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Can Next–Generation Sequencing Replace Sanger Sequencing? A Review of the Illumina Cystic Fibrosis Diagnostic Test on the MiSeqDx™ Instrument

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Introduction

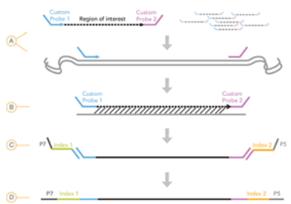
Cystic Fibrosis is a considered to be a common genetic disease, especially among the European Union (EU) and United States (US). The WHO currently states that the incidence rate in newborns is between 1 in 2000-3000 in the EU and 1 in 3500 in the US. Current practice for confirmatory diagnostic testing of individuals suspected of having cystic fibrosis typically involves the use of single-plex PCR and subsequent Sanger sequencing of numerous amplicons spanning the protein coding region of the CFTR gene. This process is both time and labor intensive.

Here we describe the development of a new next generation sequencing assay, the Illumina MiSeqDx Cystic Fibrosis Diagnostic Assay, for the detection of all known and novel CFTR variants on the MiSeqDx instrument. The assay is designed to sequence the full protein coding region of CFTR and intron-exon boundaries to detect variations within the CFTR gene as an aid in diagnosis of cystic fibrosis in genomic DNA extracted from whole blood samples. The assay is designed to also detect two deep intronic mutations (3849+10kbC>T and 1811+1.6kbA>G), and two large deletions (CFTR dele2,3, and CFTRdele22,23).

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 Diagnostic Assay allows 8 samples to be processed in less than 48 hours from extracted DNA through completed data analysis. For the library preparation, the 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. All 8 normalized samples can be pooled and 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 integrated 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 is added, the fluorescent dye imaged to identify the base and the terminator enzymatically cleaved to allow incorporation of the next 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.

Assay Performance

The Illumina MiSeqDx Cystic Fibrosis Diagnostic 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 99.98% due to No call for PolyTG/PolyT) and a call rate >99.9% when tested on >1500 HapMap and human variation samples of multiple ethnicities.

Accuracy and Reproducibility

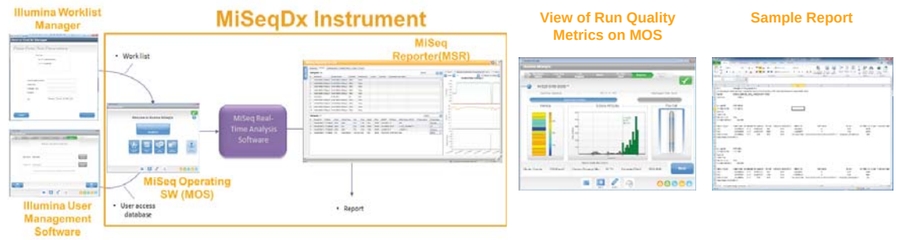
A set of 47 Coriell DNA samples, chosen to be representative of the different types of mutations within CFTR gene, were tested using the Illumina MiSeqDx Cystic Fibrosis Diagnostic assay by 3 operators on each of 3 MiSeqDx instruments. The results across 9 MiSeqDx runs indicated excellent reproducibility (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	244682	0	0	100.0%
	MiSeqDx2 Result	244682	0	0	100.0%
	MiSeqDx3 Result	244682	0	0	100.0%
Operator 2	MiSeqDx1 Result	244682	0	0	100.0%
	MiSeqDx2 Result	244682	0	0	100.0%
	MiSeqDx3 Result	244682	0	0	100.0%
Operator 3	MiSeqDx1 Result	244682	0	0	100.0%
	MiSeqDx2 Result	244681	1	0	100.0%
	MiSeqDx3 Result	244682	0	0	100.0%
Cumulative		2202137	1	0	100.0%

	Sanger			
MiSeq Data	Homozygous Reference	Heterozygous	Homozygous Alternate	N/N
Homozygous Reference	2199339	0	0	0
Heterozygous	0	1583	0	0
Homozygous Alternate	0	0	369	0
N/N	0	0	0	0

The assay is able to accurately sequence the full CFTR gene, including homopolymeric regions (for example, PolyTG/PolyT, 2183AA>G, 2184delA).

MiSeqDx Software Components



Results Summary

Parameter	Result
Content	All CFTR exons + 5-10nt intronic sequence + 2 intronic mutations + 2 large deletions
Assay Time	7 hr run, 2.5 hr hands-on
Total Run Time (DNA to data)	48hrs
Throughput	8
Accuracy	100%
Reproducibility	> 99.99%
Call Rate (per sample)	> 99.99%
DNA Input	250ng
DNA Source	Blood

Conclusions

The Illumina MiSeqDx Cystic Fibrosis Diagnostic Assay can accurately sequence the entire CFTR gene in a single run with up to 8 samples. This will provide significant workflow benefits versus current Sanger Sequencing methods. 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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