A universal array-based multiplexed test for cystic fibrosis carrier screening

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Cystic fibrosis is a multisystem autosomal recessive disorder with high carrier frequencies in caucasians and significant, but lower, carrier frequencies in other ethnicities. Based on technology that allows high detection of mutations in caucasians and significant detection in other ethnic groups, the American College of Medical Genetics (ACMG) and American College of Obstetricians and Gynecologists (ACOG) have recommended pan-ethnic cystic fibrosis carrier screening for all reproductive couples. This paper discusses carrier screening using the Tag-It™ multiplex mutation platform and the Cystic Fibrosis Mutation Detection Kit. The Tag-It cystic fibrosis assay is a multiplexed genotyping assay that detects a panel of 40 cystic fibrosis transmembrane conductance regulator mutations including the 23 mutations recommended by the ACMG and ACOG for population screening. A total of 16 additional mutations detected by the Tag-It cystic fibrosis assay may also be common. The assay method is described in detail, and its performance in a genetics reference laboratory performing high-volume cystic fibrosis carrier screening is assessed.

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Cystic fibrosis (CF) is the most common autosomal recessive disorder in the caucasian population, with an incidence of approximately one in 3200 live births and a carrier frequency of one in 25 [1]. CF is also found at somewhat lower incidence in other ethnic groups [2].

CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Carrier screening for CF using molecular testing for mutations in the CFTR gene has been recommended by the American College of Medical Genetics (ACMG) and the American College of Obstetricians and Gynecologists (ACOG) [2,3,101]. CF carriers are asymptomatic but are heterozygous for a single pathogenic CFTR mutation.

The ACMG and the ACOG originally recommended a panel of 25 mutations for CF carrier screening, plus reflex testing of four variants [2]. This panel included all mutations that were then known to occur at or greater than a frequency of 0.1% of CF alleles in any US ethnic group, based on the CF Foundation Registry of over 15,000 affected patients. In 2004, a revised panel removed two mutations from the original list [3]. I148T was removed because population screening data demonstrated that I148T is a benign variant rather than a pathogenic mutation. 1078delT was removed because screening data demonstrated that its frequency, 0.03%, fell below a minimum threshold of 0.1%.

The publication of the original ACMG/ACOG guidelines in 2001 led to a dramatic increase in the number of CF mutation analyses performed in laboratories in the USA [4]. Increases in testing volumes necessitated that higher volume reference laboratories have access to high-throughput multiplex platforms with potential automation of liquid handling and allele-calling software. This article focuses on the experience of one reference laboratory using a high-throughput multiplex assay that is suitable for high-volume CF carrier screening. In 2003, the authors validated the Tm Bioscience (Ontario, Canada) CFTR 40+4 Mutation Detection

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Table 1. Cystic fibrosis molecular tests.

Company name	Screening or genotyping	Multiplexing	Discrimination of wild type from mutan
Tm Bioscience	Genotyping	Full	Enzymatically
Celera Diagnostics/Abbott	Genotyping	Full	Enzymatically
Roche Diagnostics	Genotyping	Full	Hybridization
Innogenetics	Genotyping	Full	Hybridization
Ambion Diagnostics	Genotyping	Full	Hybridization
Tepnel	Genotyping	Full	Enzymatically
Nanogen Technologics	Screening	Partial	Hybridization
Third Wave Technologies	Screening	Partial	Enzymatically

reagents, which were then classified as analyte-specific reagents (ASRs). In 2005, Tm Bioscience Corp. received 510(k) clearance from the US Food and Drug Administration (FDA) for this kit. Since one of the authors of this paper (Jean Amos) was an early adopter of this platform, and also collaborated in studies submitted to the FDA for clearance of this kit, the authors have summarized below their experience with this 40+4 assay, which is the first (and currently only) FDA-cleared CF genotyping kit.

Market profile

ASRs are the active ingredients of a laboratory-developed assay. They are designed to complement general reagents for a specific diagnostic application and are produced under good manufacturing practices. In response to the ACMG CF carrier screening recommendations, and prior to FDA clearance of the Tm Bioscience kit, several vendors had developed ASRs for mutation analysis. These different assay methods have b described elsewhere; they differ in several ways (TABLE 1) [First, tests differ in their genotyping chemistry by whether t discriminate wild-type from mutant alleles enzymatically or hybridization. ASRs commercialized by Celera Diagr tics/Abbott, Tepnel and Third Wave Technologies discrimir alleles enzymatically, as does Tm Bioscience's CF *in vitro* d nostic (IVD) kit. ASRs commercialized by Roche Diagnos Innogenetics, Ambion Diagnostics and Nanogen discrimir by hybridization.

The commercially available CF ASRs are all multiplexe the PCR step, and are either partially multiplexed or f multiplexed at the genotyping step. Assays that are f multiplexed at the genotyping step are able, in one run identify which mutations, if any, are present, and whether

Table 2. Mutations and variants detected by the cystic fibrosis transmembrane conductance regulator 40+4 assay.

Mutation	Mutation	Mutation	Mutatior
*ΔF508	*A455E	*3849+10kbC>T	2183AA>G
*ΔI507	*1717-1G>A	*W1282X	2307insA
*G542X	*R560T	*N1303K	Y1092X[§]
*G85E	*R553X	394delTT	M1101K
*R117H	*G551D	Y122X	S1255X
I148T	*1898+1G>A	R347H	3876delA
*621+1G>T	*2184delA	V520F	3905insT
*711+1G>T	*2789+5G>A	A559T	[†]5/7/9T
1078delT	*3120+1G>A	S549N	[†]F508C
*R334W	*R1162X	S549R (T→G)	[†]I507V
*R347P	*3659delC	1898+5G→T	[†]I506V

*Mutations recommended for testing by the American College of Medical Genetics (ACMG) and American College of Obstetricians and Gynecologists (ACOG).
[†]Variants recommended for testing by the ACMG and ACOG.
[§]There are two possible mutations assayed at the Y1092X locus: a cytosine-to-guanine change and a cytosine-to-adenine change at nucleotide 3408.

mutations are heterozygous or homozygous. Assays that are partially multiplexed at the genotyping step are screening tests that indicate the presence of a mutation in the first run and then reflex to additional testing to identify which mutation(s) is present and whether it is heterozygous or homozygous. ASRs commercialized by Celera Diagnostics/Abbott, Roche Diagnostics, Innogenetics, Ambion and Tepnel, as well as Tm Bioscience's CF IVD kit, are fully multiplexed genotyping tests. The Nanogen ASR is a screening test. Fully multiplexed genotyping tests may have an advantage over screening tests in that the final results for all samples can be obtained on the first run without requiring a reflex test. In addition, genotyping tests may have a shorter turnaround time (TAT) for mutation-positive samples. Lower TAT results reach patients more quickly, which is particularly important for pregnant women undergoing prenatal screening.

Currently, CF carrier screening is performed in large reference, niche genetics and academic laboratories in which a geneticist oversees testing. However, as the testing volume grows, the authors expect this test to migrate to routine and smaller reference laboratories in order to capture testing revenue.

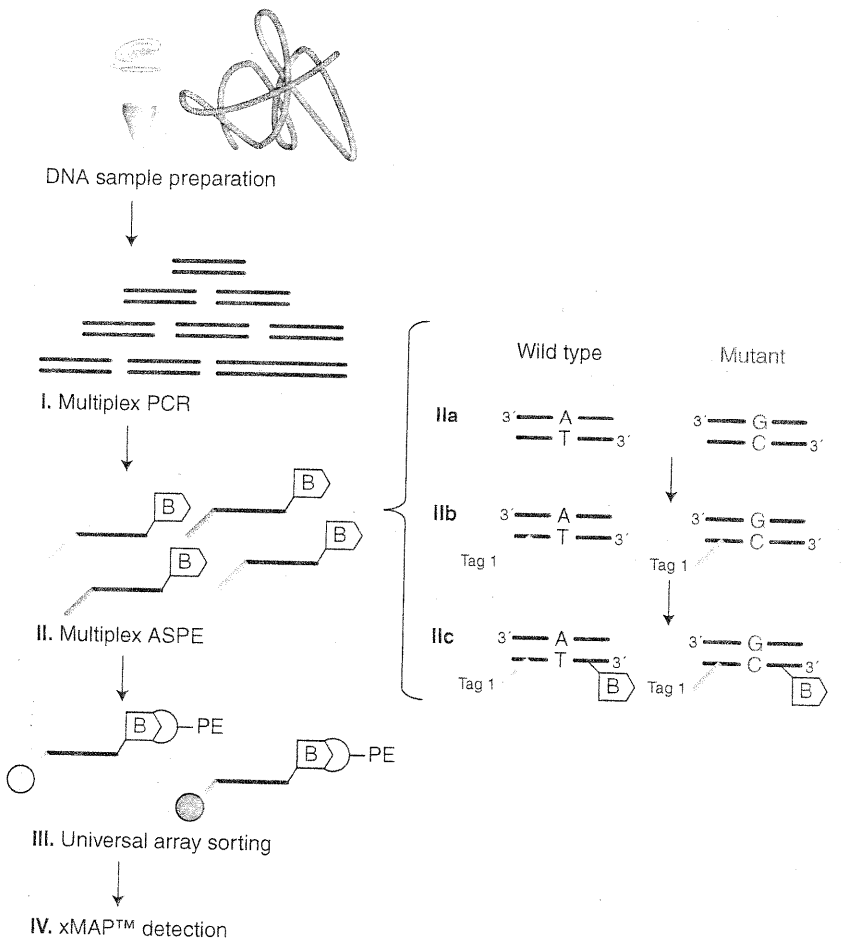


Figure 1. Schematic representation of the cystic fibrosis transmembrane conductance regulator 40+4 assay.
ASPE: Allele-specific primer extension; B: Biotin; PE: Primer extension.

Overview of content & test procedure

The Tag-It™ Cystic Fibrosis Kit produced by Tm Bioscience is used in a multiplexed genotyping assay capable of simultaneously determining the genotype at numerous loci in a single sample. The mutations assessed by the kit are described in TABLE 2. The panel includes the 23 mutations and reflex variants that currently meet the ACMG criteria for carrier screening. In addition, 15 mutations are included that were thought to be frequent, although the data may not be supportive of their use (see below).

The assay operates on a universal array platform, in which the gene-specific hybridization and discrimination process occurs separately from the array sorting. The Tag-It CF assay consists of four basic steps: multiplex PCR, multiplex allele-specific primer extension (ASPE), bead–array hybridization and Luminex xMAP™ detection and analysis (FIGURE 1). Laboratories may perform the assay over two shifts, performing DNA extraction, PCR and ASPE in the first and hybridization, detection and reporting in the second. A single operator can simultaneously handle up to four 96-well plates (almost 390 patient specimens).

After sample preparation of genomic DNA, the assay is performed using methods that have been described previously [7]. A multiplex PCR reaction is performed to amplify 16 exons in the CFTR gene. The PCR products are treated with shrimp alkaline phosphatase and exonuclease I (EXO/SAP) to inactivate any remaining nucleotides and to digest the primers. Multiplex ASPE is used to discriminate alleles. Each mutation locus has an allele-specific primer (ASP) for each of its alleles detected in the assay. For each ASP, the 3′ end of the primer is a perfect match for its allele, but will have a 3′ mismatch on any other allele of the locus. A DNA polymerase lacking 3′ exonuclease is used for extension so that this polymerase cannot extend mismatched 3′ ends. Therefore, an ASP is only extended if its target allele is present in the sample. Biotin–deoxycytidine triphosphate (dCTP) is incorporated into the extending chain if extension occurs.

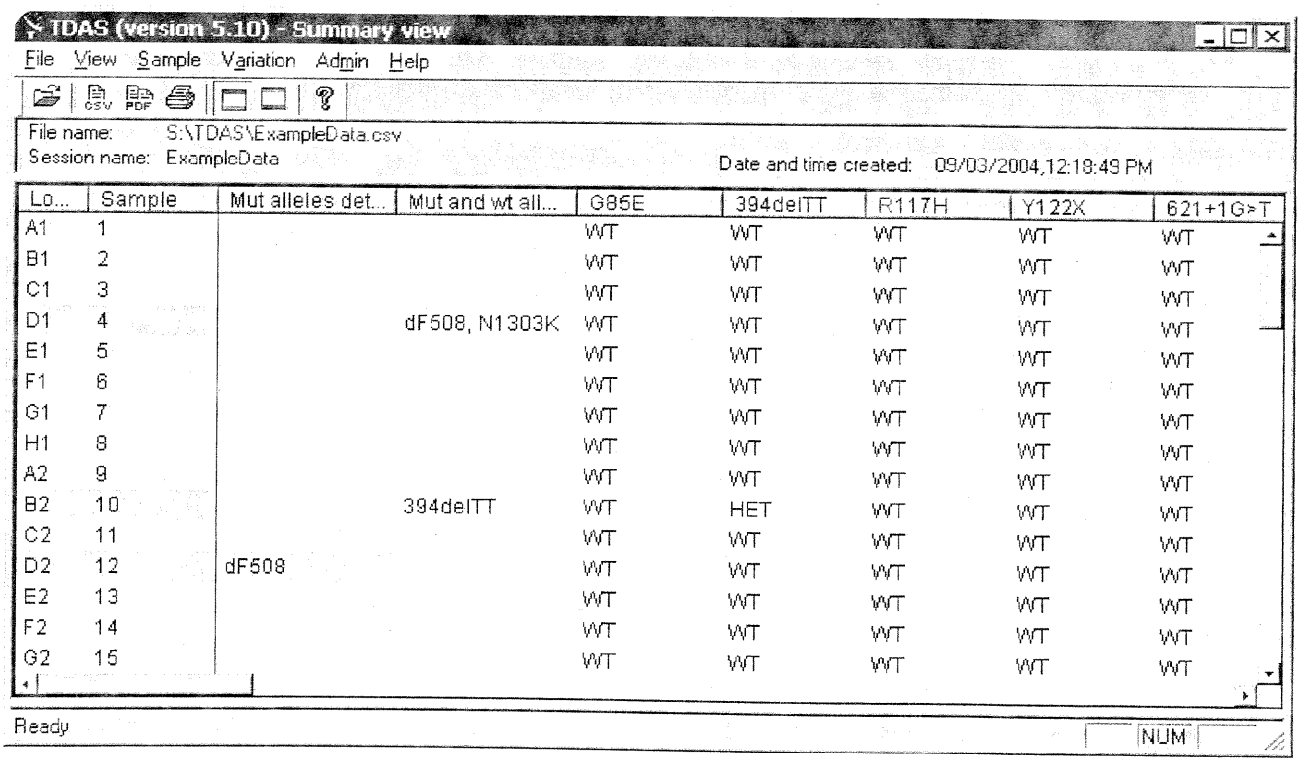
Each ASP has a unique 24-mer DNA tag at the 5′ end. These tags were designed to be different from each other and, due to their sequence content, are unlikely to hybridize to target

DNA. Furthermore, the tags were designed to hybridize to their antitag complements with no mismatch hybridization within the set of tag sequences under isothermal conditions [7]. The tags and antitags are used for array sorting of the ASPs after the ASPE reactions. The antitags are selectively coupled to microspheres (beads) that are categorized into up to 100 classes according to their spectral properties. Each antitag is associated with beads from one bead class. The liquid array sorting allows each ASP to become associated with a particular class of beads. This ultimately allows for the determination of which ASPs have extended.

The ASPE products are added to wells of a 96-well plate containing bead-immobilized antitags. A fluorescent reporter molecule (streptavidin–phycoerythrin) is bound to the biotin on the extended primers. Each tagged primer hybridizes only to its unique antitag complement; therefore, each bead class represents a specific allele, through the bead–antitag/tagged primer association.

The reaction is analyzed by the xMAP instrument developed by Luminex Corp. for bead class and associated hybridization signal intensity. Lasers interrogate hybridized microspheres individually as they pass single file in a rapidly flowing stream. According to the manufacturer, 250–1000 microspheres are interrogated per second, resulting in an analysis system capable of analyzing and reporting up to 100 different hybridization reactions in a single well of a 96-well plate in just a few moments.

The raw data file generated by the Luminex instrument is analyzed by the Tag-It Data Analysis Software (TDAS). The software analyzes all the samples in a batch and provides a summary report in the summary view screen (FIGURE 2). The summary view screen highlights any mutations detected in the sample, as well as showing the genotype calls for each mutation locus. The genotype calls are based on the mutant allelic ratios (ARs), which are calculated by dividing the net (total minus background) mean fluorescence intensity (MFI) for the mutant allele by the sum of the net MFIs for both alleles (wild-type plus mutant). The mutant AR must fall within predefined ranges for the genotype calls of 'WT' (only wild-type detected), 'HET' (both wild-type and mutant alleles detected) and 'MuD' (only mutant allele detected). Generally, these predefined ranges are approximately 0.00–0.15 for WT, 0.30–0.70 for HET and 0.70–1.00 for MuD. A MuD call is usually the result of a homozygous mutant allele, but it can also be the result of a heterozygous mutation combined with a polymorphism on the wild-type allele, which destabilizes the ASP on that allele (a rare event). 'MuD' calls for alleles other than ΔF508 can be analyzed by sequencing to confirm a homozygous mutant call. Alternatively, samples can be collected from the parents of the patient and tested in order to determine the haplotypes of the patient. In the current version of the FDA-cleared test, the I148T mutation is detected but not reported in the software.



Lo...	Sample	Mut alleles det...	Mut and wt all...	G85E	394delTT	R117H	Y122X	621+1G>T
A1	1			WT	WT	WT	WT	WT
B1	2			WT	WT	WT	WT	WT
C1	3			WT	WT	WT	WT	WT
D1	4		dF508, N1303K	WT	WT	WT	WT	WT
E1	5			WT	WT	WT	WT	WT
F1	6			WT	WT	WT	WT	WT
G1	7			WT	WT	WT	WT	WT
H1	8			WT	WT	WT	WT	WT
A2	9			WT	WT	WT	WT	WT
B2	10		394delTT	WT	HET	WT	WT	WT
C2	11			WT	WT	WT	WT	WT
D2	12	dF508		WT	WT	WT	WT	WT
E2	13			WT	WT	WT	WT	WT
F2	14			WT	WT	WT	WT	WT
G2	15			WT	WT	WT	WT	WT

Figure 2. Example Summary View Screen in the Tag-It Data Analysis Software. Samples where mutations were detected are highlighted in pink in the first two columns. Sample 4 is a compound heterozygote with the dF508 and N1303K mutations. Sample 10 is a carrier of the 394delTT mutation, and sample 12 is a probable dF508 homozygote. All other samples are wild type at all the loci tested.
HET: Both wild-type and mutant alleles detected; WT: Only wild-type detected.

Table 3. Detection of cystic fibrosis carriers in various ethnic groups.

Ethnic group	Total negative	Total heterozygous	Total screened	Frequency of carriers (%)
Non-Hispanic caucasian	14765	576	15341	3.8
Ashkenazi Jewish	73	7	80	8.8
Hispanic	3567	55	3622	1.5
African–American	2004	17	2021	0.8
Asian–American	849	5	849	0.6
Other*	781	8	789	1
Not provided	3410	143	3553	4
Total	*25449*	*811*	*26260*	*3.2*

*Other ethnicity/mix of more than one ethnicity.

In TDAS, all raw data for a given sample can be viewed by clicking on the sample identifier. All raw data for a given mutation can be viewed by clicking on the mutation identifier. Patient data and summary reports can be exported to a laboratory information system (LIS) if desired.

Cystic fibrosis carrier screening using the Tag-It cystic fibrosis assay

The Tag-It CF mutation detection reagents were used as ASRs in a laboratory-developed assay that was validated against another assay used in the authors' laboratory. These reagents are identical to those in the FDA-cleared Tag-It kit.

The Tag-It assay was used to analyze over 26,000 samples at Specialty Laboratories, Inc. for CF carrier screening during the period of December 2003 to March 2005. The expected carrier frequency of approximately 4% was seen in caucasians (TABLE 3). Although the I148T polymorphism was analyzed by the assay during most of this period, patients testing positive for this polymorphism are considered negative for CF carrier status, since I148T is not considered to be a pathogenic mutation [3]. Ethnicities were self-identified by patients. As expected, most of the identified CF carriers were self-identified as caucasians (71%).

Although the frequency of CF is highest in caucasians, it would be incorrect to assume that the testing population is overwhelmingly caucasian. In fact, the assumption that CF is solely a caucasian disease can cause delays in diagnosis that can have a negative effect on the medical outcomes of patients [8]. In these samples, approximately 30% of the CF testing referrals were from noncaucasian individuals. Approximately one in ten carriers detected were patients with noncaucasian ethnicities, indicating the importance of a pan-ethnic mutation panel. TABLE 4 presents the CFTR mutations detected in carriers. ΔF508 was the most frequent mutation, found in 70% of heterozygotes. The R117H-5T-negative complex allele was the next most frequent combination, at 10%. The detection of additional mutations outside of the 23 mutations recommended by the ACMG/ACOG was sporadic, with the exception of A559T. Three African–American carriers had the A559T mutation, and this mutation was only found in patients with this ethnicity. This mutation has been previously reported to occur at high frequency in African–Americans [9]; therefore, it may represent an important addition to screening panels to enhance the carrier detection rates in this minority group.

The use of enhanced mutation panels is controversial in some circles. The ACMG has recommended against their use, leading to potential confusion among physicians about whether mutations other than those in the minimal panel can even be considered pathogenic. It is important to note that, although some alleles first reported in patients have been found to be polymorphic, it is likely that most of the reported mutations do indeed cause disease and can be reported when detected in clinical samples without special reference. Indeed, many laboratories have developed in-house assays detecting 51–200 (or greater) mutations [10–12] and the increased sensitivity of these panels will become clearer over time. Certainly, although the inclusion of additional mutations in the Tag-It kit was justified in the FDA application and cleared without comment, the authors did not detect many additional carriers through their use. It is likely that increased mutation panels are more easily justified as a marketing device ('more is better') than by the existing data.

Expert commentary

The Tag-It CF assay is an accurate, high-throughput platform for CF carrier screening. It offers multiplexed genotyping capability and has been shown to accurately detect 40 CFTR mutations. Although multiple manipulations are required, the genotype for all the samples can be obtained in the first run. The TAT using the Tag-It assay can be as short as 24 h, from the receipt of a sample to an electronic written report. Furthermore, because the average signal-to-noise ratio is high (between 20:1 and 100:1), the repeat rate is less than 0.3%

Table 4. Cystic fibrosis transmembrane conductance regulator mutations detected in cystic fibrosis carriers.

Heterozygotes	Caucasian	Ashkenazi Jewish	Hispanic	African–American	Asian	Other*	Ethnicity not provided	Total	Frequency (%)
G85E	3	0	0	0	0	0	1	4	0.5
394delTT‡	1	0	0	0	0	0	0	1	0.1‡
R117H-5T Neg	64	0	4	0	0	0	11	79	9.9
R117H-5T Pos	4	0	0	0	0	0	4	8	1
Y122X‡	0	0	0	0	0	0	0	0	0‡
621(+1)G>T	9	0	0	0	0	0	0	9	1.1
711(+1)G>T	3	0	0	0	0	0	1	4	0.5
1078delT‡	0	0	0	0	0	0	0	0	0‡
R334W	1	0	1	0	0	0	0	2	0.2
R347H‡	2	0	0	0	0	0	0	2	0.2‡
R347P	3	0	0	0	0	0	0	3	0.4
A455E	4	0	0	0	0	0	1	5	0.6
ΔI507	3	0	0	0	0	0	0	3	0.4
ΔF508	411	6	35	10	2	6	100	570	70.3
V520F‡	0	0	0	0	0	0	0	0	0‡
1717(-1)G>A	8	0	0	0	0	0	2	10	1.2
G542X	13	0	4	0	0	0	5	22	2.7
S549N‡	0	0	0	0	0	0	0	0	0‡
S549R‡	0	0	0	0	0	0	1	1	0.1‡
G551D	8	0	0	0	0	1	3	12	1.5
R553X	7	0	1	2	1	0	1	12	1.5
A559T‡	0	0	0	3	0	0	0	3	0.4‡
R560T	2	0	0	0	0	0	1	3	0.4
1898(+1)G>A	0	0	1	0	0	0	1	2	0.2
1898(+5)G>T‡	0	0	1	0	1	0	1	3	0.4‡
2183delAA>G‡	0	0	0	0	0	0	0	0	0‡
2184delA	0	0	0	0	0	0	0	0	0
2307insA‡	0	0	0	0	0	0	0	0	0‡
2789(+5)G>A	5	0	0	0	0	0	2	7	0.9
3120+(1)G>A	0	0	2	2	0	0	3	7	0.9
Y1092X‡	0	0	0	0	0	0	0	0	0.9‡

*Other ethnicity/mix of more than one ethnicity.
‡These mutations highlighted are not included in the ACMG/ACOG panel.
ACMG: American College of Medical Genetics; ACOG: American College of Obstetricians and Gynecologists.

Table 4. Cystic fibrosis transmembrane conductance regulator mutations detected in cystic fibrosis carriers (cont.).

Heterozygotes	Caucasian	Ashkenazi Jewish	Hispanic	African–American	Asian	Other*	Ethnicity not provided	Total	Frequency (%)
R1162X	3	0	0	0	0	0	0	3	0.4
3659delC	3	0	0	0	1	0	0	4	0.5
3849(+10kb)C>T	2	0	6	0	0	0	1	9	1.1
3876delA†	0	0	0	0	0	0	0	0	0†
S1255X†	0	0	0	0	0	0	0	0	0†
3905insT†	3	0	0	0	0	0	0	3	0.4†
W1282X	7	1	0	0	0	0	2	10	1.2
N1303K	7	0	0	0	0	1	2	10	1.2
Total	*576*	*7*	*55*	*17*	*5*	*8*	*143*	*811*	
Frequency (%)	*71*	*0.9*	*6.8*	*2.1*	*0.6*	*0.9*	*17.6*		

*Other ethnicity/mix of more than one ethnicity.
†These mutations highlighted are not included in the ACMG/ACOG panel.
ACMG: American College of Medical Genetics; ACOG: American College of Obstetricians and Gynecologists.

(data not shown). Since most of the assay steps are basic liquid-handling processes, the assay is conducive to automation, which is in development. The TDAS software provides rapid and straightforward data analysis, a particularly useful feature since the results of one sample involve genotypes at many different loci.

An important feature of the Tag-It assay is flexibility. Since the array sorting is separate from the gene-specific hybridization component, additional mutations can be added to the test without the need to develop a new array. Similarly, mutations can be deleted when necessary without changing the array.

This flexibility, as well as the multiplexed genotyping capability of the Tag-It assay, could also be useful for the development of future tests on this platform. Molecular tests for many diseases could involve the analysis of multiple loci, whether it is a disease with many mutations in one gene (e.g., CF) or a multigenic or multifactorial disease (e.g., diabetes, asthma or heart disease). Multiplexed analysis will be necessary for efficient molecular testing for these diseases. Once a laboratory invests in a platform for CF testing, the same platform can be useful for other tests that are already commercially available or in the pipeline. Other assays that demand multiplex analysis are newborn screening panels and Ashkenazi Jewish genetic disease panels. The use of multiplex analysis goes beyond screening and testing for genetic diseases and could be used in other clinical areas, including microbial identification and respiratory or gastrointestinal viral panels.

Five-year view

Since publication of the 2001 guidelines, CF carrier screening has become more widespread, and the number of CF carrier tests performed will continue to grow. Identification of CF carriers could decrease the incidence of CF in the population and concomitantly increase the population carrier frequency, as has been the case for Tay–Sachs disease. Carrier screening for Tay–Sachs disease was implemented for Jewish couples in the 1970s, and the incidence of this disease has decreased by 90% [13]. A similar decrease in the incidence of CF can be expected, and the degree of the decrease will depend largely on what percentage of the population is screened and the clinical sensitivity of the CF tests.

The volume of CF testing is still expected to increase. Despite the new guidelines, it is estimated that only 20% of obstetricians in the USA are routinely referring patients for CF carrier screening [14,15]. In order to handle large volumes, laboratories that are currently using CF screening tests may switch to genotyping tests. Automation of testing will become commonplace.

In general, as a test becomes more commonly performed and more automated, larger numbers of hospital-based laboratories begin to perform the test in-house. In addition, routine, decentralized testing for CF may grow due to the availability of an FDA-cleared kit. However, internalization of CF carrier testing by hospitals also bears a responsibility for responsible reporting under the supervision of qualified clinical molecular geneticists. The ease of generating results thus becomes superseded by the complexity of interpretation.

CFTR mutation detection tests are the first example of what is likely to be an ever-increasing number of molecular tests that require high-throughput and multiplex analysis of multiple mutations. The current technologies set the stage for the evolution of the multiplex testing field, and a laboratory's choice of platform for CF testing will also likely influence how the laboratory performs other multiplex tests.

Key issues

- Cystic fibrosis (CF) is a severe autosomal recessive disease found in all ethnicities, but is especially prevalent in caucasians and Ashkenazi Jews.
- CF carrier screening has been recommended by the American College of Medical Genetics (ACMG) and the American College of Obstetricians and Gynecologists (ACOG).
- A panel of 23 mutations for CF carrier screening is recommended by the ACMG and the ACOG. These mutations occur at a frequency of at least 0.1% of CF alleles in the US population as a whole.
- The Tag-It™ CF assay is a multiplexed, US Food and Drug Administration-cleared genotyping assay that can be used for CF carrier screening. It tests for the 23 mutations recommended by the ACMG and ACOG, plus 16 other common mutations.
- The Tag-It assay can be used for high-throughput CF carrier screening because it is a fully multiplexed genotyping test with software that expedites data analysis. Furthermore, the assay could be automated for an additional increase in efficiency.
- The flexibility of the Tag-It platform could be advantageous in the evolution of CF molecular tests, offering either an expanded mutation panel or ethnicity-specific panels. In addition, the flexibility of the platform offers opportunities for use in molecular tests for other diseases.
- Decentralization of CF carrier screening will require novel approaches to responsible reporting.

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