

MiSeqDx[®] Cystic Fibrosis 139-Variant Assay

FOR IN VITRO DIAGNOSTIC USE

Catalog # DX-102-1004: 2 Runs, up to 96 Samples per Kit

Catalog # DX-102-1003: 20 Runs, up to 960 Samples per Kit

Intended Use

The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay is a qualitative *in vitro* diagnostic system used to simultaneously detect 139 clinically relevant cystic fibrosis disease-causing mutations and variants of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene in genomic DNA isolated from human peripheral whole blood specimens. The variants include those recommended in 2004 by the American College of Medical Genetics (ACMG)¹ and in 2011 by the American College of Obstetricians and Gynecologists (ACOG)². The test is intended for carrier screening in adults of reproductive age, in confirmatory diagnostic testing of newborns and children, and as an initial test to aid in the diagnosis of individuals with suspected cystic fibrosis. The results of this test are intended to be interpreted by a board-certified clinical molecular geneticist or equivalent and should be used in conjunction with other available laboratory and clinical information.

This test is not indicated for use for newborn screening, fetal diagnostic testing, pre-implantation testing, or for stand-alone diagnostic purposes.

The test is intended to be used on the Illumina MiSeqDx instrument.

Summary and Explanation of the Assay

Clinical Description

Cystic fibrosis (CF) is one of the most common genetic disorders of the Western world and the most common life-threatening autosomal recessive disorder in the non-Hispanic white population³⁻⁷. CF impacts the viscosity of mucus secretions and affects the epithelia of the respiratory tract, pancreas, intestine, hepatobiliary system, male genital tract, and the sweat glands, resulting in complex multi-organ, multisystem disease⁴⁻⁶ with the lungs being the primary organ system associated with morbidity and mortality⁸. In many cases, nutritional decline presages progression of CF lung disease; thus, a key focus of current interventional efforts is early diagnosis through newborn screening⁷, thereby facilitating timely access to vital medical services and allowing the best possible outcome for individuals with the disease^{4,7}. Although there are gender differences in survival, with median survival reported to be greater for men than for women, overall median survival is 38.3 years in the US⁸.

CFTR Variants and Incidence

The cystic fibrosis transmembrane conductance regulator gene (*CFTR*) identified in 1989 is located on the long arm of chromosome 7 and contains 27 coding exons spread over 230 kb⁴. A 6.5 kb mRNA produced by the normal allele encodes CFTR, a 1490-amino acid integral membrane protein that functions as a regulated chloride channel in the epithelial cells of multiple organs^{4,5}. More than 1900 variants of *CFTR* are currently described, the majority being point mutations⁹. The most common *CFTR* variant is the F508del allele⁵, which accounts for almost 70% of all *CFTR* variants³. However, other common *CFTR* variants often result in a CF phenotype and other CFTR-related disorders³⁻⁵.

Cystic fibrosis has a disease incidence estimated at one in 2,000 to 4,000 live births and a prevalence of approximately 30,000 individuals in the US population⁴. It occurs in all ethnic and racial populations, at various frequencies: one in 3,000 Caucasians; one in 9,200 Hispanic Americans; one in 10,900 Native Americans; one in 15,000 African Americans; and one in 31,000 Asian Americans^{4,6}. Current estimates of CFTR mutation carrier frequency by ethnicity in the US,

based on a cohort of 364,890 individuals referred for carrier testing with no family history of CF are provided in [Table 1](#).

Table 1 General Cystic Fibrosis Mutation Carrier Frequency in Different Ethnic Groups in the US¹⁰

Ethnic Group	Observed Carrier Frequency
African American	1 in 84
Ashkenazi Jewish	1 in 29
Asian	1 in 242
Caucasian	1 in 28
Hispanic	1 in 59
Jewish	1 in 32
Middle Eastern	1 in 91
Native American	1 in 70
South Asian	1 in 118
Other Ethnicity	1 in 111
> 1 Ethnicity	1 in 34
Part African American	1 in 56
Part Caucasian	1 in 32
Part Hispanic	1 in 51
Not provided	1 in 37
All individuals	1 in 38

Overview of the CFTR2 Project

The CFTR2 project is an international initiative led by a team of researchers and clinicians, and supported by a grant from National Institute of Health and the U.S. Cystic Fibrosis Foundation.^{11,12} CFTR2 is intended to provide comprehensive and expert-reviewed functional and clinical information on *CFTR* variants. In an effort to clinically validate all CF variants with allele frequencies of 0.01% and greater, 25 CF registries and clinics from around the world¹³ pooled together resources with the goal of matching clinical information from over 39,000 CF patients with the nearly 1,900 CF variants that had been recorded over the years in the CFTR1 database at the Hospital for Sick Children in Toronto.^{11,13} Clinical characteristics, such as sweat chloride concentration, lung function (FEV1% predicted), and pancreatic status were analyzed alongside *CFTR* genotype information. The systematic approach of simultaneously analyzing these variants from clinical, functional, and genetic perspectives yielded 134 unique CF-causing variants at 129 unique genomic positions (since for five positions, two nucleotide changes appear at the same position) contained in the CFTR2 database (as of August 2013). The use of a panel comprising all these variants is expected to account for 95.4% of cystic fibrosis causing alleles, and increase the identification of the couples at risk through detection of both alleles to ~91% from 72% using the ACMG recommended panel of 23 variants.

CFTR Variants in Panel

The variants reported by the MiSeqDx Cystic Fibrosis 139-Variant Assay were specifically chosen because they represent the full set of clinically validated variants classified as CF-causing in the CFTR2 database at Johns Hopkins University, a product of the CFTR2 (Clinical and Functional Translation of CFTR) initiative.

The assay tests for: 134 CF-causing variants; one ACMG recommended panel variant (R117H, classified as a Mutation of Varying Clinical Consequence, MVCC, by CFTR2); one conditionally reported modifying variant (PolyTG/PolyT); and three conditionally reported benign variants (I506V, I507V, F508C)¹⁴; for a total of 139 reported variants.

The 134 CF-causing variants correspond to 129 CF-causing variants in the CFTR2 database. The CFTR2 database includes five CF-causing variants for which the same protein level change can arise from two distinct nucleotide changes [e.g., S466X(C>A) and S466X(C>G)]. These five variants are listed according to the amino acid codon in the CFTR2 database (e.g., S466X) while the assay reports each individual variant [e.g., S466X(C>A) and S466X(C>G)]. The list of 139 variants reported by the MiSeqDx Cystic Fibrosis 139-Variant Assay is provided in [Table 2](#).

Table 2 MiSeqDx Cystic Fibrosis 139-Variant Assay Summary of Variants

[Variants are listed in genomic coordinate order; the associated nucleotide level change for each variant is in parenthesis.

Bold=ACMG-23; Italics=Conditionally reported]

M1V (c.1A>G)	T338I (c.1013C>T)	R553X (c.1657C>T)	3272-26A>G (c.3140-26A>G)
CFTRdele2,3 (c.54-5940_273+10250del21kb)	S341P (c.1021T>C)	A559T (c.1675G>A)	L1065P (c.3194T>C)
Q39X (c.115C>T)	1154insTC (c.1022_1023insTC)	R560T (c.1679G>C)	R1066C (c.3196C>T)
E60X (c.178G>T)	R347H (c.1040G>A)	R560K (c.1679G>A)	R1066H (c.3197G>A)
P67L (c.200C>T)	R347P (c.1040G>C)	1811+1.6kbA>G (c.1679+1.6kbA>G)	L1077P (c.3230T>C)
R75X (c.223C>T)	R352Q (c.1055G>A)	1812-1G>A (c.1680-1G>A)	W1089X (c.3266G>A)
G85E (c.254G>A)	1213delT (c.1081delT)	E585X (c.1753G>T)	Y1092X(C>A) (c.3276C>A)
394delTT (c.262_263delTT)	1248+1G>A (c.1116+1G>A)	1898+1G>A (c.1766+1G>A)	Y1092X(C>G) (c.3276C>G)
405+1G>A (c.273+1G>A)	1259insA (c.1127_1128insA)	1898+3A>G (c.1766+3A>G)	M1101K (c.3302T>A)
406-1G>A (c.274-1G>A)	W401X (c.1202G>A)	2143delT (c.2012delT)	E1104X (c.3310G>T)
E92X (c.274G>T)	W401X (c.1203G>A)	2183AA >G (c.2051_ 2052delAAinsG)	R1158X (c.3472C>T)
E92K (c.274G>A)	1341+1G>A (c.1209+1G>A)	2184delA (c.2052delA)	R1162X (c.3484C>T)
Q98X (c.292C>T)	1461ins4 (c.1329_ 1330insAGAT)	2184insA (c.2052_2053insA)	3659delC (c.3528delC)
457TAT>G (c.325_327delTATinsG)	A455E (c.1364C>A)	R709X (c.2125C>T)	S1196X (c.3587C>G)
D110H (c.328G>C)	1525-1G>A (c.1393-1G>A)	K710X (c.2128A>T)	W1204X (c.3611G>A)
R117C (c.349C>T)	S466X (C>A) (c.1397C>A)	2307insA (c.2175_2176insA)	W1204X (c.3612G>A)
R117H (c.350G>A)	S466X (C>G) (c.1397C>G)	L732X (c.2195T>G)	3791delC (c.3659delC)
Y122X (c.366T>A)	L467P (c.1400T>C)	2347delG (c.2215delG)	3849+10kbC>T (c.3717+12191C>T)
574delA (c.442delA)	1548delG (c.1418delG) [†]	R764X (c.2290C>T)	G1244E (c.3731G>A)
621+1G>T (c.489+1G>T)	S489X (c.1466C>A)	2585delT (c.2453delT)	3876delA (c.3744delA)
663delT (c.531delT)	S492F (c.1475C>T)	E822X (c.2464G>T)	S1251N (c.3752G>A)
G178R (c.532G>A)	Q493X (c.1477C>T)	2622+1G>A (c.2490+1G>A)	3905insT (c.3773_3774insT)
711+1G>T (c.579+1G>T)	I507del (c.1519_ 1521delATC)	E831X (c.2491G>T)	W1282X (c.3846G>A)
711+3A>G (c.579+3A>G)	F508del (c.1521_ 1523delCTT)	W846X (c.2537G>A)	4005+1G>A (c.3873+1G>A)
711+5G>A (c.579+5G>A)	1677delTA (c.1545_ 1546delTA)	R851X (c.2551C>T)	4016insT (c.3884_3885insT)
712-1G>T (c.580-1G>T)	V520F (c.1558G>T)	2711delT (c.2583delT)	N1303K (c.3909C>G)
H199Y (c.595C>T)	Q525X (c.1573C>T) [†]	2789+5G>A (c.2657+5G>A)	Q1313X (c.3937C>T)
P205S (c.613C>T)	1717-8G>A (c.1585-8G>A)	Q890X (c.2668C>T)	4209TGTT>AA (c.4077_ 4080delTGTTinsAA)
L206W (c.617T>G)	1717-1G>A (c.1585-1G>A)	L927P (c.2780T>C)	CFTRdele22,23 (c.3964-78_ 4242+577del)
Q220X (c.658C>T)	G542X (c.1624G>T)	S945L (c.2834C>T)	4382delA (c.4251delA)

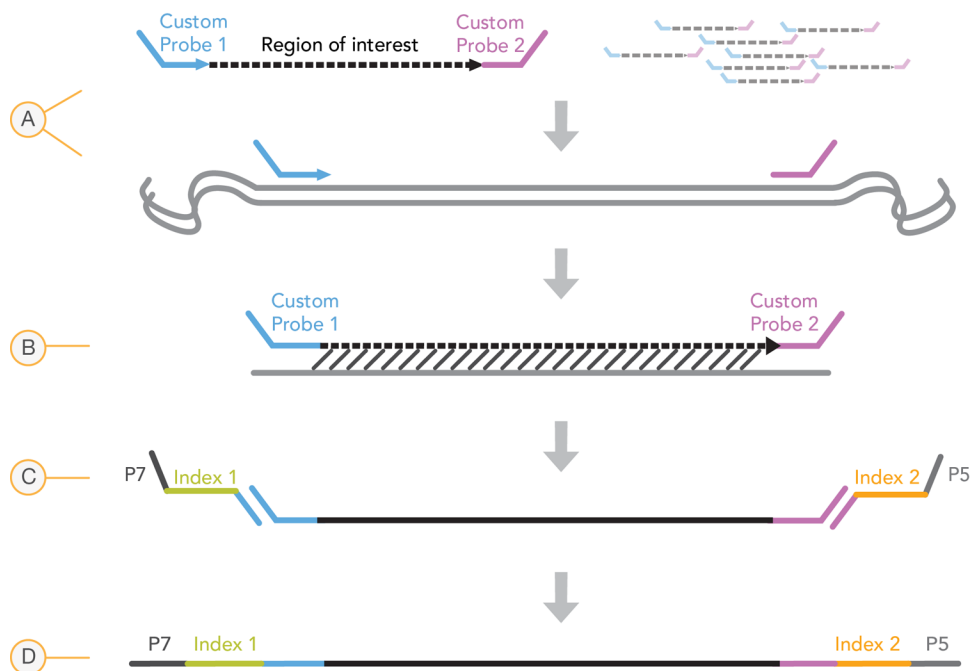
852del22 (c.720_741delAGGGAGAATGATGATGAAGTAC)	S549R (c.1645A>C)	3007delG (c.2875delG)	<i>PolyTG/PolyT</i>
1078delT (c.948delT)	S549N (c.1646G>A)	G970R (c.2908G>C)	<i>I506V (c.1516A>G)</i>
G330X (c.988G>T)	S549R (c.1647T>G)	3120G>A (c.2988G>A)	<i>I507V (c.1519A>G)</i>
R334W (c.1000C>T)	G551D (c.1652G>A)	3120+1G>A (c.2988+1G>A)	<i>F508C (c.1523T>G)</i>
I336K (c.1007T>A)	Q552X (c.1654C>T)	3121-1G>A (c.2989-1G>A)	

[†] Classified in the CFTR2 database¹² as a CF-causing variant, while the Sosnay paper¹³ classifies the variant as indeterminate. The database classification is more current and reflects the completed functional testing, which was not available at the time of the Sosnay publication.

Principles of Procedure

The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay involves two main procedures. The first is to prepare the samples for sequencing, which is called library preparation. Library preparation consists of four key steps: Hybridization, Extension-Ligation, PCR Amplification, and Library Normalization. The second procedure is to sequence the prepared sample using SBS (sequencing by synthesis) chemistry on the MiSeqDx.

Library Preparation



- A Hybridization**—The first step, Hybridization, hybridizes a pool of upstream and downstream oligonucleotides specific to the MiSeqDx Cystic Fibrosis 139-Variant Assay to the sample genomic DNA. At the end of this process, a three-step wash procedure with a filter capable of size selection removes unbound oligonucleotides from the genomic DNA.
- B Extension-Ligation**—The second step, Extension-Ligation, connects the hybridized upstream and downstream oligonucleotides. A DNA polymerase extends from the upstream oligonucleotides through the targeted region, followed by ligation to the 5' end of the downstream oligonucleotide using a DNA ligase. The result is the formation of products that contain the CF-specific oligonucleotides flanked by sequences required for amplification.
- C PCR Amplification**—The third step, PCR Amplification, amplifies the extension-ligation products using primers that add index sequences for sample multiplexing, as well as common adapters required for cluster generation on

the MiSeqDx. At the end of this process, a PCR clean-up procedure purifies the PCR products (referred to as a library).

- D Library Normalization**—The final step, Library Normalization, normalizes the quantity of each library to ensure more equal library representation in the final pooled library. At the end of this process, the pooled library is loaded onto the MiSeqDx for sequencing using SBS chemistry.

Sequencing

SBS chemistry uses a reversible-terminator method to detect single nucleotide bases as they are incorporated into growing DNA strands. During each sequencing cycle, a single fluorescently labeled deoxynucleotide triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Because all four reversible terminator-bound dNTPs (A, G, T, C) are present as single, separate molecules, natural competition minimizes incorporation bias. Base calls are made directly from signal intensity measurements during each sequencing cycle. The result is base-by-base sequencing.

Data Analysis

The first step in data analysis is called primary analysis. This process is done by Real Time Analysis (RTA) software and generates base calls and quality scoring. In the next step, called secondary analysis, the base calls generated during primary analysis are processed to produce information for each sample. Performed by MiSeq Reporter or Local Run Manager software, secondary analysis includes demultiplexing, FASTQ file generation, alignment, variant calling, and generation of VCF files containing information about variants found at specific positions in the reference genome.

Both MiSeq Reporter and Local Run Manager have identical sample analysis and reporting functionality. The main difference between the two is the method employed to interface with the MiSeqDx instrument. For more information on the differences, and to determine which software is in use, see [MiSeqDx Instrument Interface Methods on page 5](#).

- **Demultiplexing**—If the run contains multiple samples and the run has index reads, this is the first step in secondary analysis. Demultiplexing separates data from pooled samples based on the unique sequence indexes that were added during the PCR amplification step.
- **FASTQ File Generation**—After demultiplexing, MiSeq Reporter or Local Run Manager generates intermediate files in the FASTQ format, which is a text format used to represent sequences. FASTQ files contain the reads for each sample and the quality scores, excluding reads from any clusters that did not pass filter.
- **Alignment**—Alignment compares sequences against the reference to identify a relationship between the sequences and assigns a score based on regions of similarity. Aligned reads are written to files in BAM format. For the MiSeqDx Cystic Fibrosis 139-Variant Assay, a banded Smith-Waterman algorithm performs local sequence alignments to determine similar regions between two sequences.
- **Variant Calling**—This step records single nucleotide variants (SNV), insertions and deletions (indels), and other structural variants in a standardized text file called `MiSeqDxCF139VariantAssay.txt`.

For more information on the analysis workflow, see the guides for the analysis software installed with your MiSeqDx. For MiSeq Reporter, see *MiSeq Reporter Software Reference Guide (document # 15038356)*. For Local Run Manager, see *Local Run Manager Software Reference Guide for MiSeqDx (document # 1000000011880)* and the *Local Run Manager CF 139 Variant Analysis Module Workflow Guide (document # 1000000012184)*.

MiSeqDx Instrument Interface Methods

There are two different methods to interface with the MiSeqDx instrument available for the MiSeqDx Cystic Fibrosis 139-Variant Assay. The original interface method uses MiSeq Reporter software, along with Illumina Worklist Manager (IWM) and Illumina User Management Software. The new method uses Local Run Manager software.

Both MiSeq Reporter and Local Run Manager have identical sample analysis and reporting functionality.

Software Functions	Original	New
Set up a run for the MiSeqDx instrument	Illumina Worklist Manager (IWM)	Local Run Manager
Sample setup and tracking	Illumina Worklist Manager (IWM)	Local Run Manager
Control user access	Illumina User Management Software	Local Run Manager
Perform secondary analysis	MiSeq Reporter	Local Run Manager
Generate reports	MiSeq Reporter	Local Run Manager

Follow these steps to determine if Local Run Manager is in use.

- 1 Access the MiSeqDx instrument remotely.
- 2 When prompted, log in.
- 3 Make sure "Local Run Manager" displays at the top of the screen.



NOTE

If you are not prompted to log in when you access the instrument remotely, MiSeq Reporter is in use.

Limitations of the Procedure

- 1 For *in vitro* diagnostic use. The results obtained using the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay should be used and interpreted in the context of a full clinical evaluation.
- 2 The assay is designed to identify a specific subset of known variants in the *CFTR* gene, but does not include all variants identified in the *CFTR* gene. Specifically, the assay only reports amino acid level changes if these are associated with the nucleotide changes as listed in [Table 2](#). While other nucleotide level changes can lead to the same amino acid level changes, these will not be reported by the assay. Therefore, the failure to identify a variant does not guarantee that other *CFTR* variants are not present in the samples being analyzed.
- 3 Variants identified by this assay vary in frequency among different populations.
- 4 As with any hybridization-based assay, underlying polymorphisms or variants in oligonucleotide-binding regions can affect the alleles being probed and, consequently, the calls made.
- 5 The assay cannot determine whether the orientation of the PolyTG/PolyT variant is in cis/trans to the R117H variant. For patients with an R117H variant, additional testing should be performed to determine whether a PolyTG/PolyT variant, which may affect the clinical phenotype (e.g., 12-13(TG) or 5T), is in a cis/trans orientation to the R117H variant.
- 6 PolyTG/PolyT are homopolymeric regions known to be difficult to interpret with sequence-based assays due to polymerase slippage. A 0.9% (4/448) miscall rate was observed for PolyTG/PolyT results demonstrating a ± 1 TG discrepancy when compared to Sanger bi-directional sequencing ([Table 18](#)).

Product Components

The Illumina MiSeqDx Platform consists of the following:

- MiSeqDx Cystic Fibrosis 139-Variant Assay (Catalog # DX-102-1004 or DX-102-1003)
- MiSeqDx Instrument (Catalog # DX-410-1001)

Reagents

Reagents Provided

Reagents for the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay are provided by Illumina. Catalog # DX-102-1003 has been configured for 20 runs with a maximum of 48 samples per run (up to 960 samples total). Catalog # DX-102-1004 has been configured for two runs with a maximum of 48 samples per run (up to 96 samples total).

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 1

Table 3 Box 1A Pre-Amp Reagents

Component	Quantity		Fill Volume	Active Ingredients	Storage
	DX-102-1003	DX-102-1004			
CF 139-Variant Assay-Oligo Pool	10 tubes	1 tube	600 µl	Buffered aqueous solution containing oligonucleotides targeting the <i>CFTR</i> gene	-25°C to -15°C
Hybridization Buffer	10 tubes	1 tube	4.32 ml	Buffered aqueous solution containing salts and formamide	-25°C to -15°C
Extension-Ligation Mix	10 tubes	1 tube	4.8 ml	Buffered aqueous solution containing proprietary blend of DNA polymerases, DNA ligase, and dNTPs	-25°C to -15°C
Index Primers A (A501)–H (A508)	10 tubes per primer	1 tube per primer	192 µl	PCR primers with index sequences and sequencing adapters	-25°C to -15°C
Index Primers 1 (A701)–12 (A712)	10 tubes per primer	1 tube per primer	128 µl	PCR primers with index sequences and sequencing adapters	-25°C to -15°C
PCR Polymerase	10 tubes	1 tube	56 µl	Proprietary DNA polymerase	-25°C to -15°C
PCR Master Mix	10 tubes	1 tube	2.8 ml	Buffered aqueous solution containing salts and dNTPs	-25°C to -15°C

Table 4 Box 1B Post-Amp Reagents

Component	Quantity		Fill Volume	Active Ingredients	Storage
	DX-102-1003	DX-102-1004			
Library Normalization Diluent	10 tubes	1 tube	4.6 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol, and formamide	-25°C to -15°C
Library Dilution Buffer	10 tubes	1 tube	4.5 ml	Buffered aqueous solution	-25°C to -15°C
PhiX Internal Control	1 tube	1 tube	10 µl	Buffered aqueous solution containing PhiX genomic DNA	-25°C to -15°C

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 2

Table 5 Box 2 Post-Amp Reagents

Component	Quantity		Contents	Storage
	DX-102-1003	DX-102-1004		
MiSeqDx Reagent Cartridge - CF 139-Variant Assay	20 cartridges	2 cartridges	Single-use cartridge that contains cluster generation and sequencing reagents for use with the MiSeqDx, including formamide, 2-Mercaptoethanol, and < 2% DMSO	-25°C to -15°C

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 3

Table 6 Box 3A Pre-Amp Reagents

Component	Quantity		Fill Volume	Active Ingredients	Storage
	DX-102-1003	DX-102-1004			
Stringent Wash Buffer	10 bottles	1 bottle	24 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol, and formamide	2°C to 8°C
Universal Wash Buffer	10 tubes	1 tube	4.8 ml	Buffered aqueous solution containing salts	2°C to 8°C

Table 7 Box 3B Post-Amp Reagents

Component	Quantity		Fill Volume	Active Ingredients	Storage
	DX-102-1003	DX-102-1004			
PCR Clean-Up Beads	10 tubes	1 tube	5 ml	Buffered aqueous solution containing solid phase paramagnetic beads and polyethylene glycol	2°C to 8°C
Library Normalization Wash	20 tubes	2 tubes	4.8 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol, and formamide	2°C to 8°C
Library Beads	10 tubes	1 tube	1.2 ml	Buffered aqueous solution containing solid phase paramagnetic beads	2°C to 8°C
MiSeqDx Flow Cell - CF 139-Variant Assay	20 containers	2 containers	1 flow cell	Glass substrate with covalently bound oligonucleotides	2°C to 8°C

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 4

Table 8 Box 4 Post-Amp Reagents

Component	Quantity		Fill Volume	Active Ingredients	Storage
	DX-102-1003	DX-102-1004			
MiSeqDx SBS Solution (PR2) - CF 139-Variant Assay	20 bottles	2 bottles	353.1 ml	Buffered aqueous solution	2°C to 8°C

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 5

Table 9 Box 5 Pre-Amp Reagents

Component	Quantity		Fill Volume	Active Ingredients	Storage
	DX-102-1003	DX-102-1004			
Filter Plate	20 plates	2 plates	N/A	Polypropylene microtiter plate with a modified polyethersulfone membrane	15°C to 30°C

Table 10 Box 5 Post-Amp Reagents

Component	Quantity		Fill Volume	Active Ingredients	Storage
	DX-102-1003	DX-102-1004			
Elution Buffer	10 tubes	1 tube	4.8 ml	Buffered aqueous solution	15°C to 30°C
Library Storage Buffer	10 tubes	1 tube	3.5 ml	Buffered aqueous solution	15°C to 30°C

Reagents Required, Not Provided

Pre-Amp Reagents

- 10 N NaOH (prepare from tablets or use a standard solution)
- TE Buffer
- RNase/DNase-free water

Post-Amp Reagents

- 10 N NaOH (prepare from tablets or use a standard solution)
- Ethanol, 200 proof for molecular biology
- TE Buffer
- RNase/DNase-free water

Storage and Handling

- 1 Room temperature is defined as 15°C to 30°C.
- 2 The following reagents are shipped frozen and are stable when stored at -25°C to -15°C until the specified expiration date.
 - CF 139-Variant Assay-Oligo Pool
 - Hybridization Buffer
 - Extension-Ligation Mix
 - Index Primers A (A501) - H (A508)
 - Index Primers 1 (A701) - 12 (A712)
 - PCR Polymerase
 - PCR Master Mix
 - Library Normalization Diluent
 - Library Dilution Buffer
 - PhiX Internal Control
 - MiSeqDx Reagent Cartridge - CF 139-Variant Assay

Except for the reagent cartridge, the reagents are stable for a maximum of 6 freeze/thaw cycles that occur before the specified expiration date.

Do not refreeze the reagent cartridge after it has been thawed. It can be stored up to 6 hours at 2°C to 8°C.

- 3 The following reagents are shipped refrigerated and are stable when stored at 2°C to 8°C until the specified expiration date.
 - Stringent Wash Buffer
 - Universal Wash Buffer
 - PCR Clean-Up Beads
 - Library Beads
 - Library Normalization Wash
 - MiSeqDx SBS Solution (PR2) - CF 139-Variant Assay
 - MiSeqDx Flow Cell - CF 139-Variant Assay
- 4 The following reagents are shipped ambient and are stable when stored at room temperature until the specified expiration date:
 - Elution Buffer
 - Filter Plate
 - Library Storage Buffer

- 5 Changes in the physical appearance of the reagents provided can indicate deterioration of the materials. If changes in the physical appearance occur (e.g., obvious changes in reagent color or cloudiness apparent with microbial contamination), do not use the reagents.
- 6 The Hybridization Buffer, Stringent Wash Buffer, and Library Normalization Diluent reagents might form visible precipitates or crystals. Before use, vortex vigorously, and then visually inspect to ensure that no precipitates are present.
- 7 Adhere to the following best practices when handling PCR Clean-Up Beads and Library Beads:
 - The beads should never be frozen.
 - Allow the beads to reach room temperature.
 - Immediately prior to use, vortex the beads until well-suspended and color appears homogeneous.
 - Thoroughly mix the sample after the beads are added by pipetting up and down 10 times. A shaker can be used to thoroughly mix samples.
 - Incubate the bead/sample mixture at room temperature for the entire duration indicated.
 - Follow instructions when using the magnetic stand. Wait for the solution to clear before aspirating. Keep the plate on the magnetic stand when slowly aspirating the supernatant, taking care not to disturb the separated beads.
- 8 The PCR amplification plate can remain on the thermal cycler overnight, or it can be stored at 2°C to 8°C for up to 2 days. Before storing the plate at 2°C to 8°C, seal the plate well.
- 9 Do not freeze the Library Beads or mix with the Library Normalization Diluent reagent if not used immediately.
- 10 The diluted amplicon library can be stored at -25°C to -15°C for up to 14 days.
- 11 Load diluted amplicon pool onto the reagent cartridge after denaturation.

Equipment and Materials

Equipment and Materials Provided, Sold Separately

- 1 **MiSeqDx Instrument**, Catalog # DX-410-1001
- 2 **TruSeq Index Plate Fixture Kit**, Catalog # FC-130-1005
- 3 **TruSeq Index Plate Fixture & Collar Kit**, Catalog # FC-130-1007
- 4 **Index Adapter Replacement Caps**, Catalog # DX-502-1003

Equipment and Materials Required, Not Provided

Pre-Amp Equipment and Materials

- 1 **Heat Block**—One heat block for a 96 well plate is required. The heat block must meet the following performance specifications. Heat blocks with heated lids are acceptable for use.
 - Temperature Range: Ambient +5°C to 99°C
 - Temperature Regulation: $\pm 0.1^\circ\text{C}$ at 37°C; $\pm 0.4^\circ\text{C}$ at 60°C
- 2 **Sample Incubator**—One incubator (hybridization oven) is required. The incubator must meet the following performance specifications.
 - Temperature Range: 10°C to 100°C
 - Temperature Regulation: $\pm 0.2^\circ\text{C}$
- 3 **Tabletop Centrifuge**—A temperature controlled tabletop centrifuge capable of maintaining 20°C is required. (A separate centrifuge is required in the post-amp area.) Any plate centrifuge that fits a 96-well plate with filter unit and attains the designated speeds of the protocol (280 to 2400 \times g) is acceptable.
- 4 **Precision Pipettes**—One set of precision pipettes is required. (A separate set is required in the post-amp area.) The use of precision pipettes is required to ensure accurate reagent and sample delivery. Single-channel or multi-channel pipettes can be used if they are calibrated regularly and are accurate within 5% of stated volume.
- 5 **Consumables**—The following consumables are required.
 - 96-well skirted PCR plates, 0.2 ml, polypropylene, or equivalent
 - 96-well storage plates, 0.8 ml (MIDI plates)

- Solution basin, PVC, DNase, RNase-free (trough)
- Adhesive aluminum foil seal
- Appropriate PCR plate seal
- Aerosol resistant pipette tips

Post-Amp Equipment and Materials

- 1 **Thermal Cycler**—One thermal cycler is required. The thermal cycler must have a heated lid and meet the following performance specifications:
 - Temperature Control Range: 4°C to 99°C
 - Control Accuracy: $\pm 0.25^\circ\text{C}$ from 35°C to 99°C
- 2 **Microplate Shaker**—One microplate shaker is required in the post-amp lab area. The plate shaker must meet the following performance specifications:
 - Max Mixing Speed: 3000 rpm
 - Mixing Speed Range: 200 to 3000 rpm
- 3 **Tabletop Centrifuge**—One tabletop centrifuge capable of maintaining 20°C is required. (A separate centrifuge is required in the pre-amp area.) Any plate centrifuge that attains the designated speeds of the protocol (280 to 2400 \times g) is acceptable.
- 4 **Heat Block**—One heat block for tubes is required. The heat block must meet the following performance specifications.
 - Temperature Range: Ambient +5°C to 99°C
 - Temperature Regulation: $\pm 0.1^\circ\text{C}$ at 37°C; $\pm 0.4^\circ\text{C}$ at 60°C
- 5 **Magnetic Stand**—One magnetic stand for a 96 well plate is required. Better performance is seen when the magnets are on the side of the stand and not on the bottom.
- 6 **Precision Pipettes**—One set of precision pipettes is required. (A separate set is required in the pre-amp area.) The use of precision pipettes is required to ensure accurate reagent and sample delivery. Single-channel or multi-channel pipettes can be used if they are calibrated regularly and are accurate within 5% of stated volume.
- 7 **Consumables**—The following consumables are required.
 - 96-well skirted PCR plates, 0.2 ml, polypropylene, or equivalent
 - 96-well storage plates, 0.8 ml (MIDI plates)



NOTE

Make sure that the 96-well plate is fit compatible with the magnetic stand.

- Conical tubes, 15 ml
- Eppendorf microcentrifuge tubes (screw-top recommended)
- PCR eight-tube strips
- Solution basins, PVC, DNase, RNase-free (trough)
- Adhesive aluminum foil seals
- Adhesive single-use plate seals
- Aerosol resistant pipette tips

Specimen Collection, Transport, and Storage



NOTE

Handle all specimens as if they are potentially infectious agents.

- 1 Whole blood specimens collected in K₂EDTA tubes can be used.
- 2 Whole blood specimens can be stored for no longer than 7 days at room temperature, up to 30 days at 2°C to 8°C, or up to 30 days if frozen at -25°C to -15°C.
- 3 Transport whole blood for no longer than 7 days at room temperature, 30 days at 2°C to 8°C, or 30 days if frozen at -25°C to -15°C. Transportation of whole blood must comply with country, federal, state, and local regulations for the transport of etiologic agents.

- 4 No adverse effect on assay performance was observed when genomic DNA was subjected to 6 freeze/thaw cycles.
- 5 No adverse effect on assay performance was observed with whole blood specimens with elevated bilirubin, cholesterol, triglyceride, EDTA, or hemoglobin present.

Warnings and Precautions



CAUTION

Federal law restricts this device to sale by or on the order of a physician or other practitioner licensed by the law of the State in which he/she practices, to use or order the use of the device.

- 1 **Some components of this assay contain potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations.** For environmental, health, and safety information, see the SDS at support.illumina.com/sds.html. (See [Reagents on page 7](#) for more information.)
- 2 Some components of this assay contain 2-Mercaptoethanol, a reducing agent. (See [Reagents on page 7](#) for more information.) Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Use in a well-ventilated area and dispose of any containers and unused contents in accordance with applicable local governmental safety standards. For more information, contact Illumina Technical Support.
- 3 Handle all specimens as if they are potentially infectious agents.
- 4 Failure to follow the procedures as outlined may result in erroneous results or significant reduction in sample quality.
- 5 Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wear disposable gloves and laboratory coats when handling specimens and assay reagents. Wash hands thoroughly after handling specimens and assay reagents.
- 6 Do not use any assay components beyond their stated expiration date on the assay carton label. Do not interchange assay components from different assay lots. Note that assay lots are identified on the assay carton label.
- 7 Store the assay components at the specified temperature in designated pre-amplification and post-amplification areas.
- 8 Repeated freeze-thaw cycles (up to 6) of the Box 1 components will not compromise the integrity of the assay.
- 9 To prevent sample or reagent degradation, ensure that all sodium hypochlorite vapors have fully dissipated prior to starting the protocol.
- 10 Proper laboratory practices and good laboratory hygiene is required to prevent PCR products from contaminating reagents, instrumentation, and genomic DNA samples. PCR contamination may cause inaccurate and unreliable results.
- 11 To prevent contamination, ensure that pre-amplification and post-amplification areas have dedicated equipment (e.g., pipettes, pipette tips, vortexer, and centrifuge).
- 12 Avoid cross contamination. Use fresh pipette tips between samples and between dispensing reagents. Mix samples with a pipette and centrifuge the plate when indicated. Do not vortex the plates. Using aerosol-resistant tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.
- 13 Index-sample pairing must match sample information entered for the MiSeqDx run. Mismatches between the sample information and plate layout will result in loss of positive sample identification and incorrect result reporting.
- 14 Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air, impacting results.
- 15 Ensure that all ethanol is removed from the bottom of the wells during wash steps. Residual ethanol may impact results.
- 16 Adhere to the specified drying time following the magnetic stand step to ensure complete evaporation. Residual ethanol can impact the performance of subsequent reactions.
- 17 Do not mix the CF 139-Variant Assay-Oligo Pool and Hybridization Buffer for storage. When combined, the CF 139-Variant Assay-Oligo Pool becomes unstable, even when stored frozen.

- 18 The use of thermal cyclers with active cooling (e.g., Peltier, thermoelectric cooled) is not recommended for the hybridization step. The passive cooling step is critical for proper hybridization.
- 19 Always add PCR Polymerase to the PCR Master Mix just prior to use. Never store the combined working solution.
- 20 During the library normalization step, it is extremely critical to completely resuspend the library bead pellet. This is essential for achieving consistent cluster density on the MiSeqDx flow cell.
- 21 Adhere to the specified incubation times in the library normalization step. Improper incubation can affect library representation and cluster density.
- 22 Due to the number of plate transfers and subsequent potential for contamination, extreme care should be taken to ensure that well contents remain fully in the well. Do not splash contents.
- 23 The 250 ng DNA input recommendation allows for DNA quantity variation; assay performance is driven by this input level.
- 24 Sample variants with a "No Call" designation on the test report indicate that the data for that variant position did not meet defined sequencing thresholds. Variants with a "No Call" designation must not be reported unless repeat testing produces values that meet defined thresholds and are no longer designated as "No Call".

Acronyms

Table 11 Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay Acronyms

Acronym	Definition
AMP	AMplification Plate
CLP	CLean-up Plate
DAL	Diluted Amplicon Library
FPU	Filter Plate Unit
HYB	HYBridization Plate
LNP	Library Normalization Plate
NTC	No Template Control
PAL	Pooled Amplicon Library
SGP	StoraGe Plate

Procedural Notes

- 1 Illumina requires that one positive control DNA sample and a negative control (NTC or No Template Control) is included in every run, which is defined as a set of samples processed in parallel. The positive control DNA sample should be a well-characterized sample with one or more known *CFTR* variants. Illumina recommends the use of a wild type control. The wild type control should be run as a sample and should not replace the positive or negative control.
- 2 Before beginning the MiSeqDx Cystic Fibrosis 139-Variant Assay, extract and quantitate the DNA.
- 3 Any validated DNA extraction method can be used.
- 4 Quantitate the DNA using a spectrophotometer. Verify that the A260/A280 of the DNA sample is > 1.5. Normalize the DNA sample to 50 ng/μl. Each sample requires 5 μl of genomic DNA (total of 250 ng).

Sample Throughput and Index Representation

For the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay, the sample throughput per MiSeqDx run can be between 8 to 48 samples. The indexing primers used during PCR amplification must be chosen based on desired final sample throughput to ensure diversity in index sequence.



NOTE

For maximum throughput efficiency, perform library preparation for up to 96 samples, and then divide the samples into two sequencing runs with a maximum of 48 samples each. The MiSeqDx can only sequence 48 samples at a time. For MiSeq Reporter, create separate sample sheets for each set of 48 samples. For Local Run Manager, enter sample information for each set of 48 samples directly into the CF 139 Variant analysis module.

MiSeqDx uses a green LED to sequence G/T bases and a red LED to sequence A/C bases. At each cycle at least one of two nucleotides for each color channel needs to be read to ensure proper registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise registration failure could occur during sequencing of the Index Read.

See [Table 12](#) for choosing index primer combinations for 48 or 96 sample library preparations.

Table 12 Index Primer Combinations for 48-Sample or 96-Sample Sequencing Runs

Index 1 (I7) Set 1 Primers Columns 1–6	Index 1 (I7) Set 2 Primers Columns 7–12	Index 2 (I5) Primers Rows A–H
Index Primer 1 (A701)	Index Primer 6 (A706)	Index Primer A (A501)
Index Primer 2 (A702)	Index Primer 7 (A707)	Index Primer B (A502)
Index Primer 3 (A703)	Index Primer 8 (A708)	Index Primer C (A503)
Index Primer 4 (A704)	Index Primer 9 (A709)	Index Primer D (A504)
Index Primer 5 (A705)	Index Primer 11 (A711)	Index Primer E (A505)
Index Primer 10 (A710)	Index Primer 12 (A712)	Index Primer F (A506)
--	--	Index Primer G (A507)
--	--	Index Primer H (A508)

If sequencing fewer than the 48 samples in a sequencing run, select the appropriate indexes based on their sequences to maintain color balance in the green and red channels (see [Table 14](#) and [Table 15](#)). At a minimum, runs with 8 to 48 samples must include the indexing primer combinations identified in [Table 13](#).

To accurately process smaller runs, at least eight samples must be present. If six unique samples (excluding the positive and negative controls) are not available, it is acceptable to fill the run with sample replicates or any human genomic DNA sample. See [Table 13](#) for the minimal set of color-balanced indexes to use for 8-sample sequencing runs.

Table 13 Index Primer Combinations for 8-Sample Sequencing Runs

	Index Primer 1 (A701)	Index Primer 2 (A702)	Index Primer 10 (A710)
Index Primer C (A503)	Sample 1	Sample 2	Sample 3
Index Primer D (A504)	Sample 4	Sample 5	Sample 6
Index Primer E (A505)	Sample 7	Sample 8	--

Index Primer Sequences

Table 14 Index 1 (I7) Set 1 & Set 2 Primers

Index Primer	Sequence
Index Primer 1 (A701)	ATCACGAC
Index Primer 2 (A702)	ACAGTGGT
Index Primer 3 (A703)	CAGATCCA
Index Primer 4 (A704)	ACAAACGG
Index Primer 5 (A705)	ACCCAGCA
Index Primer 6 (A706)	AACCCCTC
Index Primer 7 (A707)	CCCAACCT
Index Primer 8 (A708)	CACCACAC
Index Primer 9 (A709)	GAAACCCA
Index Primer 10 (A710)	TGTGACCA
Index Primer 11 (A711)	AGGGTCAA
Index Primer 12 (A712)	AGGAGTGG

Table 15 Index 2 (I5) Primers

Index Primer	Sequence
Index Primer A (A501)	TGAACCTT
Index Primer B (A502)	TGCTAAGT
Index Primer C (A503)	TGTTCTCT
Index Primer D (A504)	TAAGACAC
Index Primer E (A505)	CTAATCGA
Index Primer F (A506)	CTAGAACA
Index Primer G (A507)	TAAGTCC
Index Primer H (A508)	TAGACCTA

Instructions for Use

Enter Run Information

MiSeq Reporter or Local Run Manager are the two software options available to set up a Cystic Fibrosis 139-Variant Assay run.

If you use MiSeq Reporter software, use Illumina Worklist Manager to generate a sample sheet.

If you use Local Run Manager software, there is no separate sample sheet. Enter run and sample setup information directly into the Local Run Manager CF 139 Variant analysis module.

For more information on the differences between MiSeq Reporter and Local Run Manager, see [MiSeqDx Instrument Interface Methods on page 5](#).

Using Illumina Worklist Manager (IWM)

MiSeqDx Sample Sheet Preparation

- 1 From the Illumina Worklist Manager Welcome screen, select **Create Worklist**.
- 2 In the Test Type field, select **CF 139-Variant Assay**.
- 3 In the Worklist Name field, enter a name for the sample sheet.
 - If the alpha-numeric reagent cartridge barcode ID is used for the sample sheet name, the MiSeq Operating Software (MOS) will find the sample sheet automatically.
 - If any other name is used for the sample sheet, the **Browse** button in the MiSeq Operating Software (MOS) can be used to locate the appropriate sample sheet.
- 4 **[Optional]** Enter a description to identify the run.
- 5 Make sure that the date matches the start date of the run.
- 6 Select **Next**.

Enter Sample Information

- 1 From the Table tab or the Plate tab, enter the following information for each sample well:
 - a **Sample ID**—Enter a unique sample ID.
 - b **Index 1 and Index 2**—Specify the index adapter that will be used for each Index Read.
- 2 **[Optional]** To record more detailed information about the samples, enter a sample name and description.
- 3 **[Optional]** To identify controls on the plate, select Negative or Positive from the **Control** drop-down menu.
- 4 Go to the Plate Graphic tab and use the **Copy to Clipboard** or **Print** option to capture an image of the sample plate.
- 5 Select **Finish**. When saving the sample sheet, the software creates both a .csv and .png file of the Plate Graphic automatically and saves them in the same location for use with experiment setup.



NOTE

Only use the Illumina Worklist Manager to edit sample sheet information. Editing outside of Illumina Worklist Manager can cause the run or the analysis to fail.

Using the Local Run Manager CF 139 Variant Analysis Module

Set Parameters

- 1 Log in to Local Run Manager.
- 2 Click **Create Run**, and select **CF 139**.
- 3 Enter a run name that identifies the run from sequencing through analysis.
Use alphanumeric characters, spaces, underscores, or dashes.
- 4 **[Optional]** Enter a run description to help identify the run.
Use alphanumeric characters.

Specify Samples for the Run

Specify samples for the run using the following options:


- **Enter samples manually**
 - 1 Select the number of sample from the Number of Samples drop-down box.
Consider the following information when you make a selection.
 - Choose the number of samples that is nearest to the number of samples you are testing. If the exact number of samples is not in the list, select the number nearest, but less than the number you are

testing, so that index diversity requirements are met. For example, if you want to test 18 samples, select 16 samples. Then add 2 additional samples. Make sure to select index adapters for extra wells.


- 2 Use the blank table on the Create Run screen.
Suggested sample wells are highlighted.

- **Import samples**—Navigate to an external file in a comma-separated values (*.csv) format. A template is available for download on the Create Run screen.

Enter Samples Manually

- 1 Enter a unique sample name in the Sample Name field.
Use alphanumeric characters, dashes, or underscores.
- 2 Right-click and select positive and negative control samples.
- 3 [Optional] Enter a sample description in the Sample Description tab.
Use alphanumeric characters, dashes, underscores, or spaces.
- 4 [Optional] Select an Index 1 adapter from the Index 1 (i7) drop-down list.
This step is optional because the i7 and i5 index combinations that autopopulate the wells highlighted already meet index diversity requirements.
- 5 [Optional] Select an Index 2 adapter from the Index 2 (i5) drop-down list.
This step is optional because the i7 and i5 index combinations that autopopulate the wells highlighted already meet index diversity requirements.
- 6 Click the  **Print** icon to display the plate layout.
- 7 Select **Print** to print the plate layout as a reference for preparing libraries.
- 8 [Optional] Click **Export** to export sample information to an external file.
- 9 Click **Save Run**.

Import Samples

- 1 Click **Import Samples** and browse to the location of the sample information file. There are two types of files you can import.
 - Click **Template** to create a new plate layout. The template file contains the correct column headings for import. Enter sample information in each column for the samples in the run. Delete example information in unused cells, and then save the file.
 - Use a file of sample information that was exported from the CF 139 Variant analysis module using the Export feature.
- 2 Click the  **Print** icon to display the plate layout.
- 3 Select **Print** to print the plate layout as a reference for preparing libraries.
- 4 Click **Save Run**.

Hybridization of Oligonucleotide Pool

Preparation

- 1 Bring the CF 139-Variant Assay-Oligo Pool, Hybridization Buffer, genomic DNA samples, and positive control sample to room temperature.
- 2 Vortex the CF 139-Variant Assay-Oligo Pool and Hybridization Buffer vigorously to make sure that all precipitates have completely dissolved, then briefly centrifuge the tubes to collect liquid.
- 3 Set a 96-well heat block to 95°C.
- 4 Pre-heat an incubator to 37°C.
- 5 Create the sample plate according to the plate graphic printed from Illumina Worklist Manager or Local Run Manager.

Procedure

- 1 Set out a new 96-well PCR plate (hereafter referred to as the **HYB** plate).
- 2 Add 5 μl of sample or control at 50 $\text{ng}/\mu\text{l}$ (250 ng total) to the appropriate wells in the **HYB** plate. Follow the generated plate layout for correct well selection.
- 3 Add 5 μl of the CF 139-Variant Assay-Oligo Pool to all sample wells.
- 4 Add 40 μl of Hybridization Buffer to each sample in the **HYB** plate. Gently pipette up and down 3–5 times to mix.
- 5 Seal the **HYB** plate and centrifuge 1000 \times g at 20°C for 1 minute.
- 6 Place the **HYB** plate in the pre-heated block at 95°C and incubate for 1 minute.
- 7 Reduce the heat block to 40°C and continue incubating until the heat block reaches 40°C (approximately 80 minutes).

Gradual cooling is critical for proper hybridization; therefore, PCR thermal cyclers with active cooling (e.g., Peltier, thermoelectric cooled) are not recommended for this process.



SAFE STOPPING POINT

After the heat block reaches 40°C, the **HYB** plate is stable holding at 40°C for 2 hours.

Removal of Unbound Oligonucleotides

Preparation

- 1 Bring Extension-Ligation Mix, Stringent Wash Buffer, and Universal Wash Buffer to room temperature, and then vortex briefly.
- 2 Assemble the filter plate assembly unit (hereafter referred to as the **FPU**) in order from top to bottom: lid, filter plate, adapter collar, and MIDI plate.
- 3 Pre-wash the filter plate membrane as follows:
 - a Add 45 μl of Stringent Wash Buffer to each well.
 - b Cover the filter plate with the lid and centrifuge at 2400 \times g at 20°C for 5 minutes.



NOTE

Check to verify that all wells of the filter plate are draining completely. If the wash buffer does not drain completely, centrifuge again at 2400 \times g at 20°C until all liquid has gone through (an additional 5–10 minutes).



CAUTION

It is critical to control the centrifuge temperature during the washing steps. If the temperature reaches 25°C or higher, the higher temperature may lead to higher stringency in primer binding. In rare cases, if samples have SNVs in primer binding regions, the higher stringency may lead to allele dropout.

Procedure

- 1 Remove the **HYB** plate from the heat block and centrifuge at 1000 \times g at 20°C for 1 minute.
- 2 Transfer the entire volume (approximately 55 μl) of each sample to the corresponding wells of the filter plate.
- 3 Cover the filter plate with the lid and centrifuge at 2400 \times g at 20°C for 5 minutes.
- 4 Wash the filter plate as follows:
 - a Add 45 μl of Stringent Wash Buffer to each sample well.
 - b Cover the filter plate with the lid and centrifuge at 2400 \times g at 20°C for 5 minutes.
- 5 Repeat the wash as described in the previous step.



NOTE

If the wash buffer does not drain completely, centrifuge again at 2400 \times g at 20°C until all liquid has gone through (an additional 5–10 minutes).

- 6 Discard all the flow-through (containing formamide), then reassemble the **FPU**.
- 7 Add 45 μl of Universal Wash Buffer to each sample well.
- 8 Cover the filter plate with the lid and centrifuge at 2400 \times g at 20°C for 10 minutes.



NOTE

Make sure that all liquid has drained after centrifugation. Repeat centrifugation if necessary.

Extension-Ligation of Bound Oligonucleotides

Procedure

- 1 Add 45 μ l of Extension-Ligation Mix to each sample well of the filter plate.
- 2 Seal the filter plate with adhesive aluminum foil, and then cover with the lid.
- 3 Incubate the **FPU** in the pre-heated 37°C incubator for 45 minutes.
- 4 While the **FPU** plate is incubating, prepare the AMP (Amplification Plate) as described in the following section.

PCR Amplification

Preparation

- 1 Prepare fresh 0.05 N NaOH.
- 2 Determine the index primers to be used according to the plate graphic printout from Illumina Worklist Manager or Local Run Manager.
- 3 Bring PCR Master Mix and the appropriate index primers to room temperature. Vortex each thawed tube to mix, and then briefly centrifuge the tubes.
- 4 Set out a new 96-well PCR plate (hereafter referred to as the **AMP** plate).
- 5 Add index primers to the AMP plate as follows:
 - a Add 4 μ l of the selected index primers [A (A501) – H (A508)] to the appropriate well in a column of the **AMP** plate.
 - b Discard the original white caps and apply new white caps.
 - c Add 4 μ l of the selected index primers [1 (A701) – 12 (A712)] to the appropriate row of the **AMP** plate. *Tips must be changed after each row to avoid index cross-contamination.*
 - d Discard the original orange caps and apply new orange caps.
- 6 Prepare the PCR Master Mix/PCR Polymerase PCR working solution as follows:
 - a Briefly centrifuge the PCR Polymerase tube before use to remove air bubbles.
 - b For 96 samples, add 56 μ l of PCR Polymerase to 2.8 ml of PCR Master Mix.
 - c Invert the prepared PCR working solution 20 times to mix.

The PCR working solution is stable at room temperature for 10 minutes.

Procedure

- 1 Remove the **FPU** from the incubator, and then remove the aluminum foil seal.
- 2 Cover the filter plate with the lid and centrifuge at 2400 \times g at 20°C for 2 minutes.
- 3 Add 25 μ l of 0.05 N NaOH to each sample well on the filter plate. Pipette the NaOH up and down 5–6 times.
- 4 Cover and incubate the filter plate at room temperature for 5 minutes.
- 5 While the filter plate is incubating, transfer 22 μ l of the PCR working solution to each well of the AMP plate containing index primers.
- 6 Transfer samples eluted from the filter to the AMP plate as follows:
 - a Pipette the samples in the first column of the filter plate up and down 5–6 times.
 - b Transfer 20 μ l from the filter plate to the corresponding column of the **AMP** plate.
 - c Gently pipette up and down 5–6 times to thoroughly combine the DNA with the PCR working solution.
 - d Transfer the remaining columns from the filter plate to the AMP plate in a similar manner. *Tips must be changed after each column to avoid index and sample cross-contamination.*
- 7 Seal the **AMP** plate and secure with a rubber roller.
- 8 Centrifuge at 1000 \times g at 20°C for 1 minute.
- 9 Transfer the **AMP** plate to the post-amplification area.
- 10 Perform PCR using the following program on a thermal cycler:
 - 95°C for 3 minutes

- 25 cycles of:
 - 95°C for 30 seconds
 - 62°C for 30 seconds
 - 72°C for 60 seconds
- 72°C for 5 minutes
- Hold at 10°C

**SAFE STOPPING POINT**

If not proceeding immediately to PCR Clean-Up, the **AMP** plate can remain on the thermal cycler overnight, or can be stored at 2°C to 8°C up to 48 hours.

PCR Clean-Up

Preparation

- 1 Bring the PCR Clean-Up Beads to room temperature.
- 2 Prepare fresh 80% ethanol from absolute ethanol.

Procedure

- 1 Centrifuge the AMP plate at 1000 × g at 20°C for 1 minute.
- 2 Set out a new MIDI plate (hereafter referred to as the **CLP** plate).
- 3 Invert PCR Clean-Up Beads 10 times. Vortex vigorously and then invert 10 more times. Visually inspect solution to ensure that beads are resuspended.
- 4 Add 45 µl of PCR Clean-Up Beads to each well of the **CLP** plate.
- 5 Transfer the entire PCR product from the AMP plate to the **CLP** plate.
- 6 Seal the **CLP** plate and shake on a microplate shaker at 1800 rpm for 2 minutes.
- 7 Incubate at room temperature without shaking for 10 minutes.
- 8 Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 9 With the **CLP** plate on the magnetic stand, carefully remove and discard the supernatant.
- 10 With the **CLP** plate on the magnetic stand, wash the beads as follows:
 - a Add 200 µl of freshly prepared 80% ethanol to each sample well.
 - b Incubate the plate on the magnetic stand for a minimum of 30 seconds or until the supernatant is clear.
 - c Carefully remove and discard the supernatant.
- 11 Repeat the wash as described in the previous step.
- 12 Use a P20 multi-channel pipette set to 20 µl to remove excess ethanol.
- 13 Remove the **CLP** plate from the magnetic stand and air-dry the beads for 10 minutes.
- 14 Add 30 µl of Elution Buffer to each sample.
- 15 Seal the **CLP** plate and shake on a microplate shaker at 1800 rpm for 2 minutes. After shaking, verify if samples were resuspended. If not, repeat this step.
- 16 Incubate at room temperature for 2 minutes.
- 17 Place the **CLP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 18 Set out a new MIDI plate (hereafter referred to as the **LNP** plate).
- 19 Transfer 20 µl of the supernatant from the **CLP** plate to the **LNP** plate.
- 20 **[Optional]** Transfer the remaining 10 µl of supernatant from the **CLP** plate to a new plate and label the plate with a run name and date. Store this plate at -25°C to -15°C until completion of the sequencing run and data analysis. The cleaned up PCR products can be used for troubleshooting efforts in the event of sample failures.

**SAFE STOPPING POINT**

If stopping at this point, seal the **LNP** plate and centrifuge at 1000 × g at 20°C for 1 minute. The plate is stable for up to 3 hours at 2°C to 8°C.

Library Normalization and Pooling

Preparation

- 1 Prepare fresh 0.1 N NaOH by adding 30 μ l of 10 N NaOH to 2970 μ l of RNase/DNase-free water.
- 2 Bring Library Normalization Diluent, Library Beads, Library Normalization Wash, and Library Dilution Buffer to room temperature.
- 3 Vortex Library Normalization Diluent vigorously and ensure that all precipitates have dissolved.
- 4 Vortex Library Beads vigorously for 1 minute with intermittent inversion until the beads are resuspended and no pellet is found at the bottom of the tube when the tube is inverted.

Procedure

- 1 Mix Library Normalization Diluent and Library Beads in a fresh 15 ml conical tube as follows:



NOTE

If processing < 24 samples, use a fresh 1.5 ml tube.

- a For 96 samples, add 4.4 ml of Library Normalization Diluent.
- b Pipette Library Beads up and down 10 times to resuspend.



NOTE

It is extremely critical to completely resuspend the library bead pellet at the bottom of the tube. The use of a P1000 ensures that the beads are homogeneously resuspended and that there is no bead mass at the bottom of the tube. This is essential for achieving consistent cluster density on the flow cell.

- c For 96 samples, pipette 800 μ l of Library Beads to the tube containing Library Normalization Diluent.
- d Mix by inverting the tube 15–20 times.
- 2 Add 45 μ l of the combined Library Normalization Diluent/Library Beads working solution to each well of the **LNP** plate containing libraries.
- 3 Seal the **LNP** plate and shake on a microplate shaker at 1,800 rpm for 30 minutes.



NOTE

If proceeding with sequencing on the same day, now is a good time to begin thawing the reagent cartridge. Follow the instructions to thaw the MiSeqDx Reagent cartridge in the section entitled [Prepare the Reagent Cartridge on page 22](#).

- 4 Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 5 With the **LNP** plate on the magnetic stand, carefully remove and discard the supernatant.
- 6 Remove the **LNP** plate from the magnetic stand and wash the beads with Library Normalization Wash as follows:
 - a Add 45 μ l of Library Normalization Wash to each sample well.
 - b Seal the **LNP** plate and shake on a microplate shaker at 1,800 rpm for 5 minutes.
 - c Place the plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
 - d Carefully remove and discard the supernatant.
- 7 Repeat the Library Normalization Wash procedure as described in the previous step.
- 8 Use a P20 multi-channel pipette set to 20 μ l to remove excess Library Normalization Wash.
- 9 Remove the **LNP** plate from the magnetic stand and add 30 μ l of 0.1 N NaOH to each well.
- 10 Seal the **LNP** plate and shake on a microplate shaker at 1,800 rpm for 5 minutes.
- 11 During the 5 minute elution, set out a new 96-well PCR plate (hereafter referred to as the **SGP** plate.)
- 12 Add 30 μ l Library Storage Buffer to each well to be used in the **SGP** plate.
- 13 After the 5-minute elution, ensure all samples in the **LNP** plate are completely resuspended. If the samples are not completely resuspended, gently pipette those samples up and down or lightly tap the plate on the bench to resuspend the beads, then shake for another 5 minutes.
- 14 Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes.
- 15 Transfer the supernatant from the **LNP** plate to the **SGP** plate. Gently pipette up and down 5 times to mix.
- 16 Seal the **SGP** plate and then centrifuge at 1,000 \times g at 20°C for 1 minute.

- 17 Vortex the Library Dilution Buffer and make sure that all the precipitates have dissolved completely.
- 18 Briefly centrifuge to collect contents.
- 19 Set out a fresh Eppendorf tube (hereafter referred to as the **PAL** [Pooled Amplicon Library] tube).
- 20 Determine the samples to be pooled for sequencing. A maximum of 48 samples can be pooled for sequencing.
- 21 Transfer 5 μ l of each library to be sequenced from the **SGP** plate, column by column, to a PCR eight-tube strip.
- 22 Combine and transfer the contents of the PCR eight-tube strip into the **PAL** tube. Mix the **PAL** tube thoroughly.
- 23 Set out 2–3 fresh Eppendorf tubes (hereafter referred to as the **DAL** [Diluted Amplicon Library] tubes).
- 24 Add 585 μ l of Library Dilution Buffer to the **DAL** tubes.
- 25 Transfer 9 μ l of **PAL** to each **DAL** tube containing Library Dilution Buffer. Pipette up and down 3–5 times to rinse the tip and to make sure the transfer is complete.

**SAFESTOPPING POINT**

If not proceeding immediately to sequencing on the MiSeqDx, the **DAL** tubes can be stored at -25°C to -15°C for up to 14 days.

Library Sequencing

Prepare for Library Sequencing

- 1 Set a heat block suitable for 1.5 ml centrifuge tubes to 96°C .
- 2 In an ice bucket, prepare an ice-water bath. Chill the Library Dilution Buffer in the ice-water bath.
- 3 Begin thawing the MiSeqDx reagent cartridge.

Prepare the Reagent Cartridge

- 1 Thaw the MiSeqDx Reagent Cartridge - CF 139-Variant Assay in a water bath containing enough room temperature laboratory-grade water to submerge the base of the reagent cartridge up to the water line printed on the reagent cartridge. Do not allow the water to exceed the maximum water line.
- 2 Allow the reagent cartridge to thaw in the room temperature water bath for approximately 1 hour or until thawed.
- 3 Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge. Make sure that no water has splashed on the top of the reagent cartridge.

Inspect the Reagent Cartridge

- 1 Invert the reagent cartridge ten times to mix the thawed reagents, and then inspect that all positions are thawed.

**NOTE**

It is critical that the reagents in the cartridge are thoroughly thawed and mixed to ensure proper sequencing.

- 2 Inspect reagents in positions 1, 2, and 4 to make sure that they are fully mixed and free of precipitates.
- 3 Gently tap the cartridge on the bench to reduce air bubbles in the reagents.

**NOTE**

The MiSeqDx sipper tubes go to the bottom of each reservoir to aspirate the reagents, so it is important that the reservoirs are free of air bubbles.

- 4 Place the reagent cartridge on ice or set aside at 2°C to 8°C (up to 6 hours) until ready to set up the run. For best results, proceed directly to loading the sample and setting up the run.

Denature and Dilute PhiX Internal Control

- 1 Prepare 0.1N NaOH by combining the following volumes in a conical tube:
 - DNase/RNase-free water (2475 μ l)
 - Stock 10 N NaOH (25 μ l)

- Invert the tube several times to mix.

**CAUTION**

Using freshly diluted NaOH is essential in order to completely denature samples for cluster generation on the MiSeqDx.

**NOTE**

If PhiX is prepared the same day as Library Normalization, the same stock of 0.1N NaOH can be used.

- Combine the following volumes to dilute the PhiX Internal Control library to 2 nM:
 - 10 nM PhiX Internal Control library (2 μ l)
 - 1X TE Buffer (8 μ l)
- Combine the following volumes to result in a 1 nM PhiX Internal Control library:
 - 2 nM PhiX Internal Control library (10 μ l)
 - 0.1 N NaOH (10 μ l)
- Vortex briefly to mix the 1 nM PhiX Internal Control library solution.
- Centrifuge the 1nM PhiX Internal Control at $280 \times g$ at 20°C for 1 minute.
- Incubate for 5 minutes at room temperature to denature the PhiX Internal Control library solution into single strands.
- Combine the following volumes in a new microcentrifuge tube to result in a 20 pM PhiX Internal Control library:
 - Denatured PhiX Internal Control library (2 μ l)
 - Pre-chilled Library Dilution Buffer (98 μ l)

**NOTE**

The denatured 20 pM PhiX Internal Control library can be stored up to 3 weeks at -25°C to -15°C as single-use aliquots.

Prepare Samples for Sequencing

- Proceed with one **DAL** tube for sequencing.
- If the **DAL** tube was stored frozen, thaw completely and mix by pipetting up and down.
- Add 6 μ l of 20 pM PhiX Internal Control to the **DAL** tube.
- Pipette up and down 3–5 times to rinse the tip and ensure complete transfer.
- Mix the **DAL** tube by vortexing the tube at top speed.
- Centrifuge the **DAL** tube at $1000 \times g$ at 20°C for 1 minute.
- Incubate the **DAL** tube on a heat block at 96°C for 2 minutes.
- After the incubation, invert the **DAL** tube 1–2 times to mix, then immediately place in the ice-water bath.
- Keep the **DAL** tube in the ice-water bath for 5 minutes.

**NOTE**

Perform the heat denaturation step immediately before loading the **DAL** tube into the MiSeqDx reagent cartridge to ensure efficient template loading on the MiSeqDx flow cell.

Load Sample Libraries onto Cartridge

- Use a separate, clean, and empty 1 ml pipette tip to pierce the foil seal over the reservoir on the reagent cartridge labeled **Load Samples**.
- Pipette 600 μ l of the **DAL** sample libraries into the **Load Samples** reservoir. Avoid touching the foil seal.
- Check for air bubbles in the reservoir after loading sample. If air bubbles are present, gently tap the cartridge on the bench to release the bubbles.
- Proceed directly to the run setup steps using the MiSeq Operating Software (MOS) interface.

Interpretation of Results

- 1 The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay is designed to detect 139 CFTR variants, including those recommended by ACMG (Table 2).
- 2 The assay report lists the sample names and genotype for each variant detected for a sample.
 - All samples are interrogated for 134 CF-causing variants and the ACMG recommended R117H variant; only detected mutant alleles are listed in the assay report.
 - The PolyTG/PolyT variant is reported only if the R117H variation is identified for a sample. For patients with an R117H variant, additional testing to determine whether a PolyTG/PolyT variant, which may affect the clinical phenotype [e.g., 12-13 (TG) or 5T] is in cis/trans orientation to the R117H variant, should be performed.



NOTE

The PolyTG/PolyT genotype is determined by the MiSeqDx Cystic Fibrosis 139-Variant Assay based on read counting of the most common genotypes. Due to the digital nature of next generation sequencing, the assay is able to achieve high accuracy from multiple observations in comparison to other sequencing-based technologies that use just a few observations.

- When a sample has homozygous F508del or I507del genotype, if one or more among the three benign polymorphisms I506V, I507V and F508C are detected, this is reported for the sample. If all three benign polymorphisms are wild type, the report indicates that I506V, I507V and F508C variants are not present for the sample.



NOTE

Because this is a sequencing-based assay, there is no interference to F508del or I507del reporting on account of the three benign polymorphisms. Hence, no corrections will be made to the detected result.

- The genotype result is reported as HET when a sample is identified as heterozygous and both wild type and mutant alleles are detected for the sample.
 - The genotype result is reported as HOM when a sample is identified as homozygous and only the mutant allele is detected for the sample.
 - If no variant is identified for a sample, the report indicates "No panel variants are detected".
- 3 The assay report provides sample call rate information for each sample. Call rate is calculated as the number of variant positions/regions that meet a pre-defined confidence value threshold divided by the total positions/regions interrogated.
 - For samples requiring conditional reporting, the additional variants interrogated are also accounted for in the call rate calculation.
 - Any variant with a pre-defined confidence value below the threshold is reported as a "No call". It is recommended that the sample be repeated.
 - 4 A sample result is considered valid only if the call rate is $\geq 99\%$. If the call rate is below 99%, the performance will be reported as "Fail", and the sample must be repeated.

NOTE: If the sample call rate is $< 50\%$, the performance will be reported as "Fail" and a "Sample Failed" comment will be indicated on the report. There will be no variant information displayed. This sample must be repeated.
 - 5 It is recommended that variants that were validated using synthetic specimens (see Accuracy Table) be verified by the user using a validated reference method prior to reporting the first patient result with those variants.
 - 6 If more than two variants are identified for a sample, it is recommended that the user verify the result by repeating the sample using the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay with a fresh gDNA extract to rule out cross-contamination of the specimen.



NOTE

Haplotype phasing should be considered when two or more variants are detected.

- 7 All variant interpretations should be made by a certified clinical molecular geneticist or equivalent following local procedures and guidelines¹⁵. Potential interpretation references include but are not limited to: CFTR2 database¹¹, Sosnay paper¹³, ACMG 2004 guidelines¹, and 2011 ACOG committee opinion². For information on how results are calculated and presented, or for a description of the contents in text file report, see the guides for the analysis software installed with your MiSeqDx. For MiSeq Reporter, see *MiSeq Reporter Software Reference Guide* (document # 15038356). For Local Run Manager, see *Local Run Manager Software Reference Guide for MiSeqDx* (document # 1000000011880) and *Local Run Manager CF 139 Variant Analysis Module Workflow Guide* (document # 1000000012184).

Quality Control Procedures

Good laboratory practices dictate that control material should be evaluated to detect differences in blood processing and technical procedures in the user's laboratory that may produce significant variability in results.

- 1 **Positive Controls**—A positive DNA control sample is required on every run. The positive control DNA sample should be a well-characterized sample with at least one known CFTR variant¹⁶. Illumina recommends the use of rotating positive controls consistent with the 2008 ACMG Technical Standards and Guidelines for CF mutation testing¹⁷ and the 2013 ACMG clinical laboratory standards for next-generation sequencing¹⁸. The positive control sample must generate the expected genotype. If the positive control generates a genotype different from what is expected, then an error in sample tracking or incorrect recording of indexing primers may have occurred. The entire assay must be repeated, starting from library preparation.
- 2 **Negative (No Template/No DNA) Control**— The use of a negative (no template/no DNA) control is required on every run to detect possible incidences of contamination. The call rate for the negative control should be less than 10%. If a negative control generates a call rate > 10%, then contamination during assay processing may have occurred. The assay is considered as having failed and the entire assay must be repeated, starting from library preparation.



NOTE

The negative control sample is reported as "Pass" if it generates a call rate ≤ 10%, and "Fail" if the call rate is > 10%. Also, just as with samples, when the call rate is < 50%, a "Sample Failed" comment will be indicated on the report.

- 3 **Wild Type Control**—The wild type DNA control sample is recommended on every run. The wild type control sample should be a well characterized sample that does not contain any CFTR variants. The wild type control sample must generate the expected genotype. If the wild type control generates a genotype different from what is expected, then an error in sample tracking or incorrect recording of indexing primers may have occurred. The entire assay must be repeated, starting from library preparation.
- 4 Prior to initial use of this product in the user's laboratory, the performance of the assay should be verified by testing a number of positive and negative samples with known performance characteristics.
- 5 All quality control requirements should be performed in accordance with local, state and/or federal regulations or accreditation requirements.

Performance Characteristics

Accuracy

Accuracy of the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay was assessed by evaluating 500 samples representing a wide variety of CFTR variants from four separate sources. The primary source of accuracy data was a clinical accuracy study conducted using a panel of 366 samples. The majority (n = 355) of samples consisted of archived, anonymized clinical gDNA specimens isolated from human blood, the remaining 11 samples were obtained from commercially available cell line specimens.

Data from this study was supplemented with accuracy data from 68 cell line samples evaluated in the reproducibility study, 14 clinical samples from the extraction method evaluation analytical study, and 52 synthetic plasmid samples. The synthetic plasmids were designed to include the genomic context of the rare variants, and contained anywhere from one to nine variants within the same construct. They were linearized, diluted to genomic DNA equivalent copy numbers, and blended with human genomic DNA samples of wild type genotype at equivalent copy numbers to mimic a heterozygous sample.

The genotyping results for 137 SNV/small InDel sites, including the PolyTG/PolyT region were compared to Sanger bi-directional sequence analysis. Two validated PCR based assays were used as the reference method for the two large deletions in the panel. Each duplex PCR assay made use of two primer sets to discriminate between wild type, heterozygous, and homozygous genotypes. One of the primer sets was designed to flank the deletion breakpoints, whereas the other amplified a region internal to the deletion. The two products were detected by size separation on an agarose gel.

The PCR assays were validated using a panel of 28 samples in all (22 samples for each deletion) consisting of cell line and blood derived genomic DNA samples and synthetic plasmids, which encompassed the WT, HET, and HOM genotypes for each large deletion. The PCR assays were confirmed to have 100% specificity and reproducibility for all samples tested, by evaluation of PCR products on an agarose gel. The accuracy of the PCR assays was confirmed using Sanger Sequencing and found to be 100% for all samples.

Accuracy was determined for each genotype through three statistical measures. Positive Agreement (PA) was calculated for each variant genotype by dividing the number of samples with agreeing variant calls by the total number of samples with that variant as identified by the reference methods. Negative Agreement (NA) was calculated across all wild type (WT) positions by dividing the number of concordant WT positions by the total number of WT positions as defined by the reference methods. Overall Agreement (OA) was calculated across all reported positions by dividing the number of concordant WT and variant positions by the total number of reported positions as determined by the reference methods.

The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay had a genotype-level PA of 100%. The NA for all WT positions was > 99.99%, and the OA for all reported positions was > 99.99%. All test results are based on initial testing.

Table 16 Overall Accuracy of the MiSeqDx Cystic Fibrosis 139-Variant Assay

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
CFTR dele2, 3	DEL	c.54-5940_ 273+10250 del21kb	500	4	1	0	495	0	0	100	100	100
E60X	SNV	c.178G>T	500	6	1	0	493	0	0	100	100	100
P67L	SNV	c.200C>T	500	1	0	1	498	0	0	100	100	100
R75X	SNV	c.223C>T	500	3	1	0	496	0	0	100	100	100
G85E	SNV	c.254G>A	500	6	2	0	492	0	0	100	100	100
394deITT	DIV	c.262_263 deITT	500	3	1	0	496	0	0	100	100	100
406-1G>A	SNV	c.274-1G>A	500	4	0	0	496	0	0	100	100	100
E92X	SNV	c.274G>T	500	0	1	1	498	0	0	100	100	100
D110H	SNV	c.328G>C	500	1	0	1	498	0	0	100	100	100
R117C	SNV	c.349C>T	500	4	0	0	496	0	0	100	100	100
R117H	SNV	c.350G>A	500	17	2	0	481	0	0	100	100	100
Y122X	SNV	c.366T>A	500	0	1	0	499	0	0	100	100	100
621+1G>T	SNV	c.489+1G>T	500	7	5	0	488	0	0	100	100	100
663deIT	DIV	c.531deIT	500	1	0	1	498	0	0	100	100	100
G178R	SNV	c.532G>A	500	1	1	0	498	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
711+1G>T	SNV	c.579+1G>T	500	3	1	0	496	0	0	100	100	100
P205S*	SNV	c.613C>T	500	1	0	1	498	0	0	100*	100	100
L206W	SNV	c.617T>G	500	8	1	0	491	0	0	100	100	100
1078delT	DIV	c.948delT	500	1	1	0	498	0	0	100	100	100
G330X	SNV	c.988G>T	500	1	1	0	498	0	0	100	100	100
R334W	SNV	c.1000C>T	500	6	1	0	493	0	0	100	100	100
I336K	SNV	c.1007T>A	500	0	1	0	499	0	0	100	100	100
1154insTC	DIV	c.1022_1023 insTC	500	0	1	0	499	0	0	100	100	100
R347H	SNV	c.1040G>A	500	6	1	1	492	0	0	100	100	100
R347P	SNV	c.1040G>C	500	3	2	0	495	0	0	100	100	100
R352Q	SNV	c.1055G>A	500	5	0	0	495	0	0	100	100	100
A455E	SNV	c.1364C>A	500	4	2	0	494	0	0	100	100	100
S466X (C>G)	SNV	c.1397C>G	500	1	0	1	498	0	0	100	100	100
1548delG	DIV	c.1418delG	500	1	0	1	498	0	0	100	100	100
Q493X	SNV	c.1477C>T	500	4	2	0	494	0	0	100	100	100
I507del	DIV	c.1519_1521 delATC	500	4	2	0	494	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
F508del	DIV	c.1521_1523 delCTT	500	84	29	0	387	0	0	100	100	100
1677delTA	DIV	c.1545_1546 delTA	500	1	0	0	499	0	0	100	100	100
V520F	SNV	c.1558G>T	500	2	0	0	498	0	0	100	100	100
1717-1G>A	SNV	c.1585-1G>A	500	4	1	0	495	0	0	100	100	100
G542X	SNV	c.1624G>T	500	12	3	0	485	0	0	100	100	100
S549N	SNV	c.1646G>A	500	2	2	1	495	0	0	100	100	100
S549R (c.1647T>G)	SNV	c.1647T>G	500	3	1	0	496	0	0	100	100	100
G551D	SNV	c.1652G>A	500	8	3	0	489	0	0	100	100	100
R553X	SNV	c.1657C>T	500	8	2	0	490	0	0	100	100	100
A559T	SNV	c.1675G>A	500	4	0	1	495	0	0	100	100	100
R560T	SNV	c.1679G>C	500	6	1	0	493	0	0	100	100	100
1812-1 G>A	SNV	c.1680-1G>A	500	0	2	0	498	0	0	100	100	100
1898+1G>A	SNV	c.1766+1G>A	500	2	1	0	497	0	0	100	100	100
2143delT	DIV	c.2012delT	500	2	1	0	497	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
2183AA>G	DIV	c.2051_2052delAAinsG	500	3	1	0	496	0	0	100	100	100
2184insA	DIV	c.2052_2053insA	500	3	0	1	496	0	0	100	100	100
2184delA	DIV	c.2052delA	500	1	1	0	498	0	0	100	100	100
R709X	SNV	c.2125C>T	500	1	0	2	497	0	0	100	100	100
K710X	SNV	c.2128A>T	500	3	0	0	497	0	0	100	100	100
2307insA	DIV	c.2175_2176insA	500	3	0	2	495	0	0	100	100	100
R764X	SNV	c.2290C>T	500	1	0	2	497	0	0	100	100	100
W846X	SNV	c.2537G>A	500	0	1	0	499	0	0	100	100	100
2789+5G>A	SNV	c.2657+5G>A	500	9	1	0	490	0	0	100	100	100
Q890X	SNV	c.2668C>T	500	1	0	0	499	0	0	100	100	100
3120G>A	SNV	c.2988G>A	500	1	0	0	499	0	0	100	100	100
3120+1G>A	SNV	c.2988+1G>A	500	7	1	0	492	0	0	100	100	100
3272-26A>G	SNV	c.3140-26A>G	500	0	1	0	499	0	0	100	100	100
R1066C	SNV	c.3196C>T	500	6	0	0	494	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
R1066H	SNV	c.3197G>A	500	1	0	1	498	0	0	100	100	100
W1089X	SNV	c.3266G>A	500	4	0	0	496	0	0	100	100	100
Y1092X (C>A)	SNV	c.3276C>A	500	3	1	0	496	0	0	100	100	100
M1101K	SNV	c.3302T>A	500	2	2	0	496	0	0	100	100	100
R1158X	SNV	c.3472C>T	500	7	1	0	492	0	0	100	100	100
R1162X	SNV	c.3484C>T	500	5	1	0	494	0	0	100	100	100
3659delC	DIV	c.3528delC	500	4	1	0	495	0	0	100	100	100
S1196X	SNV	c.3587C>G	500	1	0	0	499	0	0	100	100	100
3791delC	DIV	c.3659delC	500	2	0	0	498	0	0	100	100	100
3849+10kbC>T	SNV	c.3717+12 191C>T	500	11	2	0	487	0	0	100	100	100
3876delA	DIV	c.3744delA	500	6	1	0	493	0	0	100	100	100
S1251N	SNV	c.3752G>A	500	1	0	1	498	0	0	100	100	100
3905insT	DIV	c.3773_3 774insT	500	3	1	0	496	0	0	100	100	100
W1282X	SNV	c.3846G>A	500	9	1	0	490	0	0	100	100	100
N1303K	SNV	c.3909C>G	500	9	1	0	490	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
CFTR dele22,23 [§]	DEL	c.3964-78_ 4242+577del	500	1	0	1	498	1 [§]	0	100	99.80	99.80
M1V	SNV	c.1A>G	500	0	0	1	499	0	0	100	100	100
Q39X	SNV	c.115C>T	500	0	0	1	499	0	0	100	100	100
405+1 G>A	SNV	c.273+1G>A	500	0	0	1	499	0	0	100	100	100
E92K	SNV	c.274G>A	500	0	0	1	499	0	0	100	100	100
Q98X	SNV	c.292C>T	500	0	0	2	498	0	0	100	100	100
457TAT>G	DIV	c.325_327 delTATinsG	500	0	0	1	499	0	0	100	100	100
574delA	DIV	c.442delA	500	0	0	2	498	0	0	100	100	100
711+3A>G	SNV	c.579+3A>G	500	0	0	1	499	0	0	100	100	100
711+5 G>A	SNV	c.579+5G>A	500	0	0	1	499	0	0	100	100	100
712-1 G>T	SNV	c.580-1G>T	500	0	0	1	499	0	0	100	100	100
H199Y	SNV	c.595C>T	500	0	0	1	499	0	0	100	100	100
Q220X	SNV	c.658C>T	500	0	0	1	499	0	0	100	100	100
852del22	DIV	c.720741 delAGGG AGAAT GATGAT GAAGTAC	500	0	0	1	499	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
T338I	SNV	c.1013C>T	500	0	0	1	499	0	0	100	100	100
S341P	SNV	c.1021T>C	500	0	0	1	499	0	0	100	100	100
1213delT	DIV	c.1081delT	500	0	0	1	499	0	0	100	100	100
1248+1G>A	SNV	c.1116+1G>A	500	0	0	1	499	0	0	100	100	100
1259insA	DIV	c.1127_1 128insA	500	0	0	2	498	0	0	100	100	100
W401X (c.1202G>A)	SNV	c.1202G>A	500	0	0	1	499	0	0	100	100	100
W401X (c.1203G>A)	SNV	c.1203G>A	500	0	0	1	499	0	0	100	100	100
1341+1G>A	SNV	c.1209+1G>A	500	0	0	2	498	0	0	100	100	100
1461ins4	DIV	c.1329_ 1330ins AGAT	500	0	0	1	499	0	0	100	100	100
1525-1G>A	SNV	c.1393-1G>A	500	0	0	1	499	0	0	100	100	100
S466X (C>A)	SNV	c.1397C>A	500	0	0	1	499	0	0	100	100	100
L467P	SNV	c.1400T>C	500	0	0	1	499	0	0	100	100	100
S489X	SNV	1466C>A	500	0	0	2	498	0	0	100	100	100
S492F	SNV	c.1475C>T	500	0	0	1	499	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
Q525X	SNV	c.1573C>T	500	0	0	1	499	0	0	100	100	100
1717-8G>A	SNV	c.1585-8G>A	500	0	0	1	499	0	0	100	100	100
S549R (c.1645A>C)	SNV	c.1645A>C	500	0	0	1	499	0	0	100	100	100
Q552X	SNV	c.1654C>T	500	0	0	1	499	0	0	100	100	100
R560K	SNV	c.1679G>A	500	0	0	1	499	0	0	100	100	100
1811+1.6kb A>G	SNV	c.1679+1.6 kbA>G	500	0	0	1	499	0	0	100	100	100
E585X	SNV	c.1753G>T	500	0	0	1	499	0	0	100	100	100
1898+3A>G	SNV	c.1766+3A>G	500	0	0	1	499	0	0	100	100	100
L732X	SNV	c.2195T>G	500	0	0	2	498	0	0	100	100	100
2347delG	DIV	c.2215delG	500	0	0	2	498	0	0	100	100	100
2585delT	DIV	c.2453delT	500	0	0	2	498	0	0	100	100	100
E822X	SNV	c.2464G>T	500	0	0	2	498	0	0	100	100	100
2622+1G>A [¶]	SNV	c.2490+1G>T [¶]	500	0	0	2	498	0	0	100	100	100
E831X	SNV	c.2491G>T	500	0	0	1	499	0	0	100	100	100
R851X	SNV	c.2551C>T	500	0	0	1	499	0	0	100	100	100
2711delT	DIV	c.2583delT	500	0	0	1	499	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
L927P	SNV	c.2780T>C	500	0	0	1	499	0	0	100	100	100
S945L	SNV	c.2834C>T	500	0	0	1	499	0	0	100	100	100
3007delG	DIV	c.2875delG	500	0	0	1	499	0	0	100	100	100
G970R	SNV	c.2908G>C	500	0	0	1	499	0	0	100	100	100
3121-1G>A	SNV	c.2989-1G>A	500	0	0	1	499	0	0	100	100	100
L1065P	SNV	c.3194T>C	500	0	0	1	499	0	0	100	100	100
L1077P^	SNV	c.3230T>C	500	0	0	1	499	0^	0	100	100	100
Y1092X (C>G)	SNV	c.3276C>G	500	0	0	1	499	0	0	100	100	100
E1104X	SNV	c.3310G>T	500	0	0	1	499	0	0	100	100	100
W1204X (c.3611G>A)	SNV	c.3611G>A	500	0	0	1	499	0	0	100	100	100
W1204X (c.3612G>A)	SNV	c.3612G>A	500	0	0	1	499	0	0	100	100	100
G1244E	SNV	c.3731G>A	500	0	0	1	499	0	0	100	100	100
4005+1G>A	SNV	c.3873+1G>A	500	0	0	1	499	0	0	100	100	100
4016insT	DIV	c.3884_3 885insT	500	0	0	1	499	0	0	100	100	100
Q1313X	SNV	c.3937C>T	500	0	0	1	499	0	0	100	100	100

Variant (Common Name)	Variant Type	cDNA Name	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
				Clinical Samples	Cell Line Samples	Synthetic Samples						
4209TG TT>AA	DIV	c.4077_4080delT GTTinsAA	500	0	0	1	499	0	0	100	100	100
4382delA	DIV	c.4251delA	500	0	0	1	499	0	0	100	100	100
PolyTG/ PolyT ^ε	PolyTGPolyT	c.1210-12T[5_9]	19	17	2	0	0	0	0	100	N/A	100
I506V [‡]	SNV	c.1516A>G	1	0	0	0	1	0	0	N/A	100	100
I507V [‡]	SNV	c.1519A>G	1	0	0	0	1	0	0	N/A	100	100
F508C [‡]	SNV	c.1523T>G	1	0	0	0	1	0	0	N/A	100	100
Total			67522	557			66965	1	0	100	> 99.99	> 99.99

DIV is an acronym for Deletion/Insertion Variant.

* The Sanger report indicated the P205S variant as heterozygous for the clinical sample. A review of the Sanger trace data however indicated that the variant was in fact homozygous and incorrectly reported. The MiSeqDx reported the variant as homozygous.

§ A synthetic sample heterozygous for exon 8 was reported as heterozygous for the variant CFTR dele22, 23. Further investigation revealed that this result was likely from low-level contamination.

^ The original synthetic heterozygous specimen was determined to be improperly prepared. When it was subsequently tested after it was reprepared, using the same plasmid, it was detected.

ε When R117H is positive, the PolyTG/PolyT variant is additionally reported.

‡ In the case of one homozygous F508del variant, three additional wild type bases (i.e., variants I506V, I507V, F508C) that were not identified in the specimen were additionally reported.

¶ The original validation study for the assay included 2 synthetic samples containing the nucleotide change c.2490+1G>T for variant 2622+1 G>A (data are included in this table). A second validation study was later done with a synthetic sample containing the nucleotide change c.2490+1G>A to support the actual nucleotide change (c.2490+1G>A) associated with the variant.

Table 17 Accuracy of the MiSeqDx Cystic Fibrosis 139-Variant Assay for I506V, I507V, and F508C.

Variant (Common Name)	Total Calls per Variant	Positive Calls (Variants)			Negative Calls (Wild Type)	# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
		Clinical Samples	Cell Line Samples	Synthetic Samples						
I506V	500	7	0	0	493	0	0	100	100	100
I507V	500	0	1	0	499	0	0	100	100	100
F508C	500	1	1	0	498	0	0	100	100	100

Table 18 Accuracy of the MiSeqDx Cystic Fibrosis 139-Variant Assay for PolyTG/PolyT Variants

PolyTGPolyT Genotype	# Clinical Samples	# Cell Line Samples	# Synthetic Samples	# Miscalls	# No Calls*	% Accuracy
(TG)9(T)7/(TG)11(T)7	2	0	0	0	1	50
(TG)9(T)9/(TG)10(T)7	1	0	0	0	0	100
(TG)9(T)9/(TG)11(T)7	5	1	0	0	0	100
(TG)9(T)9/(TG)11(T)9	1	0	0	0	0	100
(TG)10(T)7/(TG)10(T)7	25	8	0	0	0	100
(TG)10(T)7/(TG)10(T)9	39	16	0	0	0	100
(TG)10(T)7/(TG)11(T)5	2	0	0	0	0	100
(TG)10(T)7/(TG)11(T)7	72	11	0	0	0	100
(TG)10(T)7/(TG)12(T)5	1	0	0	0	0	100
(TG)10(T)7/(TG)12(T)7	10	1	0	0	1	90.9
(TG)10(T)9/(TG)10(T)9	7	6	0	0	0	100
(TG)10(T)9/(TG)11(T)5	5	0	0	0	0	100

PolyTGPolyT Genotype	# Clinical Samples	# Cell Line Samples	# Synthetic Samples	# Miscalls	# No Calls*	% Accuracy
(TG)10(T)9/(TG)11(T)7	76	20	0	0	0	100
(TG)10(T)9/(TG)11(T)9	3	0	0	0	0	100
(TG)10(T)9/(TG)12(T)5	3	2	0	0	0	100
(TG)10(T)9/(TG)12(T)7	13	0	0	0	1	92.3
(TG)11(T)5/(TG)11(T)7	6	0	0	1	0	83.3
(TG)11(T)7/(TG)11(T)7	52	8	0	0	0	100
(TG)11(T)7/(TG)11(T)9 [^]	2	1	0	3 [^]	0	0
(TG)11(T)7/(TG)12(T)5	2	0	0	0	0	100
(TG)11(T)7/(TG)12(T)7	37	3	0	0	0	100
(TG)11(T)9/(TG)12(T)7	3	0	0	0	0	100
(TG)12(T)7/(TG)12(T)7	2	2	0	0	0	100
Total**	448			4	3	98.44

* Samples were not retested.

[^] One of the discordant results was from the reproducibility study. The PolyTG/PolyT result for the sample was concordant across all 18 replicates, but discordant with Sanger bi-directional sequencing.

** The total sample count for the PolyTG/PolyT variant is 448 because all synthetic samples (n = 52) were built by blending linearized plasmids with one of two cell line samples, which were part of the reproducibility study. Since reporting the PolyTG/PolyT variant for these additional synthetic samples would result in the variant being over-reported, the synthetic samples were excluded from this analysis.

Reproducibility

The reproducibility of the MiSeqDx Cystic Fibrosis System was determined through a blinded study using three trial sites and two operators at each site. Two well-characterized panels of 46 samples each were tested by each of the operators at each site for a total of 810 calls per site. The panels contained a mix of genomic DNA from lymphoblastoid cell lines with known variants in the *CFTR* gene, as well as leukocyte-depleted blood spiked with lymphoblastoid cell lines with known variants in the *CFTR* gene. The blood samples were provided to allow incorporation of the extraction steps used to prepare gDNA that serves as the primary input for the assay workflow.

The sample pass rate, defined as the number of samples passing QC metrics on the first attempt, was 99.9%.

The genotype-level Positive Agreement for all variants was 99.77%. The Negative Agreement for all WT positions was 99.88% and the Overall Agreement for all reported positions was 99.88%. All test results are based on initial testing. No repeat testing was done for the reproducibility study.

Table 19 Reproducibility of the MiSeqDx Cystic Fibrosis 139-Variant Assay

Panel	Sample #	Sample Genotype	Variants	Total Calls per Site	Positive Agreeing Calls (Variants)			Negative Agreeing Calls (Wild Type)			# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
					Site 1	Site 2	Site 3	Site 1	Site 2	Site 3					
A	1	S549N (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	2	1812-1 G>A (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	3	Q493X/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	4*	F508del/2184delA (HET)		810	12	12	12	797	798	798	0	1*	100	100	100
A	5^	Y122X/R1158X (HET)		810	12	10	12	798	665	798	0	135^	94.44	94.44	94.44
A	6	F508del/2183AA>G (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	7	R75X (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	8	I507del/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	9**	F508del/W1282X (HET)		810	12	11	12	798	797	798	2**	0	97.22	99.96	99.92

Panel	Sample #	Sample Genotype	Variants	Total Calls per Site	Positive Agreeing Calls (Variants)			Negative Agreeing Calls (Wild Type)			# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
					Site 1	Site 2	Site 3	Site 1	Site 2	Site 3					
A	10**	F508del/3272-26A>G (HET)		810	12	11	12	798	797	798	2**	0	97.22	99.96	99.92
A	11	F508del/3849+10kbC>T (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	12	621+1G>T/3120+1G>A (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	13	E60X/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	14	M1101K (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	15	M1101K (HOM)		810	6	6	6	804	804	804	0	0	100	100	100
A	16	F508del (HOM)	I506V, I507V, F508C not present	828	6	6	6	822	822	822	0	0	100	100	100
A	17	F508del/3659delC (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	18	R117H/F508del (HET)	(TG)10 (T)9/ (TG)12 (T)5	816	18	18	18	798	798	798	0	0	100	100	100
A	19	621+1G>T/711+1G>T (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	20	G85E/621+1G>T (HET)		810	12	12	12	798	798	798	0	0	100	100	100

Panel	Sample #	Sample Genotype	Variants	Total Calls per Site	Positive Agreeing Calls (Variants)			Negative Agreeing Calls (Wild Type)			# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
					Site 1	Site 2	Site 3	Site 1	Site 2	Site 3					
A	21	A455E/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	22	F508del/R560T (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	23	F508del/Y1092X (C>A) (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	24	N1303K (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	25	G542X (HOM)		810	6	6	6	804	804	804	0	0	100	100	100
A	26	G542X (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	27	G551D/R553X (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	28	3849+10kbC>T (HOM)		810	6	6	6	804	804	804	0	0	100	100	100
A	29	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
A	30	F508del (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	31	1717-1G>A (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	32	R1162X (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	33	R347P/G551D (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	34	R334W (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	35	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
A	36	G85E (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	37	I336K (HET)		810	6	6	6	804	804	804	0	0	100	100	100

Panel	Sample #	Sample Genotype	Variants	Total Calls per Site	Positive Agreeing Calls (Variants)			Negative Agreeing Calls (Wild Type)			# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
					Site 1	Site 2	Site 3	Site 1	Site 2	Site 3					
A	38	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
A	39	F508del/3849+10kbC>T (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	40	621+1G>T/3120+1G>A (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	41	F508del/3659delC (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	42	R117H/F508del (HET)	(TG)10 (T)9/ (TG)12 (T)5	816	18	18	18	798	798	798	0	0	100	100	100
A	43	G85E/621+1G>T (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	44	A455E/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
A	45	N1303K (HET)		810	6	6	6	804	804	804	0	0	100	100	100
A	46	G551D/R553X (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	47	2789+5G>A (HOM)		810	6	6	6	804	804	804	0	0	100	100	100
B	48	CFTR dele2, 3/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	49	F508del/1898+1G>A (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	50	WT		810	0	0	0	810	810	810	0	0	N/A	100	100

Panel	Sample #	Sample Genotype	Variants	Total Calls per Site	Positive Agreeing Calls (Variants)			Negative Agreeing Calls (Wild Type)			# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
					Site 1	Site 2	Site 3	Site 1	Site 2	Site 3					
B	51	F508del/2143delT (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	52	3876delA (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	53	3905insT (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	54	394delTT (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	55	F508del (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	56	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	57	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	58	F508del (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	59	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	60	L206W (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	61	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	62	G330X (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	63	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	64	R347H (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	65	1078delT (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	66	G178R/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	67	S549R (c.1647T>G) (HET)		810	6	6	6	804	804	804	0	0	100	100	100

Panel	Sample #	Sample Genotype	Variants	Total Calls per Site	Positive Agreeing Calls (Variants)			Negative Agreeing Calls (Wild Type)			# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
					Site 1	Site 2	Site 3	Site 1	Site 2	Site 3					
B	68	S549N (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	69	W846X (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	70	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	71	E92X/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	72 ^s	621+1G>T/1154insTC (HET)		810	12	12	12	798	798	797	0	1 ^s	100	99.96	99.96
B	73	G542X (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	74	F508del (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	75 [^]	F508del (HET)		810	6	5	6	804	670	804	0	135 [^]	94.44	94.44	94.44
B	76	F508del (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	77	621+1G>T/A455E (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	78	1812-1 G>A (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	79	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	80	F508del/R553X (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	81	F508del/G551D (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	82	R347P/F508del (HET)		810	12	12	12	798	798	798	0	0	100	100	100

Panel	Sample #	Sample Genotype	Variants	Total Calls per Site	Positive Agreeing Calls (Variants)			Negative Agreeing Calls (Wild Type)			# Miscalls	# No Calls	Positive Agreement (%)	Negative Agreement (%)	Overall Agreement (%)
					Site 1	Site 2	Site 3	Site 1	Site 2	Site 3					
B	83	R117H/F508del (HET)	(TG)10 (T)9/ (TG)12 (T)5	816	18	18	18	798	798	798	0	0	100	100	100
B	84	I507del (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	85	2789+5G>A (HOM)		810	6	6	6	804	804	804	0	0	100	100	100
B	86 [§]	CFTR dele2, 3/F508del (HET)		810	12	12	12	798	797	798	0	1 [§]	100	99.96	99.96
B	87	F508del/1898+1G>A (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	88	WT		810	0	0	0	810	810	810	0	0	N/A	100	100
B	89	F508del/2143delT (HET)		810	12	12	12	798	798	798	0	0	100	100	100
B	90	3905insT (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	91	394delTT (HET)		810	6	6	6	804	804	804	0	0	100	100	100
B	92	F508del (HET)		810	6	6	6	804	804	804	0	0	100	100	100
Total				74556	2209			221182			4	273	99.77	99.88	99.88

* The wild type location corresponding to the N1303K variant for one replicate resulted in a No Call due to insufficient coverage.

^ One replicate of samples 5 and 75 had a 0% call rate. Further investigation indicates that samples may not have been added to the sample plate prior to library preparation because the sample volumes remaining in the tubes were consistent with no volume having been removed.

** Evidence indicates that samples 9 and 10 were likely switched by the operator prior to library preparation.

§ The wild type location corresponding to the M1V variant for one replicate of each of two samples resulted in a No Call due to insufficient coverage.

Table 20 Supplemental Information on Reproducibility Study Variants

Variation (Common Name)	Variant Type	CFTR Gene Region
PolyTG/PolyT	Compound DIV*	Intron 9
2183AA>G	Compound DIV*	Exon 14
CFTR dele2, 3	DEL	Intron1-Intron3
1154insTC	DIV*	Exon 8
I507del	DIV*	Exon 11
F508del	DIV*	Exon 11
2143delT	DIV*	Exon 14
3659delC	DIV*	Exon 22
3876delA	DIV*	Exon 23
394delTT	DIV in homopolymeric region*	Exon 3
1078delT	DIV in homopolymeric region*	Exon 8
2184delA	DIV in homopolymeric region*	Exon 14
3905insT	DIV in homopolymeric region*	Exon 23
E60X	SNV	Exon 3
R75X	SNV	Exon 3
G85E	SNV	Exon 3
E92X	SNV	Exon 4
R117H	SNV	Exon 4
Y122X	SNV	Exon 4
621+1G>T	SNV	Intron 4
G178R	SNV	Exon 5
711+1G>T	SNV	Intron 5
L206W	SNV	Exon 6
G330X	SNV	Exon 8
R334W	SNV	Exon 8
I336K	SNV	Exon 8
R347P	SNV	Exon 8
R347H	SNV	Exon 8
A455E	SNV	Exon 10

Variation (Common Name)	Variant Type	CFTR Gene Region
Q493X	SNV	Exon 11
1717-1G>A	SNV	Intron 11
G542X	SNV	Exon 12
S549N	SNV	Exon 12
S549R (c.1647T>G)	SNV	Exon 12
G551D	SNV	Exon 12
R553X	SNV	Exon 12
R560T	SNV	Exon 12
1812-1 G>A	SNV	Intron 12
1898+1G>A	SNV	Intron 13
W846X	SNV	Exon 15
2789+5G>A	SNV	Intron 16
3120+1G>A	SNV	Intron 18
3272-26A>G	SNV	Intron 19
Y1092X (C>A)	SNV	Exon 20
M1101K	SNV	Exon 20
R1158X	SNV	Exon 22
R1162X	SNV	Exon 22
3849+10kbC>T	SNV	Intron 22
W1282X	SNV	Exon 23
N1303K	SNV	Exon 24

* DIV is an acronym for Deletion/Insertion Variant.

DNA Extraction

Three commonly used, commercially available extraction methods representing magnetic bead extraction, alcohol precipitation, and silica filter column isolation methods were evaluated using EDTA anti-coagulated whole blood. A total of 14 unique blood samples were used in the study representing wild type and three mutant genotypes (three samples with F508del, one sample with I506V, and one sample with D110H). The three DNA extraction methods were tested independently by two different operators who each performed three runs per extraction method. Each extraction was performed by each operator on different days. The DNA concentration and A260/A280 ratio of the extracted gDNA samples was determined using spectrophotometry. The total sample size for each extraction method in this study was 168 (14 samples x 2 operators/extraction method x 3 runs/operator x 2 replicates/extracted gDNA sample).

Extraction Method	Number of Samples Tested	Call Rate	Accuracy	Sample First Pass Rate*
Alcohol Precipitation	168	100%	100%	100%
Silica Filter Column Isolation	168	100%	100%	100%
Magnetic Bead Extraction	168	100%	100%	100%

* Percent of samples having call rate of $\geq 99\%$ in first run.

DNA Input

The DNA input range of the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay was evaluated by performing a serial dilution study using 14 representative DNA samples containing 16 unique CF variants. Each sample was tested in duplicate at 9 DNA input levels ranging from 1250 ng to 1 ng (1250 ng, 500 ng, 250 ng, 100 ng, 50 ng, 25 ng, 10 ng, 5 ng, and 1 ng). For determination of accuracy, sample genotypes were compared to bidirectional Sanger sequencing data and the deletions were compared to PCR assay. 1250 ng and 25 ng were identified as the upper and lower bound for DNA input respectively as they had $\geq 95\%$ sample first pass rate with no incorrect calls (100% accuracy and call rate).

DNA inputs of 1250 ng, 250 ng, and 100 ng were further tested with 4 representative DNA samples and at least 20 replicates per DNA input level for each sample ($n=4 \times 20=80$ samples), while the lower bound of 25 ng was tested with 14 samples, 20 replicates for each sample ($n=14 \times 20=280$ samples). The accuracy and sample first pass rate was 100% at all DNA input levels.

The results indicate that the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay can be used in the DNA input range from 1250 ng to 25 ng to produce accurate results.

Interfering Substances

To assess the impact of interfering substances on the Illumina MiSeqDx Cystic Fibrosis System, the performance of the assay was evaluated in the presence and absence of potential interferents. Eight whole blood specimens were tested in the study including three CF positive samples with unique genotypes. Four endogenous interfering substances (bilirubin, cholesterol, hemoglobin, and triglyceride) were tested by spiking them into blood specimens prior to DNA extraction. The concentration limits for each substance are shown in the following table. Additionally, to assess interference resulting from blood collection (short draw), EDTA was spiked into blood samples, and to assess interference resulting from sample preparation, the final wash buffer from a silica filter column isolation method was added to purified genomic DNA.

The MiSeqDx Cystic Fibrosis 139-Variant Assay achieved 100% call rate for all samples tested, and 100% reproducibility in genotype calls between samples in the presence and absence of interfering substances.

To assess the impact of multiplexing index primer interference, a cross contamination study using two samples, each with unique homozygous genotypes at four different genomic positions, and two respective index primers was performed. No change in variant calling was observed with contamination levels $< 40\%$. The sample genotype became heterozygous when contamination levels were $\geq 40\%$.

No interference was observed from any of the endogenous or exogenous interferents.

Test Substance	Total Number of Replicates	Concentration Tested in Blood (Upper Limit)	Concentration Tested in Blood (Lower Limit)	Call Rate
Bilirubin	16	684 $\mu\text{mol/L}$	137 $\mu\text{mol/L}$	100%

Test Substance	Total Number of Replicates	Concentration Tested in Blood (Upper Limit)	Concentration Tested in Blood (Lower Limit)	Call Rate
Cholesterol	16	13 mmol/L	2.6 mmol/L	100%
Hemoglobin	16	2 g/L	0.4 g/L	100%
Triglyceride	16	37 mmol/L	7.4 mmol/L	100%
EDTA	16	7.0 mg/mL	2.8 mg/mL	100%

Sample Indexing

Sample index primers are used in the assay to assign a unique barcode to each sample DNA, allowing the ability to pool multiple samples together into a single sequencing run. A total of 96 samples indexes were tested using eight unique DNA samples to verify the ability of the assay to consistently make a genotyping call for a given sample across different indexing primer combinations. Each sample was tested with 12 different indexing primer combinations. Sample results were compared against bidirectional Sanger sequencing data for all positions/variants except the two large deletions, which were confirmed using a duplex PCR assay. Reproducibility and accuracy were 100% for all sample/index primer combinations.

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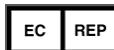
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Contact Information



Illumina
 5200 Illumina Way
 San Diego, California 92122 U.S.A.
 +1.800.809.ILMN (4566)
 +1.858.202.4566 (outside North America)
 techsupport@illumina.com
www.illumina.com



Illumina Netherlands B.V.
 Freddy van Riemsdijkweg 15
 5657 EE Eindhoven
 The Netherlands

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