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

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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 the defective gene. 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 rate of cystic fibrosis carrier testing and reduce the residual risk among other demographic groups, non-Caucasian non-Caucasian groups still have rich mutations that current genetic testing panels will not detect. The CFTR2 project (cbfz.com) has systematically reported many CF-causing mutations and their functional effects. Here we describe the development of a 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-Amplion workflow is used for targeted amplification of CFTR gene. The process involves hybridization of CFTR 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.

Assay Workflow
The MiSeqDx Cystic Fibrosis Carrier Screening Assay allows up to 48 samples to be processed in less than 48 hours from extracted DNA through completed data analysis. For the library preparation, up to 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 qseq flow cell 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 LED. 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 2x60 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 40mers and 12 unique 7mers. The MiSeqDx Cystic Fibrosis Carrier Screening assay allows up to 48 samples to be multiplexed on a single sequencing flow cell.

To demonstrate that sample indexing has no impact on the sequencing result, 470 unique samples were tested across 2 different indexes, 15 unique samples across 3 indexes, and 9 unique samples across 47 indexes. In each the results were 100% reproducible across all index tests.

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 Poly(T) 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 Collin 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 instrument. 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).

Detection of Challenging Regions
- Two-Large Deletions
  - PolyTG and PolyT regions in Intron 9
  - Deletion name
    - CFTR dele2, 3 21kb
    - CFTR dele22, 23 1.5kb
  - Poly(TG) and Poly(T) regions
  - Insertion/deletions in homopolymeric regions
  - Insertion-cum-deletion in the same region
  - A 22-bp deletion

Results Summary

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<th>Parameter</th>
<th>DNA Source</th>
<th>DNA Input</th>
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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 cystic fibrosis carrier screening. 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.

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