Technical Validation of a Tm Biosciences Luminex-Based Multiplex Assay for Detecting the American College of Medical Genetics Recommended Cystic Fibrosis Mutation Panel

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The American College of Medical Genetics (ACMG) and the American College of Obstetrics and Gynecology have recommended population-based carrier screening for cystic fibrosis to include 23 mutations and 5 polymorphisms in the cystic fibrosis transmembrane regulator gene (CFTR). We estimate 20% of all pregnant women are being tested for their CF carrier status. We assessed two commercially available analyte-specific reagents (ASRs) capable of testing all 25 mutations of the original ACMG-recommended panel, Tag-It CFTR 40 + 4 Luminex-based reagent from Tm Biosciences, and our current assay platform, CF Genotyper V. 3.0 from Abbott/Celera. Blinded testing using genomic controls containing known CFTR mutations demonstrated that the Tag-It platform detected all mutations on the ACMG-recommended panel. We next performed a platform comparison with 1029 consecutive patient samples. There were no discrepant results in 1029 consecutive analyses between the two platforms, yielding an impressive figure of >25,000 individual genotypes without error for both platforms. In conclusion, both the Abbott/Celera ASR reagent and the Luminex-based Tag-It CF ASR reagent are appropriate for use in the clinical laboratory. (J Mol Diagn 2006, 8:371–375; DOI: 10.2353/jmoldx.2006.050115)

Cystic fibrosis (CF) is the most common genetic disease resulting in shortened life expectancy in Caucasians.¹ Classical CF is a multisystem disorder with recurrent pulmonary infections leading to pulmonary hypertension and respiratory failure, exocrine pancreatic insufficiency, congenital absence of the vas deferens in males, and excessive chloride secretions by the sweat glands.¹ Standard therapy consists of pulmonary therapy with in-

haled mucolytics combined with chest percussion and postural drainage, antibiotic therapy for the recurrent pneumonias, and oral digestive enzyme administration.² Despite these treatments, most CF patients experience recurrent illnesses and progressive disability. The average life expectancy in the United States is 35 years. Other than heart-lung transplant, there is no definitive therapy for CF.^{2,3}

In the United States, CF occurs with a frequency of 1 in 3600^{4,5} births to Caucasian couples. Approximately 1 in 28 Caucasians is a carrier for CF.⁶ Almost all cases of CF are caused by mutations in the *CF transmembrane regulator (CFTR)* gene. Thus far more than 1400 different CF mutations have been described. These mutations are curated in the CF mutation database maintained by Toronto Sick Children's Hospital.⁷ Many of these mutations occur in single families. Only a handful of mutations occur with a frequency of >1:20,000 individuals.⁷

In March 2001, the American College of Medical Genetics (ACMG) published guidelines for population-based screening for CF.⁵ Using pooled data, a criterion of any mutation occurring in >0.1% of CF chromosomes was used for inclusion in the recommended screening panel. On that basis, 25 mutations for the initial screening panel were chosen (see Table 1). Subsequently, the panel recommendations were modified because I148T, an original panel mutation, was determined to be a polymorphism and not a CF mutation based on its presence at a >150-fold incidence in a screened population than in CF patients.^{8–11} Another mutation, 1078delT, was found to be present in 0.06% of CF chromosomes and in only 1 in 55,867 screened individuals and was therefore also dropped from the recommended panel.^{12,13} Screening Caucasian individuals using the ACMG-recommended panel will identify approximately 88% of non-Ashkenazi Jewish (AJ) carriers and approximately 95% of AJ CF carriers.⁶

In September 2001, the American College of Obstetrics and Gynecology recommended that CF carrier detection be offered to all Caucasian couples (including

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Table 1. List of Mutations Detected by the Tm Tag-It 40 + 4 CF Reagent

Revised ACMG panel	Removed from original panel	Poly-morphisms in revised panel	Supplementary mutations in Tag-It
ΔF508	I148T	5/7/9T	394delTT*
ΔI507	1078delT	F508C	Y122X
G542X		I507V	R347H*
G85E		I506V	V520F*
R117H			A559T
W1282X			S549N*
621+1G>T			S549R (T>G)*
711+1G>T			1898+5G>T
N1303K			2183AA>G
R334W			2307insA
R347P			Y1092X
A455E			M1101K
1717-1G>A			S1255X
R560T			3876delA*
R553X			3905insT*
G551D			
1898+1G>A			
2184delA			
2789+5G>A			
3120+1G>A			
R1162X			
3659delC			
3849+10kbC>T			

*Mutations detected by the ABI Celera Analyte Specific Reagent.

Ashkenazi Jews) and that couples of other races be informed of the availability of CF carrier detection.⁵ Following that recommendation, the volume of screening tests soared.⁸ Therefore it was vital to have testing platforms capable of automated, high throughput, and accurate testing to accommodate the increased demand for CF carrier testing.

Initially, we evaluated three platforms for their ability to accurately detect CF carriers using a 1000-sample benchmark comparison. We found that all three platforms, the CF Gold Lipa strips (Roche Molecular Biochemicals, Pleasanton, CA), the oligonucleotide ligation assay (OLA) CF Genotyper Version 3.0 (Celera/Abbott, Abbott Park, IL), and a proprietary chip-based assay, were capable of accurately performing genotyping for the 25 mutations on the original ACMG panel without errors in 1000 sample comparisons.^{8,15} We are currently using the Abbott/Celera OLA ASR reagent in a laboratory-validated test for *CFTR* carrier detection.

Recently, the Luminex bead system has been used to develop reagents for multiplex molecular analyses. This study describes the technical validation of such a product, the Tag-It CF 40 + 4 ASR reagent, for its ability to detect the 25 *CFTR* mutations and 4 polymorphisms included in the original ACMG recommendation.

Materials and Methods

DNA Preparation

DNA was extracted on an automated Qiagen BioRobot M90 system (Qiagen Inc., Valencia, CA) according to

manufacturer's protocol. The DNA concentration ranged from 10 to 30 ng/μL. The DNA was diluted fivefold in molecular biology-grade water before genotyping. No DNA quantitation was performed for either platform.

TagIt *CFTR* 40 + 4 Assay

Tm Bioscience (Toronto, Canada) supplied the Tag-It *CFTR* 40 + 4 ASR reagents including PCR Primer Mix, Allele-Specific Primer Extension (ASPE) Mix, Bead Mix, and Wash Buffer. The Bead Mix contains 86 different bead types. Each bead type has a unique fluorescent signature for bead identification and an oligonucleotide "Tag" for specific ASPE product hybridization. Each ASPE primer is 5' tailed with a specific oligonucleotide ("anti-Tag") complementary to a specific oligonucleotide "Tag" on a particular colored bead. Platinum *Taq*DNA Polymerase for PCR, Platinum *GenoTYPE* Tsp DNA Polymerase for ASPE, and streptavidin-conjugated R-phycoerythrin reporter dye were purchased from Invitrogen (Carlsbad, CA). Shrimp alkaline phosphatase and exonuclease I were purchased from USB (Cleveland, OH). Genotyping procedures were performed according to the Tm Bioscience's recommended protocol. Briefly, for each sample, 2 μL of genomic DNA was amplified in a single-tube 16-plex PCR. The amplicon sizes range from 179 to 465 bp. The PCR products were treated with shrimp alkaline phosphatase to remove the 5' phosphates of any unincorporated nucleotides and exonuclease I to digest any unincorporated primers. A 2.5-μL aliquot of the treated PCR product was used in the ASPE reaction containing biotin-labeled dCTP and 86 primers containing sequences specific for each allele assayed and also a specific 3' tag sequence for subsequent bead attachment. The ASPE products were hybridized to the Bead Mix, followed by filtration to remove the unhybridized primers and free biotin-labeled dCTP. The bead-captured ASPE products were incubated with streptavidin-conjugated R-phycoerythrin reporter dye. Samples were read on the Luminex 100 xMAP Instrument (Austin, TX), and signal was generated for each of the 40 mutations and 4 variants and their corresponding wild-type alleles. For each sample, these fluorescence values were analyzed automatically by The Tag-It Data Analysis Software (Tm Biosciences) to determine whether the wild-type and/or mutant alleles for each of the variations had been detected.

OLA

The OLA assay was performed using the Abbott/Celera CF Genotyper V. 3.0 reagent as described previously.^{8,12} The number of samples tested and the number of times each sample was tested appear in Results.

Results

Table 1 contains a list of mutations detected by the *CFTR* 40 + 4 detection system. The 23 mutations and

Table 2. List of Mutant Genomic DNA Samples Used for Validation

G85E/WT	R117H/WT	I148T/WT	621+1G>T/WT
711+1G>T/WT	1078delT/WT	R334W/WT	R347P/WT
A455E/WT	Δ I507/WT	Δ F508/ Δ F508	1717-1G>A/WT
G542X/WT	G551D/WT	R553X/WT	R560T/WT
1898+1G>A/WT	2184delA/WT	2789+5G>A/WT	3120+1G>A/WT
R1162X/WT	3659delC/WT	3849+10kbC>T/WT	W1282X/WT
N1303K/WT	3905insT/WT	S549R/WT	V520F/WT
394delTT/WT	3876delA/WT	Δ F508/I507V	Δ F508/I506V
F508C/WT	Δ F508/WT		

four polymorphisms in the revised recommended ACMG panel are shown in columns 1 and 3. The two mutations in the initial ACMG panel that were removed in the second recommendation are in column 2. Column 4 contains mutations claimed to be detected by the Tag-It kit but are not part of the ACMG-recommended screening panel. The asterisked mutations in column 4 represent the additional mutations that are also present in the Abbott/Celera ASR reagent. There are no mutations detected by the Abbott/Celera OLA reagent that are not in the Tm Tag-It kit.

Initially, we created three 96-well "validation plates" that contained genomic DNA of known genotypes including homozygotes for common CF mutations, compound heterozygotes for common CF mutations, simple heterozygous carriers, control blanks, and interspersed wild-type samples. Table 2 is a list of the 33 different genomic samples contained in each plate. As specified in our validation protocol, two plates were analyzed by one individual operator, and the third plate was analyzed by a second operator. The operators were blinded to the identity of the samples until after the analysis was completed. The Tag-It system successfully identified the correct genotype in all samples. Because we did not have genomic controls for any of the mutations excluded from the ACMG-recommended panels, we could not assess the ability of the Tag-It system to detect those mutations in genomic DNA.

Because this is a qualitative assay, the usual measurements of within run and between run precision are not applicable. The same control samples run six times on the same plate all yielded the identical genotype for all alleles. In this manner, the within run precision could be considered 100%. However, the actual measurements of relative fluorescent units did vary slightly within the run. Because the final genotype for each allele is the result of a ratio measurement between the wild-type and mutant alleles, these slight variations had no effect on the genotyping results. Analogously, the between run precision was demonstrated by running the 96 samples of the validation plates three times over the period of 10 days. Once again, there were no discrepant genotyping results, although there was some variation in the actual relative fluorescent unit values.

Subsequently, we subjected 1029 DNA samples submitted for CF genotyping to the clinical laboratory for parallel analysis with our current assay, the Abbott/Celera OLA ASR reagent. The results were compared, and there were no discrepant results between the two platforms.

Table 3 is a compilation of detected genotypes for these 1029 samples. Four heterozygotes for I148T are included in Table 3 even though these results were not reported, because the table is illustrative of the technical accuracy of the assays and not their clinical relevance. The Tm ASR automatically reports the 5T/7T/9T status when R117H is present. Both patients with R117H in this series were negative for the 5T allele.

Assay Robustness

In addition to accuracy, an assay must be sufficiently robust to allow results to be determined in a timely fashion without excessive need to repeat analyses. Our laboratory has a threshold of 5% as a maximum acceptable assay failure rate.¹⁴ Table 3 shows a comparison of the two platforms in terms of assay failure rate, indicating acceptable robustness with only eight failures for the Tag-It system and six failures for the OLA system in the 1029 samples. Five samples failed in both methods, indicating that there was a problem with the input sample rather than with the assay itself. If these five failures are removed from the series, then the failure rates become three per 1000 for the Tag-It system and 1 per 1000 for the OLA system.

Automated Allele Assignment

Both the Tag-It and OLA systems have software to make provisional genotype assignments. These assignments

Table 3. Results of 1029 Consecutive Samples Analyzed by Tag-It and Abbott/Celera OLA

Genotype	No. of CF chromosomes detected*
WT/WT	993
Δ F508	20
R117H	2
G551D	2
G542X	2
Δ I507	1
R1162X	1
3849+10kbC>T	1
W1282X	1
N1303K	1
Δ F508/R117H	1
I148T*	4*

*I148T is a polymorphism and not a CF chromosome and has been eliminated from the recommended panel.

can be reviewed by directors for accuracy. In the event the software is unable to make an automated assignment, the analyses can be reviewed. Sometimes the reviewer can determine why the program was unable to complete the genotype assignment, and a manual assignment can be made. Table 3 shows that in only 10 cases were manual calls required in the Tag-It system; eight cases required manual calls in the OLA system. The approximately 99% automated call rate for both assays is quite acceptable.

Conclusions

Because clinical molecular genetic carrier testing often occurs in pregnant women, there is pressure on the laboratory to provide timely and accurate results. Because most individuals will have only a single test during their lifetime and vital reproductive decisions may be based on the results, a premium must be placed on accuracy. For that reason, we have implemented a 1000-sample benchmark test before the introduction of a new platform into our laboratory.¹⁴ Previously, the OLA ASR reagent had passed this benchmark test, and now the Tag-It CF 40 + 4 test has similarly flawless results. These data give the laboratorian confidence that either of these platforms can provide accurate results in a CF carrier detection program.

Both the OLA and Tag-It systems are amenable to 96-well microtiter plate formats and automation using automated liquid handling systems. In our laboratory, both platforms require no manual pipetting or loading steps, leading to a virtual elimination of human error in post-DNA preparative processes. The Tag-It system requires an approximately 7.5-hour processing time after PCR, so this can be accomplished in a single work shift. There are no manual steps involved. The Luminex 100 xMAP instrument has an autosampler and is capable of processing approximately 140 samples/hour. A more detailed description of the assay procedures has been previously published in an article regarding the Tm Biosciences Ashkenazi Jewish Analyte Specific Reagent.¹⁶

Luminex bead-based genotyping systems represent a promising new platform for multiplex mutation or single nucleotide polymorphism testing. Unlike the OLA system, which requires expensive instrumentation (automated DNA sequencing instruments) and sophisticated operators for maintenance, the Luminex instruments are relatively inexpensive and easy to operate and maintain. With 100 separate identifiable beads available, a theoretical maximum of 50 different mutations can be assayed simultaneously on current platforms. This number is more than sufficient for the recommended CF screening panels and testing panels for thrombophilia (factor V Leiden, factor II prothrombin, and methylene tetrahydrofolate reductase) and for the diseases prevalent among individuals of Ashkenazi Jewish descent.

This study demonstrates that both the Tag-It and the OLA systems are capable of accurate multiplex analyses

of the 25 mutations and four polymorphisms present in the original ACMG recommendation. In fact, the Tag-It kit has recently received Food and Drug Administration clearance as an *in vitro* diagnostic testing device. An impressive 25,000+ successive genotyping reactions were performed without error by each platform. These data allow the clinical laboratory a choice in platforms to use for CF testing.

Of note is that no additional mutations to the 23 that constitute the ACMG-recommended panel were detected in these 1029 patients. This is not unexpected because even 2184delA, which remains on the ACMG panel, was observed at a frequency of one in 23,943 patients in our laboratory.⁸ By definition, all of the additional mutations detected by the Tag-It system will be seen at an even lower frequency in a pan-ethnic U.S. population. Therefore, it is not surprising that no carriers for these rare mutations were detected in a series of 1029 patient samples. We have no data regarding the ethnicity of the 1029 patients. We are a national laboratory and would expect the ethnicities observed in our patient series to reflect the ethnicities in any U.S. pan-ethnic screening program. This also calls into question the utility of adding these rare mutations to screening panels aimed at the general American population.

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