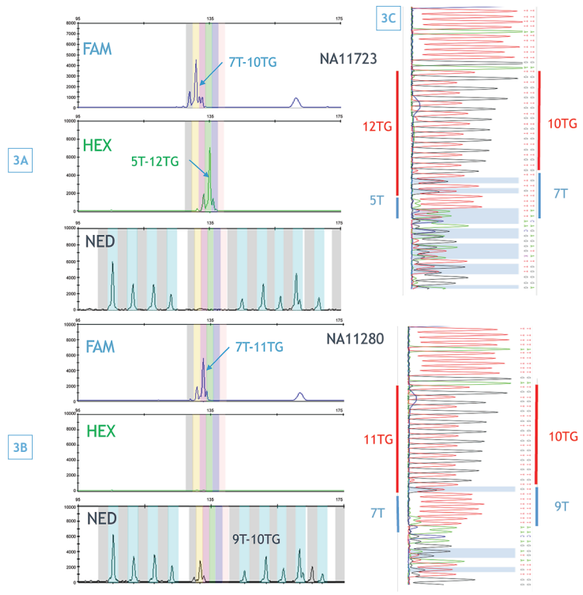

Figure 3. The Poly-T/TG Tract (IVS8) is Accurately Genotyped and Phased using a Novel Assay Design. Dye-specific peaks flag poly-T repeat length : 5T (HEX), 7T (FAM) and 9T (NED). Peak size (bp) determines the poly-TG size and phases both tracts. **A)** NA11723 has both 5T and 7T. The 5T phases in the 12TG bin and the 7T phases in the 10TG bin. **B)** NA11280 has both a 7T and 9T. The 7T is phased with 11TG and the 9T is phased with 10TG. The other peaks in the NED channel (A & B) correspond to the wt/mut peaks of the remaining 30 alleles **C)** Sanger sequencing was used to determine sizing truth for the IVS8 tract.

4A

Concordance with comparator assay			ASGN 32-plex *CFTR* PCR/CE			Zygosity Concordance
			homozygous		heterozygous	
			wt	mut	wt/mut	
ASGN Sanger Sequencing	homozygous	wt	4288	-	-	4288/4288 (100%)
		mut	-	8	-	8/8 (100%)
	heterozygous	wt/mut	-	-	42/42	84/84 (100%)

4B

Sample name	Poly-T	Poly-TG
NA11723	5T/7T	12TG/10TG
NA20242		11TG/10TG
NA13591	5T/9T	12TG/10TG
NA11277	7T/7T	10TG/10TG
NA11859		
NA11860		
NA12585		
NA12926		
NA07857		10TG/11TG
NA12444		
NA12785		
NA12961		
NA18801		
NA18802		
NA20737		
NA20745		
NA21069		
#RU0003		
RU0008		

Sample name	Poly-T	Poly-TG
CD00003	7T/7T	11TG/11TG
CD00007		
CD00014		
NA12878		
NA13033		
NA13423		
NA13664		
NA20239		
NA20741		
NA20836		
NA20915		
NA20925		
RU0002		
RU0007		
RU0009		
RU0010		
RU0011		
NA06891		10TG/12TG
RU0004		

Sample name	Poly-T	Poly-TG
NA06906	7T/7T	11TG/12TG
NA07464		
NA12960		
RU0005		
RU0006		12TG/12TG
NA20837	7T/9T	10TG/10TG
CD00012		
GM04345		
NA00999		
NA11278		
NA11284		
NA11370		
NA18803		
NA21551		
NA21847		
NA04025		10TG/11TG
NA08338		
CD00008		11TG/10TG
CD00009		
NA00897		
NA07441		
NA07732		
NA11275		
NA11280		
NA11288		
NA11472		
NA18800		
NA20924		
NA21080		
NA22063		
RU0001		
NA01531	9T/9T	10TG/10TG
NA11283		
NA11496		
NA18886		

Figure 4. The Prototype Assay was 100% Concordant with Comparator Method. Cell lines (n=73) were sequenced to determine truth for each of the 32 alleles covered in the assay. **A)** The assay was 100% concordant for both WT and MUT alleles observed (26 mut cell lines + 6 mut gBlocks)† and also to published data. **B)** Poly-T/TG sizing and phasing was 100% concordant with comparator for each of the cell lines and with published results for IVS8 where available (n=43)[6].

#RU00xx: Asuragen's internal cell lines.

†[G85E, N1303K, P5L, S945L, R1070Q and L206W]: No Coriell cell line DNA available for these variants.

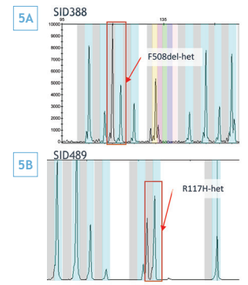

Figure 5. Analysis of 124 Presumed Normal WB Donors Identified Four Mutant Alleles. Of the four carriers, three had an F508del mutant allele **A)** which is the most common *CFTR* mutation. **B)** One carrier had an R117H mutant allele. All results were confirmed by sequencing. A carrier frequency of one in 31 (3.2%) was observed for the 32 allele set which is similar to published carrier rate observed in the US population (~3% or one in 35)[7]. **C)** The genotypic distribution of poly-T/TG suggests 7T/7T and 11TG/11TG are most common. All the F508del carriers (3/3) also had a 9T allele which corroborates the observations from literature[3]. The donor with a heterozygous R117H mutant allele did not have a 5T.

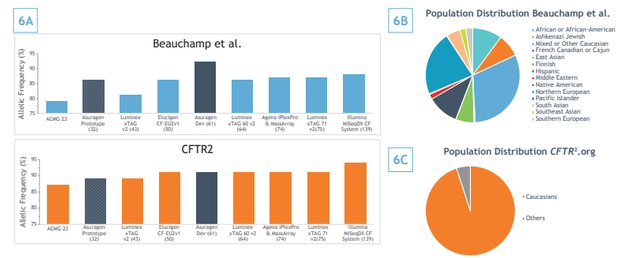

Figure 6. The Prototype 32-plex Assay Covers 86% of Pathogenic Alleles as Reported by Beauchamp et al.[5] A) Allele frequency by Beauchamp et al. (2019) or CFTR2 covered by various on-market *CFTR* kits compared with the prototype ("Asuragen Prototype"). This prototype is in active development towards 61 variants ("Asuragen Dev") that would cover >92% allele frequency based on a more ethnically diverse population reported by Beauchamp et al. **B)** compared to CFTR2[8] **C)**.

Conclusions

- We developed and evaluated a 32-variant PCR/CE *CFTR* assay that called 4672 *CFTR* (wt+mut) alleles from 73 cell lines with 100% agreement to reference genotypes.
- The assay accurately resolved SNVs, indels and repetitive sequences, and included a novel strategy for dye-specific coding and fragment sizing to genotype poly-T/TG.
- Genotyping was accomplished from a single input, 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 86% of the pathogenic variants reported in the US population and all mutations recommended by ACMG/ACOG guidelines. This coverage is similar to existing *CFTR* assay kits, but has a simpler procedure, fewer handling steps, and a single-tube configuration.

References

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