P-033 A performance evaluation of the iPLEX Pro *CFTR* assay using dried blood spots from the CDC cystic fibrosis repository

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Background

For newborn screening of cystic fibrosis (CF) there are several different assays that can be used for second-tier molecular screening of infants with elevated immunoreactive trypsinogen (IRT). The Illinois Department of Public Health (IDHP)[1] is using the iPLEX Pro *CFTR* Panel, which uses MassARRAY technology to interrogate 74 variants in the cystic fibrosis transmembrane receptor gene (*CFTR)* , including the 23 that are recommended by ACMG/ACOG[2]. As a service to state public health laboratories like IDPH, the CDC provides dried blood spots (DBS) for proficiency testing (PT) of assays used in newborn screening. In order for these PT materials to be universally valuable, CDC tries to ensure that they work well and provide expected results with assays used by newborn screening programs. The iPLEX Pro was tested with panel variants available in CDC repository samples.

Project Objective

To assess the accuracy and reliability of *CFTR* genotyping when using the iPLEX Pro *CFTR* assay on DBS from the CDC cystic fibrosis dried blood spot repository and DNA extraction methods used by at least one newborn screening laboratory.

Methods

Sample preparation

- CDC's cystic fibrosis dried blood spot (DBS) repository is composed of whole blood collected from consented patients with cystic fibrosis and carrier parent(s). All samples were spotted onto filter paper, dried and stored with desiccant at -20° C.
- A 3.2 mm size punch was removed from each of 88 repository DBS and placed into wells of a 96-well microplate for DNA extraction. Punches removed from a single DBS were used for each of three different extraction methods.
- Each of the extraction methods included washing the DBS with buffer (as specified) and eluting the DNA in buffer or water by heating at 99°C or 99.5°C for 30 minutes.

  Method 1 (Extracta DBS): Single wash and elution using Extracta DBS buffer
  Method 2 (Qiagen S2): Single wash and elution using Qiagen S2 buffer
  Method 3 (Illinois Method): Single wash with PBS/KOH buffer and water elution
- CF Panel III Controls (Cat. # G115; Maine Molecular Quality Controls, Inc. (MMQCI)) were used as a source of synthetic *CFTR* DNA for variants unavailable in the CDC repository. DNA was extracted from 200µl of each control set (a, b, c and d) using the QIAamp DNA mini kit (Cat. # 51306; QIAGEN, Inc.).

Genotyping[3]

- All DNA samples were processed using the iPLEX genotyping assay chemistry (according to manufacturer protocol). Completed reactions were spotted onto a 96 format SpectroCHIP using the MassARRAY Chip Prep Module (CPM) and analyzed using the MassARRAY Analyzer. The Typer 4 software (v.4.1.83) was used to make genotype calls. Below are the steps of the genotyping process[4]:

  Step 1: PCR setup (requires 3 multiplex reactions for each sample)
  Step 2: Shrimp alkaline phosphatase (SAP) treatment of PCR to dephosphorylate unincorporated dNTPs
  Step 3: Single base extension reaction
  Step 4: Sample clean-up and chip spotting using CPM
  Step 5: MALDI-TOF mass spectrometry
  8 hours to complete protocol and obtain results

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Results

70 of the 74 *CFTR* Variants included on the iPLEX Pro Cystic Fibrosis Panel were tested using CDC CF DBS samples and MMQCI synthetic controls

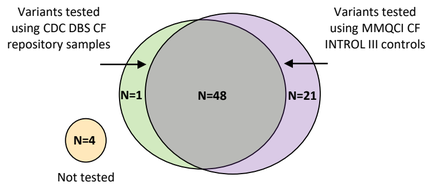


Figure 1. 70 of the 74 iPLEX Pro *CFTR* panel variants were tested for genotype accuracy. Collectively, samples within the CDC DBS CF repository contain 66% (49/74) of the iPLEX *CFTR* panel variants. MMQCI synthetic DNA controls were also used to include testing of an addition 28% (21/74) of variants. 4 of the 74 variants were not tested (6%).

Multiplex Panel 1 (24 Variants)		Multiplex Panel 2 (27 Variants)			Multiplex Panel 3 (23 Variants)	
G542X (N=4)	3905insT (N=2)	**W1282X (N=5)**	935delA (N=1)	1078delT	**F508del (N=31)**	2055del9->A (N=2)
R117H (N=4)	1677delTA (N=2)	**R1162X (N=1)**	CFTRdele2,3 3' (N=2)	G551S	**711+1G->T (N=1)**	F508C (N=1)
I507del (N=2)	2183AA->G (N=2)	**A455E (N=2)**	R1158X (N=1)	G1349D	**R334W (N=3)**	A559T (N=2)
S549N (N=2)	3876delA (N=3)	**N1303K (N=4)**	Poly T T5/T7 (N=88)[a]		**R347P (N=2)**	CFTRdele2,3 5' (N=2)
3791delC (N=1)	2307insA (N=1)	**G551D (N=6)**	Y122X		**2184delA (N=1)**	Poly T T7/T9 (N=88)[a]
E60X (N=2)	3199del6	**R553X (N=2)**	G178R		**G85E (N=1)**	G1244E (N=1)
R75X (N=3)	S549R_1645A->C	**2789+5G->A (N=3)**	I506V		**3120+1G->A (N=1)**	S549R-1647T->G
406-1G->A (N=1)	K710X	**3849+10kb C->T (N=3)**	M1101K		**621+1G->T (N=2)**	R347H
Q890X (N=2)	G330X	**1717-1G->A (N=3)**	R117C		**3659delC (N=2)**	V520F
W1089X (N=2)	S1196X	**R560T**	S1255P		**1898+1G-> A (N=2)**	S1251N
D1152H (N=1)	2143delT	L206W (N=1)	S1255X		394delTT (N=1)	R1162Q
R1066C (N=1)	I507V	Y1092X (N=2)	1898+5G->T		Q493X (N=2)	R560K

[a] These two assays contribute to genotyping of a single variant

Table 1. Genotyping of all 74 *CFTR* variants on the iPLEX panel requires three multiplex reactions for each sample. Colors denote whether a variant is present among the CDC samples only (green), both the CDC and MMCQI controls (gray), MMCQI controls only (purple) or not present in available samples (yellow). The number of alleles genotyped for each of the variants (in gray or green) is provided in parenthesis. Only 1 allele was genotyped for variants in purple. ACMG/ACOG recommended variants are shown in bold.

After applying user calls, the panel call rates for DBS DNA samples extracted using any of the methods was ≥ 99.9%

	After Automatic Software Calls		After Manual Calls by User		
	% Call Rate of Panel	(# uncalled / # possible calls)	% Call Rate of Panel	(# uncalled / # possible calls)	
Extracta DBS	99.5	10 / 2024	99.9	2 / 2024	Plex 1
Qiagen S2	99.4	12 / 2001	99.9	3 / 2001	
Illinois Method	99.9	4 / 3312	100.0	0 / 3312	
Extracta DBS	99.4	12 / 2133	99.9	2 / 2133	Plex 2
Qiagen S2	99.4	15 / 2376	99.9	3 / 2376	
Illinois Method	99.3	28 / 3888	100.0	0/ 3888	
Extracta DBS	99.9	1 / 2024	100.0	0 / 2024	Plex 3
Qiagen S2	99.9	2 / 1978	99.9	2 / 1978	
Illinois Method	100.0	0 / 3312	No manual calls required		

	Avg. % Call Rate	Avg. % Call Rate
Extracta DBS	99.6	99.9
Qiagen S2	99.6	99.9
Illinois Method	99.7	100.0

Table 2. Shown here are the % call rates ((# called variants x # samples)/ (# total variants x # samples)) for each of the 3 multiplex panels (Plex 1, 2, 3) after automatic software calls and manual user calls. If a call was not possible after manual analysis the samples was repeated. Upon repeat, all uncalled variants resolved. The average % call rates for all 3 plex panels are provided for each of the DNA extraction methods.

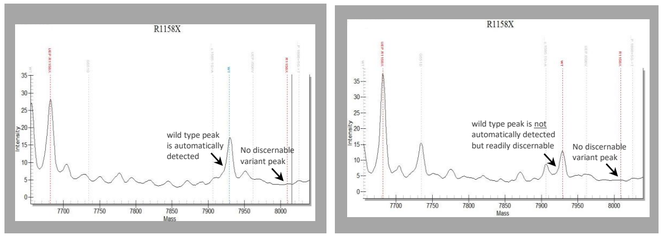
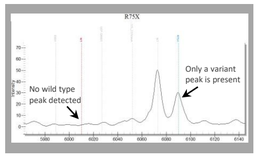


Figure 2. The R1158X *CFTR* variant contributed to a number of uncalled genotypes by the Typer 4 software. Shown is an example of data that resulted in an automatic genotype call (left panel) and a no call (right panel). Because there is a clear wild type peak and no variant peak for the no call sample (left panel) , a manual call can be confidently assigned for this sample and several others like it.

Among the 237 *CFTR* variant alleles tested only 1 genotyped incorrectly (R75X) due to a rare polymorphism in the genomic region of the variant



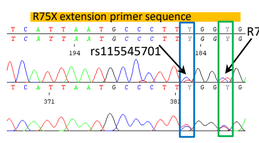


Figure 3. One of the samples in the CDC repository was incorrectly genotyped as a R75X homozygote rather than a heterozygote (note that there is no peak for the wild type allele; left panel). Sanger sequencing of the same sample confirms that this sample is a heterozygote (green box ; right panel). The sample also contains a second variant 3 nucleotides upstream (blue box, right panel) that likely disrupts hybridization of the R75X extension primer (in yellow; right panel), resulting in the incorrect call.

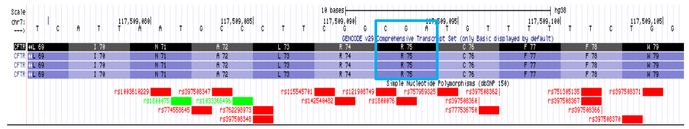


Figure 4. The R75X genomic region of *CFTR* contains numerous, single nucleotide polymorphisms (SNPs; shown in red and green; R75 in blue box). This genomic complexity contributes to difficulties in R75X genotyping for any hybridization-based molecular assay. Given the flexibility of the iPLEX assay chemistry, a second extension primer designed to the opposite strand could be included in a separate multiplex reaction to potentially eliminate incorrect genotype calls for this variant.

Conclusions

- 94% of all variants on the iPLEX *CFTR* panel were tested for genotyping accuracy using either CDC DBS repository samples or synthetic controls
- Of the 237 variant alleles genotyped only 1, R75X, was called inaccurately (for all extraction methods) due to a rare SNP (rsrs115545701; MAF = 0.005) located in the extension primer sequence
- Using automatic software calls only, the average call rate for any one of the DNA extraction methods was at least 99.6%
- 99.9% of variants were able to be called after manual analysis and the few, remaining uncalled variants were resolved upon repeat
- A 100% call rate was achieved using as little as 0.2ng of DNA per reaction
- Based on average % call rates, all DNA extraction methods performed equally
  - Small differences in % call rates between the DNA extraction methods (for each multiplex panel) were not significant and likely due to minor, technical processing issues
- Use of the Illinois DNA extraction method may offer newborn screening laboratories a cost savings by eliminating the need for commercially purchased DNA extraction reagents

References

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