AMP 2012
Abstract G42

Reducing Residual Risk in CF Carrier Screening - Using the illumina MiSeqDx™ for Cystic Fibrosis Carrier Screening

M. Chelliserry, R.E. Lenta, J.C. San, W.M. Goldstein, and E.J. Peters,
Illumina Inc., 5200 Illumina Way, San Diego, CA 92122 USA

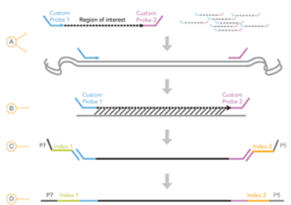
Introduction

Cystic Fibrosis (CF) is one of the most common genetic diseases in the US, with approximately 1 in 29 Caucasian individuals being a carrier for causative CF mutations. While CF is most common in individuals of Caucasian descent, ACOG/ACMG has recommended that carrier screening be offered to all couples who are considering having a child. In order to increase the detection rate of cystic fibrosis carriers and reduce the residual risk among other demographic groups, genotyping-based tests have been developed with larger mutation panels beyond the 23 ACOG/ACMG-recommended mutations. Despite this effort, non-Caucasian groups still have risk mutations that current genotyping panels will not detect. The CFTR2 project (cftr2.com) has systematically examined reported CF-causing mutations and their functional effects. Here we describe the development of a new next generation sequencing based assay, the Illumina MiSeqDx Cystic Fibrosis Carrier Screening Assay, for the detection of CFTR mutations on the MiSeqDx instrument. The test is designed to detect the 162 CFTR mutations listed in the CFTR2 database and is intended to identify an individual's CF carrier status in genomic DNA extracted from whole blood. The results of the test are intended for interpretation by a certified clinical geneticist or equivalent. This test is not indicated for fetal diagnostic testing, for preimplantation testing or for stand-alone diagnostic purposes.

Assay Technology

The assay technology involves targeted amplification of the CFTR gene followed by automated sequencing by synthesis on the MiSeqDx instrument. TruSeq Custom Amplicon workflow is used for targeted amplification of CFTR gene. The process involves hybridization of CF oligos to unfragmented genomic DNA followed by extension and ligation to form DNA templates containing regions of interest flanked by universal primer sequences. Using indexed primers supplied with the kit, the DNA templates are then PCR amplified, pooled into a single tube and sequenced on the MiSeqDx system.

Library Preparation method for MiSeqDx Cystic Fibrosis Assay



A Hybridization of custom oligonucleotide probes
B Extension and ligation
C Addition of indices and sequencing adapters by PCR
D Final amplicon ready for sequencing with Miseq

Assay Workflow

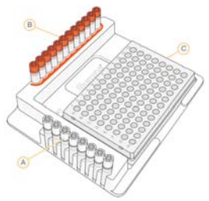
The MiSeqDx Cystic Fibrosis Carrier Screening Assay allows upto 48 samples to be processed in less than 48 hours from extracted DNA through completed data analysis. For the library preparation, upto 96 samples can be processed from extracted DNA to normalized samples (ready to be loaded on the sequencing instrument) within 7 hrs with less than 2.5 hrs of hands on time. Up to 48 samples can be pooled and genotyped/ sequenced in a single MiSeqDx run.

Sequencing on the MiSeqDx instrument

- The Illumina MiSeqDx system is a bench top personal sequencer which utilizes Sequencing by Synthesis (SBS) technology. The MiSeqDx has an integrated fluidics architecture and a built-in CPU, which enables cluster generation, sequencing, data analysis, and mutation report generation to be performed on a single instrument.
- After library preparation, the pooled, normalized and indexed library is loaded on to the MiSeq reagent cartridge which contains all of the reagents required for cluster generation and SBS. The library is first hybridized, then covalently attached through bridge amplification onto the flow cell surface, and amplified to generate millions of clusters which can then be sequenced using SBS.
- The SBS process uses four fluorescently labeled nucleotides; during each sequencing cycle, a single dNTP with reversible terminator nucleotide.
- The imaging subcomponent of the instrument consists of two cameras and two LEDs. Each LED is able to capture fluorescence in two channels (530 nm and 660 nm), which together allow for the system to recognize the four base pairs.
- The Illumina MiSeqDx Cystic Fibrosis System performs paired end 2 x 150 cycle sequencing to allow sequencing for 150 cycles from both directions, and 2x8 cycle sequencing to determine the sequences of both indexes in each cluster.

Sample Indexing/Multiplexing

The PCR primers include index sequences for sample multiplexing. There are 8 unique i5 primers and 12 unique i7 primers. The MiSeqDx Cystic Fibrosis Carrier Screening assay allows up to 48 samples to be multiplexed on a single sequencing flow cell.



i7 Index PCR Primer	Index Sequence
A701	ATCACGAC
A702	ACAGTGGT
A703	CAGATCCA
A704	ACAAACGG
A705	ACCCAGCA
A706	AACCCCTC
A707	CCCAACCT
A708	CACCACAC
A709	GAAACCCA
A710	TGTGACCA
A711	AGGGTCAA
A712	AGGAGTGG

A i5 primers (white caps)
B i7 primers (orange caps)
C **AMP** plate

i5 Index PCR Primer	Index Sequence
A501	TGAACCTT
A502	TGCTAAGT
A503	TGTTCTCT
A504	TAAGACAC
A505	CTAACTCG
A506	CTAGAACA
A507	TAAGTTCC
A508	TAGACCTA

To demonstrate that sample indexing has no impact on the sequencing result, 470 unique samples were tested across 2 different indices, 15 unique samples across 3 indices, and 3 unique samples across 47 indices. In each the results were 100% reproducible across all indices tested.

Assay Performance

The Illumina MiSeqDx Cystic Fibrosis Carrier Screening Assay had an average call rate of 99.99% when tested on N=400 unrelated blood samples (393 samples had a 100% call rate, 7 samples had a call rate of 99.35% due to No call for PolyTG) and a call rate >99.9% when tested on >1500 HapMap and human variation samples of multiple ethnicities. The performance of the assay was verified for all variants in the panel; for the rare mutations for which no samples were available, the performance was confirmed by using synthetic, plasmid-based DNA samples.

Accuracy and Reproducibility

A set of 47 Coriell DNA samples, chosen to be representative of the different types of mutations within CFTR gene, were tested using the Illumina MiSeqDx Cystic Fibrosis Carrier Screening assay by 3 operators on each of 3 MiSeqDx instruments. The results across 9 MiSeqDx runs indicated excellent reproducibility (100%) and accuracy (100%) when compared to results obtained with bi-directional Sanger sequencing (for all variations except the 2 large deletions) and PCR assay (for the 2 large deletions).

		#Correct Calls	#No calls	#Mis calls	%Reproducibility
Operator 1	MiSeqDx1 Result	7614	0	0	100.0%
	MiSeqDx2 Result	7614	0	0	100.0%
	MiSeqDx3 Result	7614	0	0	100.0%
Operator 2	MiSeqDx1 Result	7614	0	0	100.0%
	MiSeqDx2 Result	7614	0	0	100.0%
	MiSeqDx3 Result	7614	0	0	100.0%
Operator 3	MiSeqDx1 Result	7614	0	0	100.0%
	MiSeqDx2 Result	7613	1	0	99.987%
	MiSeqDx3 Result	7614	0	0	100.0%
Cumulative		68525	1	0	99.999%

Detection of Challenging Regions

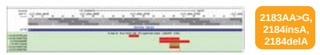
- Two Large Deletions

Deletion name	Length of Deleted Region
CFTR dele2, 3	21kb
CFTR dele22, 23	1.5kb

- PolyTG and PolyT regions in Intron 9

ATCTTTTATTTTTGA**TGTGTGTGTGTGTGTGTGTG**TTTTTTAACAGGGATTTG

- Insertion/deletions in homopolymeric regions



- Insertion-cum-deletion in the same region

```
Reference:  AA-AACAAT
Hap1:       AAG--CAAT
```

- A 22-bp deletion

Mutation	rs ID	cDNA Name (HGVS)
852del22	rs121908804	c.720_741delAGGGAGAATGATGATGAAGTAC

MiSeq Data	Sanger			
	Homozygous Reference	Heterozygous	Homozygous Alternate	N/N
Homozygous Reference	67419	0	0	0
Heterozygous	0	944	0	0
Homozygous Alternate	0	0	153	0
N/N	0	0	0	0

Performance of the Assay in sequencing across Challenging regions was verified through testing 1073 samples; this included runs at low (0.1x recommended DNA input) and high (5x recommended DNA input).

	N	#Correct Calls	#No calls	#Mis calls	%Reproducibility	% Accuracy
Large Deletion 1	104	104	0	0	100.0%	100.00%
PolyTG/PolyT	1073	1060	13*	0	98.8%	100.00%
Indels in homopolymeric regions (394delTT, 2143delT, 2184delA, 3905insT)	56	56	0	0	100.0%	100.00%
Insertion-cum-deletion (2183delAA>G)	104	104	0	0	100.0%	100.00%

*12/13 No calls were in the same sample tested at 25ng DNA input (0.1x) and 1/13 was in a sample tested at 1250ng (5x) DNA input.

Results Summary

Parameter	Result
Content	162 variations in CFTR2
Assay Time	7 hr run, 2.5 hr hands-on
Total Run Time (DNA to data)	48hrs
Throughput	48
Accuracy	100%
Reproducibility	> 99.99%
Call Rate (per sample)	> 99.99%
DNA Input	250ng
DNA Source	Blood

Conclusions

The Illumina MiSeqDx Cystic Fibrosis Carrier Screening allows genotyping of 162 variations in the CFTR2 panel in a simple, high throughput workflow with a short hands on time. The high accuracy, reproducibility, and the comprehensive nature of the panel will make it a useful tool to determine a subject's CF carrier status. A broad panel of clinically relevant mutations based upon the CFTR2 database will reduce the residual risk associated with current panels. The design of the instrument and the preliminary performance of the Illumina assay make this system appropriate for clinical use.*

* In development, not available for commercial sale.

illumina®