Development of a Comprehensive Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene Synthetic DNA Panel

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INTRODUCTION

Molecular testing for cystic fibrosis is complicated by the existence of over 1000 mutations that have been identified in the CFTR gene, most of which occur at frequencies of less than 0.1%[1]. Additionally, mutations occur with differing prevalence based on race and ethnicity[2]. This information has led to development of CFTR assays that detect mutations beyond the 23 recommended by ACOG/ACMG. Currently, commercial prenatal screening tests that detect nearly 100 mutations are available. The need for well-qualified materials to perform expanded assay development and quality control for cystic fibrosis testing has been recognized[3]. This need is especially acute for rare mutations which are not widely available in the form of patient samples. DNA samples from cell lines and synthetic materials do not cover all the mutations detected by commercially available CFTR assays[4]. In addition to the array of known rare individual mutations, there are complex compound heterozygotes that have been described. Here we describe the performance of synthetic CFTR constructs which can produce results identical to genomic DNA in DNA high resolution melt analysis (HRMA) and thus allow development of assays targeted to mutations where no genomic material is available.

MATERIALS AND METHODS

One hundred and six of 207 planned CFTR construct DNAs were made to cover 173 clinically relevant targets and corresponding normal sequences (Table 1). Constructs were designed by selecting 600 bp of sequence from each CFTR exon and adjacent introns based on an NCBI reference genome and mutant sequences as listed in the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca). The reference DNA sequences and the single-mutation variants were synthesized and cloned into a commercially available plasmid vector by Gene Oracle (Mountain View, CA), and the resulting DNA constructs were confirmed by double-stranded sequence analysis. Performance of the constructs in HRMA was assessed by testing with in-house developed CFTR assays designed for scanning exons and/or genotyping the 23 ACOG/ACGM recommended mutations. Genotyping assays targeted 60-100 bps while scanning assays targeted 150-200 bps. CFTR HRMA genotyping assays included small amplicon and unlabeled probe approaches. Construct DNAs were used at 0.132 pg per reaction which corresponds to the number of target copies in 100 ng of genomic DNA per reaction. Assays were performed in duplicate or triplicate on the Roche LightCycler® 480 over multiple runs and operators. HRMA was performed in several different software programs, including the Roche LightCycler® 480 Software, in-house developed software, and a prototype version of MeltWizard (Courtesy of Carl Wittwer, University of Utah).

Table 1. List of 173 CFTR mutations targeted in the synthetic constructs.

M1V	711+3A>G	5/7/9T	R560K	2622+1G>A	W1089X
R31C	G178R	A455E	R560T	E831X	Y1092X
Q39X	712-1G>T	1461ins4	1811+1.6bbA->G	W846X	M1101K
CFTRdele2,3	L206W	1525-1G>A	1812-1G>A	R851X	E1104X
E60X	L227R	S466X	Y569D	2711delT	D1152H
P67L	852delG22	L467P	G576A	2789+2insA	R1158X
R74W	H199Y	1548delG	D579G	2789+5G>A	R1162L
R75X	P205S	G480C	E585X	Q890X	R1162X
R75Q	Q220X	S489X	1898+1G>A	2869insG	S1196X
G85E	935delA	S492F	1898+3A>G	L927P	3659delC
394delTT	delF311	Q493X	1898+5G>T	S945L	W1204X
405+1G>A	G330X	I506V	D614G	3007delG	3791delC
405+3A>C	R334W	I507V	G622D	G970R	I1234V
406-1G>A	I336K	del507	2055del9>A	5977F	5123JSR
E92K	T338I	F508C	R668C	3120+1G>A	3849+10kbC>T
E92X	S341P	delF508	2143delT	3120G>A	G1244E
Q98X	R347P	1677delTA	2183AA>G	3121-1G>A	3876delA
444delA	R347H	V520F	2183delAA	L997F	S1251N
457TAT>G	R352Q	Q525X	2184insA	3199del6	S1255X
D110H	Q359K	1717-1G>A	2184delA	I1027T	3905insT
R117H	T360K	1717-8G>A	2307insA	3272-26A>G	D1270N
R117C	S364P	G542X	2347delG	F1052V	W1282X
Y122X	1078delT	S549N	R709X	L1065P	4005+1G>A
574delA	1154insTC	S549R	K710X	R1066H	ins T at 4016
I148T	1213delT	G551D	L732X	R1066C	N1303K
621+1G>T	1248+1G>A	Q552X	V754M	G1069R	Q1313X
663delT	1259insA	R553X	R764X	R1070W	4209TGTT>AA
711+5G>A	W401X	L558S	2585delT	R1070Q	CFTRdele22,23
711+1G>T	1341+1G>A	A559T	E822X	L1077P	4382delA

RESULTS

The constructs containing a single mutation were evaluated for genotyping patterns. Constructs were formulated to produce simple heterozygote, compound heterozygote, and additional combinations of multiple mutations. All constructs were evaluated using HRMA. For example, a simple heterozygote was produced by mixing mutant and wild type constructs at equal concentrations. Due to the uniform size of the constructs and the presence of a single or few mutations per construct, the formulations produce the expected results in HRMA. Constructs were tested with genotyping HRMA assays that included small amplicon (Figure 1) and unlabeled probe assays (Figure 1 and 2). Constructs were also tested using HRMA scanning assays (Figure 3).

Constructs were also mixed by combining multiple targets of a single type (i.e. normal, homozygous variants, or heterozygous) in a single formulation demonstrating the ability to construct a more complex synthetic DNA that represents greater genomic content. Mutation patterns were easily resolvable in HRMA for individual targets using this formulation (Figure 4).

RESULTS (continued)

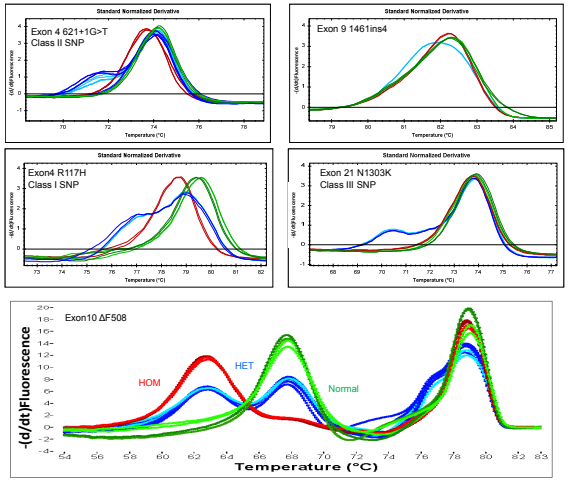

Figure 1. Genotyping Assays
Normal DNA (green) HRM patterns are shown with homozygotes (red) and heterozygotes (blue) for four different loci. The top four graphs are from small amplicon assays. Both genomic (dark green and blue) and construct DNAs (light green, light blue, and red) were used in each assay and their melt patterns fall within the variation seen for a given genotype regardless of DNA source. The bottom graph is from an unlabeled probe assay. All three genotypes show distinct probe melts, and the construct DNA (light colors) probe melts match those of the genomic (dark color) DNAs.

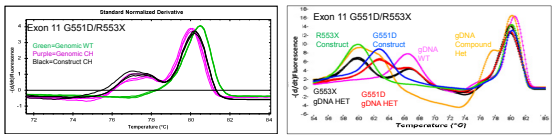

Figure 2. Compound Heterozygote Example
A compound heterozygote (CH) for the exon 11 G551D and R553X mutations was made synthetically using constructs. The melt pattern produced by the construct DNA mix matches that of the genomic compound heterozygote DNA (left image). Both genomic and compound heterozygote melt patterns are distinct from wild-type. The homozygous constructs also function as controls in an unlabeled probe assay for the compound mutation (right image).

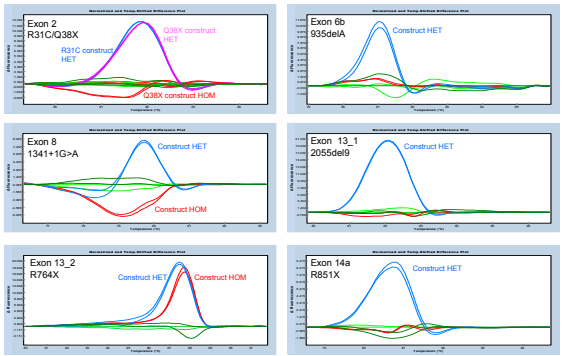

Figure 3. Performance of Synthetic Constructs in a CFTR Scanning Assay
Synthetic constructs were tested using an in-house developed CFTR scanning assay which canvasses a larger target sequence than the genotyping assay. Data are displayed using fluorescence difference plots. Detection of heterozygotes is the goal of a scanning assay and the synthetic constructs can easily be distinguished from normal DNA whether the source is genomic (dark green) or synthetic (light green), demonstrating that they can be used to evaluate assay performance. A total of twelve assays are shown over the two panes in this figure.

RESULTS (continued)

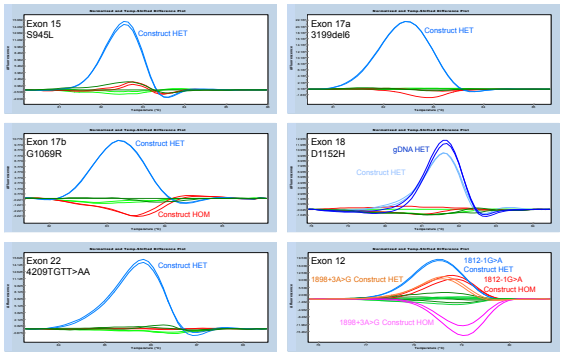

Figure 3 continued.

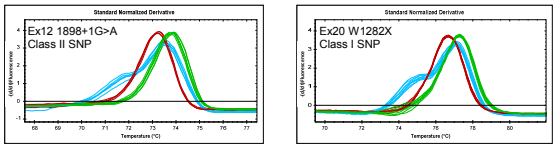

Figure 4. Constructing Mixing Example
Three synthetic construct mixes were prepared (normal, homozygote, and heterozygote), each covering 27 genotyping assay targets. Construct mixes were tested in individual assays and HRMA patterns were compared to those generated from genomic DNA and individual synthetic construct DNA. Assay results of mixed constructs, individual construct, and genomic DNA are presented for two genotyping assays above. The melt pattern produced for each genotype falls within a narrow distribution regardless of the source of the DNA (genomic, single construct, or construct mix) demonstrating that synthetic constructs covering multiple mutations can be formulated as a single mix.

CONCLUSIONS

The synthetic DNA constructs have been tested by HRMA for genotyping accuracy, reproducibility, homogeneity, and robustness. By combining different mutation constructs, every possible compound heterozygote can be constructed. The design was flexible enough to encompass regions that would perform well for both HRMA scanning and genotyping assays. The constructs performed well in HRMA genotyping assays that were designed for small amplicon or that used unlabeled probes. The constructs can be formulated by type to produce complex mixes that cover multiple targets. Synthetic constructs can be made quickly and serve as a source of highly reproducible material for assay development. This construct panel will provide sound analytical verification for both HRMA scanning and genotyping CFTR DNA assay development.

REFERENCES

1. Goetzinger and Cahill. 2010. An Update on Cystic Fibrosis Screening. *Clin Lab Med* 30: 533–543.
2. Monaghan et al. 2004. Preconception and prenatal cystic fibrosis carrier screening of African Americans reveals unanticipated frequencies for specific mutations. *Genet Med* 6(3):141–4.
3. Berwouts et al. 2008. Evaluation and Use of a Synthetic Quality Control Material, including in the European External Quality Assessment Scheme for Cystic Fibrosis. *Hum Mutat* 29(8): 1063-1070.
4. Pratt et al. 2009. Development of Genomic Reference Materials for Cystic Fibrosis Genetic Testing. *J Mol Diagn* 11:186-193.

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