Use of the MassARRAY® Mass Spectrometry Platform for High-Throughput and Accurate Detection of a 105 Member Cystic Fibrosis-Causing Mutation Panel

J Stoerker[1], J Tynan[2], L Cagasan[2], R McCullough[2], T Goodman[1], H Walline[1], C-R Vibat[3], D van den Boom[3], R Mueller[2], DH Farkas[1]

[1] Sequenom Center for Molecular Medicine (SCMM), 301 Michigan St. NE, Grand Rapids, MI. | [2] SCMM, 3595 John Hopkins Court, San Diego, CA. | [3] Sequenom, 3595 John Hopkins Court, San Diego, CA.

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Abstract

Introduction: Cystic fibrosis (CF) is the most common life-limiting, autosomal recessive disease among people of European heritage. CF affects the exocrine glands of the lungs, liver, pancreas, and intestines, causing progressive disability due to multisystem failure. CF is caused by one or more mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. More than a thousand mutations are known; 23 are recommended for screening and some labs analyze many more than that.

Methods: We developed a high throughput, multiplexed, 105 member CFTR mutation panel based on detection with the Sequenom MassARRAY system. The test was optimized using control DNAs with known CFTR genotypes. The test was verified by analyzing a "Challenge Panel" of 96 blinded DNA samples provided by the Mayo Clinic from CF patients with previously characterized mutations. Further verification was done using genomic and synthetic DNA controls that contained all of the mutations in the panel.

Results: The "Challenge Panel" contained 39 of the 105 mutations in the panel. Mutations were identified in all but one CF mutation-positive sample using the MassARRAY test (that one sample was inadequate for assay). There were no false-positive results in the no-mutation control samples. Several mutations in the "Challenge Panel" were near neighbors of panel mutations; all were genotyped correctly thus demonstrating the assay's specificity.

Conclusion: The high throughput nature of the MassARRAY platform enables analysis of up to 700 samples per shift on one system. Thus, this MassARRAY-based CFTR test is ideal for clinical laboratory-based screening of a large panel of CF-causing mutations by providing accessible, cost-effective and accurate testing.

Introduction

Cystic fibrosis is an autosomal recessive disease caused by cystic fibrosis transmembrane regulator (CFTR) gene mutations. Over 1,500 mutations, most with a <0.1% population frequency, have been described since the discovery of the ΔF508 mutation in 1989.[1, 2] Professional groups recommend carrier screening using a core panel of 23 mutations which generates various coverages based on ethnicity. Often it is not straightforward to assign a single ethnicity to an individual, thus expanded CF mutation panels may provide greater pan-ethnic detection rates. We were interested in offering a laboratory developed test (LDT) for enhanced CFTR mutation screening and present here one element of the implementation of such a test: verification (with feasibility, optimization, verification and validation being the full range of necessary steps for implementation of an LDT).

Using a "challenge panel" described below, we verified an enhanced CFTR mutation screening panel that was based on multiplex PCR and mutation-specific single base extension (SBE) reactions to distinguish wild type and mutant alleles. Matrix Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) mass spectrometry was used to detect the resultant extension products.

We report here a facet of the verification phase for an LDT consistent with one recently proposed by the College of American Pathologists,3 an enhanced mutation panel for CF screening, and the suitability of mass spectrometry as a platform for multiplex detection and clinical laboratory developed tests. The successful verification allowed progression to the validation phase (to be reported separately) and ultimately the availability of an LDT for 103 mutations and five variants in the CFTR gene (numbers slightly modified during Validation from the numbers provided at the time of submission of this abstract).

Materials and Methods

Challenge Panel

A "Challenge Panel" (CP) was obtained from colleagues at the Mayo Clinic.[4] The panel is comprised of multiple examples of each of the 23 mutations and "reflex" targets included in the ACMG/ACOG panel (at least 3 examples of each) and the additional 15 mutations comprising the Tm-Bioscience CFTR 40+4 Tag-It® Mutation Detection Kit (Toronto, ON). In addition, the CP includes several samples with mutations that could potentially interfere with the detection of mutations in the MassARRAY panel, e.g., 711+3A>G, R75Q, R334Q, and many but not all of the variations included in the Tm-Bioscience CF panel.

Samples bearing common and rare CFTR mutations were collected and de-identified according to the provisions of 45CFR46. The samples were subjected to whole genome amplification (WGA) using the Repli-G® kit from Qiagen, Inc. (Valencia, CA). All samples were diluted to 50ng/µL.

MassARRAY Mass Spectrometry CFTR Genotyping Assay

Instrumentation:
- MassARRAY Analyzer, Sequenom, Inc. (San Diego, CA)
- MassARRAY Nanodispenser RS1000, Sequenom, Inc.

Software:
- MassARRAY Analyzer Workstation, Sequenom, Inc.

Specific Reagents:
- PCR primers, IDT, Inc. (Coralville, IA)
- Extend primers, IDT, Inc.

A list of the CFTR mutations and variants in the MassARRAY CFTR Genotyping Panel is shown in Table 1. A diagram of the CFTR MassARRAY Genotyping Assay is shown in Figure 1.

Figure 1. *Diagram of MALDI-TOF MS CFTR Genotyping Assay (post DNA extraction)*

1. *PCR: Multiplex PCR with CFTR specific amplification (8 multiplexed groups)*
2. *Shrimp Alkaline Phosphate (SAP) dephosphorylation of unincorporated dNTPs*
3. *Single Base Extension: single base extension primer; Thermo Sequenase™; A,C,G, T terminators*
4. *Example assay for ΔF508:*

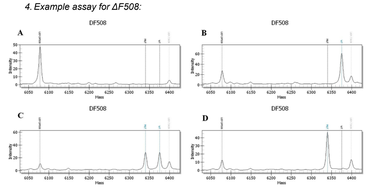
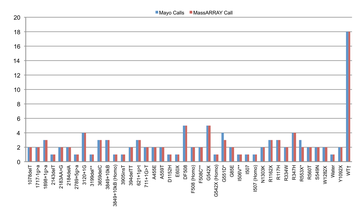


Figure 1*. Mass spectra of ΔF508 assay.* ***(A)*** *"No amplification" control shows no extension of the extension primer to the expected Wild Type (WT) or mutant (MUT) positions.* ***(B)*** *An example of a specimen WT for the ΔF508 mutation; only the WT base "A" was added resulting in the peak labeled WT.* ***(C)*** *An example of a specimen heterozygous for ΔF508; both the WT base, "A," and mutant base, "T," were added to the extension primer.* ***(D)*** *An example of a specimen homozygous mutant for ΔF508; only the mutant base "T" was added to the extension primer. UEP, unextended primer.*

Figure 2: *Concordance by mutation and variant.*



** A compound heterozygous sample from the CP (R553X; G551D) was inadequate therefore the SCMM was unable to generate a genotype.*

† Samples genotyped as wild type by the MassARRAY test may contain mutations/variants not interrogated by the panel: R75Q, V520I, F994C, G662D, I1234V, D110H, G576A, 711+3A>G, 2789+2insA, R1162Q, M348V, 3850-1G>A, I148T, R334Q, S1235R, D433Y, D1305E, D1270N, Rl47W, and R347S.

Table 1*. Mutations in MassARRAY CFTR Genotyping Panel and Nucleotide Changes Detected*

mutation name	Nucleotide change
712-1G>T	G to T at 712-1
Y1092X(C>A-G)	C to G or A at 3408
G330X	G to T at 1120
S1196X	C to G at 3719
DF508-I506V#1	A or G at 1648
2307insA	insertion of A after 2307
R75X-Q#1	C to T at 355
406-1G>A	G to A at 406- 1
457TAT>G	TAT to G at 457
S1251N	G to A 3884
2043delG	deletion of G at 2043
1949del84	deletion of 84 bp from 1949
3171delC	deletion of C at 3171
R1158X	C to T at 3604
P574H	C to A at 1853
Q552X	C to T at 1786
N1303K (C>A-G)	C to G or A at 4041
621+1G>T	G to T at 621+ 1
2869insG	insertion of G after 2869
R352Q	G to A at 1187
S549N-R(T>G)#1	T to G at 1779
Q1238X	C to T at 3844
S549N-R(T>G)#2	T to G at 1779
L206W	T to G at 749
2183delAA>G	deletion of AA at 2183
Y122X	T to A at 498
Q493X	C to T at 1609
E92X	G to T at 406
2108delA	deletion of A at 2108
1677delTA	deletion of TA from 1677
S1255X	C to A at 3896 and A to G at 3739
936delTA	deletion of TA from 936
D1152H	G to C at 3586
3659delC	deletion of C at 3659

mutation name	Nucleotide change
1288insTA	Insertion of TA at 1285 Or Insertion of AT at 1284
R764X	C to T at 2422
A455E	C to A at 1496
852del22	deletion of 22 bp from 852
W1282X	G to A at 3978
2789+5G>A	G to A at 2789+ 5
Q890X	C to T at 2800
P205S	C to T at 745
CFTRdele2,3	deletion of exons 2 and 3
R553X	C to T at 1789
3876delA	deletion of A at 3876
DI507-I507V	deletion of 3 bp between 1648 and 1653
711+5G>A	G to A at 711+ 5
2055del9>A	deletion of 9 bp CTCAAAACT to A at 2055
2184delA	deletion of A at 2184
1078delT	deletion of T at 1078
663delT	deletion of T at 663
R1162X	C to T at 3616
3199del6	deletion of ATAGTG from 3199
3849+10kbC>T	C to T in a 6.2 kb EcoRI fragment 10 kb from 19
1717-1G>A	G to A at 1717- 1
G178R	G to A at 664
E60X	G to T at 310
444delA	deletion of A at 444
C524X	C to A at 1704
R334W	C to T at 1132
R560T	G to C at 1811
3791delC	deletion of C at 3791
1898+1G->A-T-C	G to A T or C at 1898+ 1
G480C	G to T at 1570
935delA	deletion of A at 935
405+1G>A	G to A at 405+ 1
711+1G>T	G to T at 711+ 1
3120+1G>A	G to A at 3120+ 1

mutation name	Nucleotide change
DF311	deletion of 3 bp between 1059 and 1069
T338I	C to T at 1145
R117C-H#1	C to T at 481
1812-1G>A	G to A at 1812- 1
I506T-F508C#1	T to G at 1655
3667del4	deletion of 4 bp from 3667
I506T-F508C#2	T to G at 1655
G551D	G to A at 1784
K710X	A to T at 2260
296(+2)T>A	T to A at 296+ 2
W1089X	G to A at 3398
R117C-H#2	C to T at 481
G85E	G to A at 386
Q359K	C to A at 1207 and C to A at 1211
2143delT	deletion of T at 2143
W1204Xv2	G to A at 3743
574delA	deletion of A at 574
T360K	C to A at 1207 and C to A at 1211
DF508-I506V#2	A or G at 1648
R709X	C to T at 2257
A559T	G to A at 1807
394delTT	deletion of TT from 394
R347P/H	G to A at 1172
CFTR_WT_E3	wt
H199Y	C to T at 727
G542X	G to T at 1756
CFTR_WT_E2	wt
L997F	G or C at 3123
M1101K/R	T to G or A at 3434
1898+5G>T	G to T at 1898+ 5
405+3A>C	A to C at 405+ 3
2184insA	insertion of A after 2184
3905insT	insertion of T after 3905
4016insT	insertion of T at 4016

Table 2: *Concordance between MassARRAY-generated genotype and Challenge Panel genotype*

Identifier	Allele 1	Allele 2	Genotype
1	2184delA	wt	2184delA/WT
2	R560T	wt	R560T/WT
3	wt	wt	WT/WT
4	I507	wt	I507V
5	DF508	F508C	DF508ΔF508C
6	W1282X	G542X	W1282X/G542X
7	R1162X	wt	R1162X/WT
8	2143delT	wt	2143delT/WT
9	D1152H	wt	D1152H/WT
10	G542X	wt	G542X/WT
11	1078delT	wt	1078delT/WT
12	A559T	G551D	G551D/A559T
13	wt	wt	WT/WT
14	711+3a>g	wt	WT/WT
15	3849+10	3849+10	3849+10kB/3849+10kB
16	I506V	wt	I506V/WT
17	N1303K	wt	N1303K/WT
18	2789+2insA	wt	WT/WT
19	R553X	wt	R553X/WT
20	wt	wt	WT/WT
21	3905insT	wt	3905insT/WT
22	DF508	R347P	DF508/R347P
23	3659delC	wt	3659delC/WT
24	R1162Q	wt	WT/WT
25	M348V	wt	WT/WT
26	R334W	wt	R334W/WT
27	3120+1g>a	wt	3120+1G>A/WT
28	I148T	wt	WT/WT
29	1717-1g>a	3850-1g>a	1717-1G>A/WT
30	wt	wt	WT/WT
31	1898+1g>a	wt	1898+1G/WT
32	wt	wt	WT/WT
33	G542X	G542X	G542X/G542X

Identifier	Allele 1	Allele 2	Genotype
34	3659delC	R117H	3659delC/R117H
35	3849+10	W1282X	W1282X/3849+10
36	711+1g>t	wt	711+1G>T/WT
37	A455E	wt	A455E/WT
38	DF508	R334Q	DF508/WT
39	wt	wt	WT/WT
40	1717-1g>a	R553X	R553X/1717
41	A455E	wt	A455E/WT
42	R334W	R117H	R334W/R117H
43	R1162X	wt	R1162X/WT
44	DF508	DF508	DF508/DF508
45	621+3a>g	wt	WT/WT
46	R553X	G551D	INADEQUATE
47	2183AA>G	wt	2183delAA>G/WT
48	I148T	wt	WT/WT
49	R347P	G542X	G542X/R347P
50	394delTT	wt	394delTT/WT
51	3120+1g>a	wt	3120+1G/WT
52	N1303K	S1235R	N1303K/WT
53	R75Q	wt	WT/WT
54	I507	I507	DI507/DI507
55	711+1g>t	wt	711+1G/WT
56	1898+1g>a	wt	1898+1G/WT
57	DF508	2789+5g>a	2769/DF508
58	R560T	wt	R560T/WT
59	R347H	wt	D1152H
60	G542X	621+1g>t	621+1G/G542X
61	G551D	wt	G551D/WT
62	621+1g>t	wt	621+1G/WT
63	R1162X	3120+1g>a	3120/R1162X
64	G85E	wt	G85E/WT
65	1898+1g>a	wt	1898+1G/WT
66	2184delA	wt	2184delA/WT

Identifier	Allele 1	Allele 2	Genotype
67	1078delT	wt	1078delT/WT
68	3849+10kb	wt	3849+10kB/WT
69	DF508	3659delC	3659/DF508
70	V520I	wt	WT/WT
71	3120+1g>a	wt	3120/WT
72	Y1092X	wt	Y1092X/WT
73	F994C	wt	WT/WT
74	G576A	wt	WT/WT
75	S549N	G551D	S549N/G551D
76	Y1092X	wt	Y1092X/WT
77	621+1g>t	wt	621+1G/WT
78	2183AA>G	wt	2183delAA/WT
79	G85E	wt	G85E/WT
80	3840+10kb	wt	3849+10kB/WT
81	394delTT	wt	394delTT/WT
82	G662D	wt	WT/WT
83	S549N	F508C	S549N/F508C
84	I1234V	I1234V	WT/WT
85	I148T (3199del6)	D110H	3199del6/WT
86	G576A	D443Y	WT/WT
87	R347H	wt	R347P/WT
88	D1305E	wt	WT/WT
89	R117H	wt	R117H/WT
90	A559T	wt	A559T/WT
91	DF508	DF508	DF508/DF508
92	R347W	D1270N	WT/WT
93	DF508	D1270N	DF508/WT
94	E60X	wt	E60X/WT
95	R347S	wt	WT/WT
96 (NTC)	N/A	N/A	INADEQUATE

Black = mMutations common to both panels Blue = variants
Red = potentially interfering alterations NTC = "no template control"

Results

Comparison of MassARRAY CFTR Genotyping Results with Challenge Panel Genotypes

There were 96 samples present committed to testing. One of the samples, #96, was a "no template control" and was correctly identified as inadequate for study due to the absence of interpretable mass spectra. MassARRAY-generated genotyping results are shown in Figure 2 and Table 2. One sample, #46, was also identified by MassARRAY Mass Spectrometry CFTR Genotyping as inadequate (the expected genotype was a compound heterozygote of R553X and G551D alleles). For the remaining 94 samples, definitive genotyping results were generated by the MassARRAY CFTR System and there were no discrepancies with expected results of the CP.

Of the mutations and variants present in the CP, 36 were also in the MassARRAY genotyping panel and all 36 were detected (Figure 2). Figure 2 also shows the agreement between the expected findings and the MassARRAY data.

Table 2 shows the concordance between the MassARRAY-generated data and the CP genotypes. Concordance for specimens of adequate quality was 100%.

Even though some of the mutations in the CP are closely related by position and nature of the mutation to those in the MassARRAY panel, none induced an incorrect allele assignment using the MassARRAY method. For example, R75Q in sample #53, 711+3A>G in sample#14 and R1162Q in sample #24 showed no tendency to cause false positives for R75X, 711+1G>T and R1162X, respectively.

Discussion

Laboratory developed tests (LDT) may be offered by clinical laboratories to the clinical community only after rigorous feasibility, optimization, verification and validation. We were interested in development of an LDT with a relatively high mutation density to enhance the pan-ethnic coverage for CF carrier screening. Extensive assay design was performed during the feasibility and optimization phases to perfect the test. Once optimized, the test was confirmed as "working" after undergoing a Verification phase, presented in this study. A mass spectrometry-based MassARRAY panel consisting of 105 CFTR mutations and variants, depending on multiplex PCR and single base extension reactions, was used to confirm CFTR genotypes of specimens provided in a "Challenge Panel." While it is arguable if the verification phase must be undertaken with "blinded" specimens, their use in this study added rigor to the process and confidence in our design.

Results, as summarized in Table 2 and Figure 2, were excellent showing high concordance between our nascent test and the challenge panel. These findings demonstrated that this now optimized and verified test could be moved to the next phase of the process: Validation (manuscript in preparation).

Last, this study demonstrated the suitability of mass spectrometry for use in LDTs requiring medium target density. High density arrays are highly suitable for biomarker discovery while lower density platforms are well matched to detection of small numbers of interesting clinical targets. "Mid-level" density panels are relevant in applications such as CF screening, oncology, pharmacogenetics, etc. and there are numerous examples.[5-9] Mass spec-based detection is a vehicle in which large numbers of different species, e.g., mutant vs. normal CF alleles; oncogene panels, pharmacogenetic targets etc., can be resolved. Panel "customization" occurs prior to detection via biochemical manipulation of target nucleic acids for interrogation, thus providing flexibility for the ultimate offering by clinical laboratories of LDTs.

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