

MiSeqDx[®] Cystic Fibrosis Clinical Sequencing Assay

FOR IN VITRO DIAGNOSTIC USE

Catalog # DX-102-1001: 6 Runs, up to 48 Samples per Kit

Intended Use

The Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay is a targeted sequencing *in vitro* diagnostic system that resequences the protein coding regions and intron/exon boundaries of the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene in genomic DNA isolated from human peripheral whole blood specimens collected in K₂EDTA. The test detects single nucleotide variants, and small indels within the region sequenced, and additionally reports on two deep intronic mutations and two large deletions. The test is intended to be used on the Illumina MiSeqDx Instrument.

The test is intended to be used as an aid in the diagnosis of individuals with suspected cystic fibrosis (CF). This assay is most appropriate when the patient has an atypical or non-classic presentation of CF or when other mutation panels have failed to identify both causative mutations. The results of the test are intended to be interpreted by a board-certified clinical molecular geneticist or equivalent and should be used in conjunction with other available information including clinical symptoms, other diagnostic tests, and family history.

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

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 population¹⁻⁵. 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 disease²⁻⁴ with the lungs being the primary organ system associated with morbidity and mortality⁶. In many cases, nutritional decline presages progression of CF lung disease; thus, a key focus of current interventional efforts is early diagnosis through newborn screening⁵. This early diagnosis facilitates timely access to important medical services and allows the best possible outcome for individuals with the disease^{2,5}. 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 US⁶.

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 kb². 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^{2,3}. More than 1900 variants of *CFTR* are currently described, the majority being point mutations⁷. The most common *CFTR* variant is the F508del allele³, which accounts for almost 70% of all *CFTR* variants¹. However, other common *CFTR* variants often result in a CF phenotype and other CFTR-related disorders¹⁻³.

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 population². 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^{2,4}. However, assigning any one ethnicity to an affected individual is becoming more difficult⁸. 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 US⁹

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

Assay Design

All protein coding regions in the *CFTR* gene including 10 nt of flanking intronic sequence are detected for all exons except for three (exons 7, 10, and 20). For Exon 7 and exon 10, only 5 nt of flanking intronic sequence is included at the 5' end of the exon to avoid proximal homopolymeric indels. For Exon 20, 30nt of flanking intronic sequence is included at the 5' end of the exon to enable detection of the mutation 3272-26A>G. In addition, the assay also detects ~100 nt of flanking sequence at the 5' and 3' UTRs, 2 deep intronic mutations (1811+1.6kbA>G, 3489+10kbC>T), 2 large deletions (CFTRdele2,3, CFTRdele22,23) and the PolyTG/PolyT region. The full coverage of the assay is shown in the genomic coordinate positions listed in [Table 2](#).



NOTE:

Limitations exist for detection of deletions at specific genomic locations within the sequenced regions of this assay (see [Limitations of the Procedure on page 6](#)).

Table 2 MiSeqDx Cystic Fibrosis Clinical Sequencing Assay Genomic Coordinate Coverage

	hg19 Genomic coordinate start (chr7)	hg19 Genomic coordinate stop (chr7)	Length (base pair)
CFTR_Exon 1	117120041	117120211	171
CFTR_Exon 2	117144297	117144427	131
CFTR_Exon 3	117149078	117149206	129
CFTR_Exon 4	117170943	117171178	236
CFTR_Exon 5	117174320	117174429	110
CFTR_Exon 6	117175292	117175475	184
CFTR_Exon 7 [^]	117176597	117176737	141

	hg19 Genomic coordinate start (chr7)	hg19 Genomic coordinate stop (chr7)	Length (base pair)
CFTR_Exon 8	117180144	117180410	267
CFTR_Exon 9	117182060	117182172	113
CFTR_Exon 10 [^]	117188690	117188887	198
CFTR_Exon 11	117199508	117199719	212
CFTR_Exon 12	117227783	117227897	115
CFTR_Intron 12 [*]	117229516	117229526	11
CFTR_Exon 13	117230397	117230503	107
CFTR_Exon 14	117231978	117232721	744
CFTR_Exon 15	117234974	117235122	149
CFTR_Exon 16	117242870	117242927	58
CFTR_Exon 17	117243576	117243846	271
CFTR_Exon 18	117246718	117246817	100
CFTR_Exon 19	117250563	117250733	171
CFTR_Exon 20 [‡]	117251605	117251872	268
CFTR_Exon 21	117254657	117254777	121
CFTR_Exon 22	117267566	117267834	269
CFTR_Intron 22 [*]	117280010	117280020	11
CFTR_Exon 23	117282482	117282657	176
CFTR_Exon 24	117292886	117292995	110
CFTR_Exon 25	117304732	117304924	193
CFTR_Exon 26	117305503	117305628	126
CFTR_Exon 27	117306952	117307262	311
Total Bases			5203^{**}

[^] For Exon 7 and Exon 10, only 5nt of flanking intronic sequence is included upstream of the exon to avoid homopolymeric stretches in these regions. In the case of Exon 10, this is the PolyT/Poly TG region in Intron 9. This region is treated specially and separately.

^{*} For the deep intronic mutations, 5 nucleotides flanking the SNV on either side are also included.

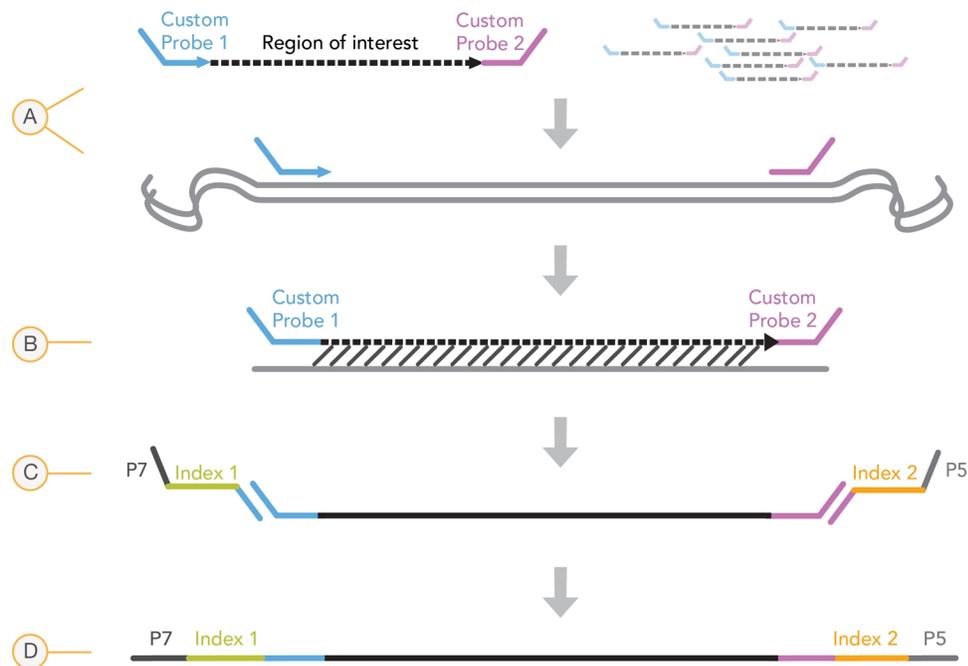
[‡] For Exon 20, 30nt of flanking intronic sequence is included at the 5' end of the exon to enable detection of the mutation 3272-26A>G.

^{**} With the two large deletions and the PolyTG/PolyT regions, the total positions/regions is 5206.

Principles of Procedure

The Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing 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 Clinical Sequencing 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 Cystic Fibrosis Clinical Sequencing 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 MiSeqDxCFClinicalSequencingAssay.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 Clinical Sequencing Analysis Module Workflow Guide (document # 1000000012185)*.

MiSeqDx Instrument Interface Methods

There are two different methods to interface with the MiSeqDx instrument available for the MiSeqDx Cystic Fibrosis Clinical Sequencing 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 The assay sequences the following regions within the CFTR gene:
 - a All protein coding regions in the CFTR gene across 27 exons
 - b Between 5 to 10 bases of flanking intronic sequence
 - c One hundred nucleotides of intronic sequence at the 5' and 3' untranslated regions
 - d Two deep intronic mutations (1811+1.6kbA>G, 3489+10kbC>T)
 - e The PolyTG/PolyT sequence located in intron 9
 - f A total of 5206 positions/regions of the possible 188,702 base pairs in the gene.
- 2 For *in vitro* diagnostic use. The results obtained using the Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay should be used and interpreted in the context of a full clinical evaluation.
- 3 The assay is designed to sequence protein coding regions and intron/exon boundaries of the CFTR gene and does not include all intronic regions and large deletions. Thus, an overall "wild-type" result does not guarantee that other cystic fibrosis transmembrane conductance regulator (*CFTR*) mutations/variants are not present in the samples being analyzed.
 - The assay is designed to detect two specific large deletions: CFTRdele2,3 and CFTRdele22,23. The assay cannot detect or report on other large deletions. This assay is only validated for insertions and deletions up to and including 3 bp in size.
- 4 All insertions/deletions are left aligned in homopolymeric regions as opposed to right aligned following HGVS nomenclature. For example, the variant c.313delA (with sequence context GAATC) is identified as a G-ATC deletion, but the deletion is reported in dbSNP as a GA-TC deletion. An exception to this are the 135 CF variations listed in CFTR2 as disease-causing (based on variant database version 04/10/2012). All the indels in homopolymeric regions within this set of variations are reported to match the expected variant reporting as per CFTR2¹⁰.
- 5 The assay has a limitation in detecting deletions at specific genomic locations within the sequenced regions. Genomic coordinates that the assay cannot report deletions are listed in Table 3. The assay cannot detect deletions that include the base or bases in the limitation column.

Table 3 Genomic Coordinates where Deletions Cannot be Detected

CFTR gene region	hg19 Genomic coordinates (chr7)
CFTR_Exon1	117120041; 117120211
CFTR_Exon3	117149091
CFTR_Exon4	117170953-117170954*; 117171082
CFTR_Exon5	117174362
CFTR_Exon6	117175417
CFTR_Exon7	117176621
CFTR_Exon8	117180176-117180177*
CFTR_Exon9	117182126
CFTR_Exon10	117188771

CFTR gene region	hg19 Genomic coordinates (chr7)
CFTR_Exon11	117199544-117199545*; 117199697
CFTR_Exon12	117227802
CFTR_Exon14	117232106-117232107*; 117232466-117232467*; 117232609
CFTR_Exon17	117243705; 117243843
CFTR_Exon18	117246751
CFTR_Exon19	117250688
CFTR_Exon20	117251788
CFTR_Exon22	117267721
CFTR_Exon23	117282597
CFTR_Exon24	117292953
CFTR_Exon25	117304740-117304741*; 117304869
CFTR_Exon26	117305518
CFTR_Exon27	117307178

* Only deletions that include both bases listed here cannot be detected. For example, in Exon8, only deletions ≥ 2 bp that include the bases at both genomic coordinates 117180176 and 117180177 cannot be detected. A single base deletion at 117180176 or 117180177 can be detected.

- a If the impacted coordinate listed in [Table 3](#) is the left-most base within a homopolymeric region, a deletion at any other position within the homopolymeric stretch cannot be detected because it cannot be distinguished from a deletion at the impacted coordinate.
- b The assay cannot detect a total of five variants listed in the ClinVar clinical database (accessed database version December 2014). These five specific variants are included in [Table 4](#). This assay limitation does not impact any variants listed in the Cystic Fibrosis database, CFTR2 (database version 04/10/2012).

Table 4 Known Variants not Detected by the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay[^]

Variant #	ClinVar ID	CFTR gene region	Genomic location (chr 7)	cDNA Name (HGVS)	Protein Name (HGVS)	rs ID
1	RCV000046424	CFTR_Exon3	117149091	c.168delA	p.Glu56Aspfs	rs397508269
2	RCV000046687	CFTR_Exon17	117243703-117243704*	c.2775_2776delTT	p.Leu926Alafs	rs397508433
3	RCV000046688	CFTR_Exon17	117243705	c.2777delT	p.Leu926Cysfs	rs397508434
4	RCV000046782	CFTR_Exon19	117250690*	c.3106delA	p.Thr1036Profss	rs397508497
5	RCV000046857	CFTR_Exon20	117251789*	c.3294delG	p.Trp1098Cysfs	rs397508534

[^] No frequency data was available for any of the variants.

* In these cases, the impacted coordinates fall within a homopolymeric region.

- 6 Variants identified by this assay vary in frequency among different populations. It is not possible to validate all combinations of variants that could be detected in the *CFTR* gene by this assay. It is recommended that novel and rare variants be confirmed by the user using a validated reference method.
- 7 As with any hybridization-based assay, underlying polymorphisms, mutations, insertions, or deletions in oligonucleotide-binding regions can affect the alleles being probed and, consequently, the calls made.
- 8 For complex variants where a deletion and insertion occur at the same site, the assay can report it as two separate variants in close proximity. Phasing of variants is not evaluated and other possible solutions to the detected sequence must be considered. See [Table 5](#) for an example of a complex variant of this nature.

Table 5 Complex Variant, Example

Sequence Context (Reference)	GAAGAAATT
Observed Sequence for Variant	GAAT--ATT
Variant Expected	Deletion of GAA, Insertion of T (both changes on the same chromosome)
Variant(s) Reported by the Assay	SNP (G>T); Deletion of AA

- 9 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 Instrument System 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. This assay cannot determine whether variants are in cis/trans to other variants.

- 10 The assay cannot determine whether the orientation of the PolyTG/PolyT variant is in cis/trans to other variants. 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 a cis/trans orientation should be performed.
PolyTG/PolyT are homopolymeric regions known to be difficult to sequence due to polymerase slippage.
- 11 This assay runs exclusively in an 8-plex format. If 6 clinical samples are not available, excluding the positive and negative controls, then include other human genomic DNA samples to fill the run.

Product Components

The Illumina MiSeqDx Platform consists of the following:

- MiSeqDx Cystic Fibrosis Clinical Sequencing Assay (Catalog # DX-102-1001)
- MiSeqDx Instrument (Catalog # DX-410-1001)

Reagents

Reagents Provided

Reagents for the Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay (catalog # DX-102-1001) are provided by Illumina. This kit has been configured for six runs with a maximum of eight samples per run (up to 48 samples total).

MiSeqDx Cystic Fibrosis Clinical Sequencing Assay, Box 1

Table 6 Box 1A Pre-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
CF Clinical Sequencing Assay-Oligo Pool	1 tube	600 µl	Buffered aqueous solution containing oligonucleotides targeting the <i>CFTR</i> gene	-25°C to -15°C
Hybridization Buffer	1 tube	4.32 ml	Buffered aqueous solution containing salts and formamide	-25°C to -15°C
Extension-Ligation Mix	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 C (A503), D (A504), and E (A505)	1 tube per primer	192 µl	PCR primers with index sequences and sequencing adapters	-25°C to -15°C
Index Primers 1 (A701), 2 (A702), and 10 (A710)	1 tube per primer	128 µl	PCR primers with index sequences and sequencing adapters	-25°C to -15°C
PCR Polymerase	1 tube	56 µl	Proprietary DNA polymerase	-25°C to -15°C
PCR Master Mix	1 tube	2.8 ml	Buffered aqueous solution containing salts and dNTPs	-25°C to -15°C

Table 7 Box 1B Post-Amp Reagents

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

MiSeqDx Cystic Fibrosis Clinical Sequencing Assay, Box 2

Table 8 Box 2 Post-Amp Reagents

Component	Quantity	Contents	Storage
MiSeqDx Reagent Cartridge - CF Clinical Sequencing Assay	6 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 Clinical Sequencing Assay, Box 3

Table 9 Box 3A Pre-Amp Reagents

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

Table 10 Box 3B Post-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
PCR Clean-Up Beads	1 tube	5 ml	Buffered aqueous solution containing solid phase paramagnetic beads and polyethylene glycol	2°C to 8°C

Component	Quantity	Fill Volume	Active Ingredients	Storage
Library Normalization Wash	2 tubes	4.8 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide	2°C to 8°C
Library Beads	1 tube	1.2 ml	Buffered aqueous solution containing solid phase paramagnetic beads	2°C to 8°C
MiSeqDx Flow Cell - CF Clinical Sequencing Assay	6 containers	1 flow cell	Glass substrate with covalently bound oligonucleotides	2°C to 8°C

MiSeqDx Cystic Fibrosis Clinical Sequencing Assay, Box 4

Table 11 Box 4 Post-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
MiSeqDx SBS Solution (PR2) - CF Clinical Sequencing Assay	6 bottles	353.1 ml	Buffered aqueous solution	2°C to 8°C

MiSeqDx Cystic Fibrosis Clinical Sequencing Assay, Box 5

Table 12 Box 5 Pre-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
Filter Plate	6 plates	N/A	Polypropylene microtiter plate with a modified polyethersulfone membrane	15°C to 30°C

Table 13 Box 5 Post-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
Elution Buffer	1 tube	4.8 ml	Buffered aqueous solution	15°C to 30°C
Library Storage Buffer	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 Clinical Sequencing Assay-Oligo Pool
- Hybridization Buffer
- Extension-Ligation Mix
- Index Primers C (A503), D (A504), and E (A505)
- Index Primers 1 (A701), 2 (A702), and 10 (A710)
- PCR Polymerase
- PCR Master Mix
- Library Normalization Diluent
- Library Dilution Buffer
- PhiX Internal Control
- MiSeqDx Reagent Cartridge - CF Clinical Sequencing 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 Clinical Sequencing Assay
 - MiSeqDx Flow Cell - CF Clinical Sequencing 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 pooled amplicon library can be stored at -25°C to -15°C for up to 3 days.
- 11 Load freshly prepared diluted amplicon pool onto the reagent cartridge.

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 8](#) for more information.)
- 2 Some components of this assay contain 2-Mercaptoethanol, a reducing agent. (See [Reagents on page 8](#) 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 the sample sheet exactly. Mismatches between the sample sheet 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 Clinical Sequencing Assay-Oligo Pool and Hybridization Buffer for storage. When combined, the CF Clinical Sequencing 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.

Acronyms

Table 14 Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay Acronyms

Acronym	Definition
AMP	AMplification Plate

Acronym	Definition
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 Clinical Sequencing 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 Clinical Sequencing Assay, the sample throughput per MiSeqDx run is 8 samples. The indexing primers used during PCR amplification must be chosen based on desired final sample throughput to ensure diversity in index sequence.

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 need 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.

Use the following minimal set of color-balanced indices for eight-sample sequencing runs:

Table 15 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	--

If six unique samples (excluding the positive and negative controls) are not available, it is acceptable to fill the run with replicates of any human genomic DNA sample.

Instructions for Use

Enter Run Information

MiSeq Reporter or Local Run Manager are the two software options available to set up a Cystic Fibrosis Clinical Sequencing 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 Clinical Sequencing analysis module.

For more information on the differences between MiSeq Reporter and Local Run Manager, see [MiSeqDx Instrument Interface Methods on page 1](#).

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 Clinical Sequencing 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 Clinical Sequencing Analysis Module

Set Parameters

- 1 Log in to Local Run Manager.
- 2 Click **Create Run**, and select **CF Clinical**.
- 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**— 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 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 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 Workflow 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 Clinical Sequencing Assay-Oligo Pool, Hybridization Buffer, genomic DNA samples, and positive control sample to room temperature.
- 2 Vortex the CF Clinical Sequencing 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 ng/ μl (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 Clinical Sequencing 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.



SAFESTOPPING 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 $45 \mu\text{l}$ 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 according to the sample sheet as follows:
 - a Add $4 \mu\text{l}$ of the index primers C (A503), D (A504), and E (A505) to the appropriate wells in a column of the **AMP** plate.
 - b Discard the original white caps and apply new white caps.
 - c Add $4 \mu\text{l}$ of the selected index primers 1 (A701), 2 (A702), and 10 (A710) to the appropriate wells in a 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 Add $5.6 \mu\text{l}$ of PCR Polymerase to $280 \mu\text{l}$ of PCR Master Mix.
 - c Invert the prepared PCR working solution 20 times to mix.

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 $25 \mu\text{l}$ 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 $22 \mu\text{l}$ 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 $20 \mu\text{l}$ 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

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, and Library Normalization Wash 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 1.5 ml tube as follows:
 - a Add 394 μ l 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 Pipette 72 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 1800 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 1800 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 1800 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 1000 \times g at 20°C for 1 minute.



SAFE STOPPING POINT

If not proceeding immediately to Library Pooling and subsequent sequencing on the MiSeqDx, store the sealed **SGP** plate at -25°C to -15°C for up to 3 days.

Library Pooling

Prepare for Library Pooling

- 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 Clinical Sequencing 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 µl)
 - Stock 10 N NaOH (25 µl)
- 2 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.

- 3 Combine the following volumes to dilute the PhiX Internal Control library to 2 nM:
 - 10 nM PhiX Internal Control library (2 µl)
 - 1X TE Buffer (8 µl)
- 4 Combine the following volumes to result in a 1 nM PhiX Internal Control library:
 - 2 nM PhiX Internal Control library (10 µl)
 - 0.1 N NaOH (10 µl)
- 5 Vortex briefly to mix the 1 nM PhiX Internal Control library solution.
- 6 Centrifuge the 1nM PhiX Internal Control at 280 × g at 20°C for 1 minute.

- 7 Incubate for 5 minutes at room temperature to denature the PhiX Internal Control library solution into single strands.
- 8 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

- 1 Bring Library Dilution Buffer to room temperature. Vortex Library Dilution Buffer and ensure that all precipitates have dissolved completely.
- 2 If the **SGP** plate was stored frozen, thaw the **SGP** plate at room temperature.
- 3 Centrifuge the **SGP** plate at 1000 \times g at 20°C for 1 minute.
- 4 Set out a fresh Eppendorf tube (hereafter referred to as the **PAL** [Pooled Amplicon Library] tube).
- 5 If the **SGP** plate was stored frozen, mix each library to be sequenced by pipetting up and down 3–5 times.
- 6 Transfer 5 μ l of each library to be sequenced from the **SGP** plate to a PCR eight-tube strip. Seal **SGP** with an adhesive plate seal and set aside.

**NOTE**

After use, store the sealed **SGP** plate at -25°C to -15°C. The sealed **SGP** plate is stable for up to 3 days.

- 7 Combine and transfer the contents of the PCR eight-tube strip into the **PAL** tube. Mix the **PAL** tube thoroughly by vortexing.
- 8 Set out a fresh Eppendorf tube (hereafter referred to as the **DAL** [Diluted Amplicon Library] tube).
- 9 Add 585 μ l of Library Dilution Buffer to the **DAL** tube.
- 10 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.
- 11 Transfer 9 μ l of **PAL** to the **DAL** tube containing Library Dilution Buffer. Pipette up and down 3–5 times to rinse the tip and ensure complete transfer.
- 12 Mix the **DAL** tube by vortexing the tube at top speed.
- 13 Centrifuge the **DAL** tube at 1000 \times g at 20°C for 1 minute.
- 14 Incubate the **DAL** tube on a heat block at 96°C for 2 minutes.
- 15 After the incubation, invert the **DAL** tube 1–2 times to mix, then immediately place in the ice-water bath.
- 16 Keep the **DAL** tube in the ice-water bath for 5 minutes.

Load Sample Libraries onto Cartridge

- 1 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**.
- 2 Pipette 600 μ l of the **DAL** sample libraries into the **Load Samples** reservoir. Avoid touching the foil seal.
- 3 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.
- 4 Proceed directly to the run setup steps using the MiSeq Operating Software (MOS) interface.

Interpretation of Results

The Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay is designed to sequence all protein coding regions in the *CFTR* gene across the 27 exons, between 5 to 30 bases of flanking intronic sequence, ~100 nt of flanking sequence at the 5' and 3' UTRs, and 2 deep intronic mutations (1811+1.6kbA>G, 3489+10kbC>T). The exact regions sequenced are listed in [Table 2](#). Additionally the assay also reports on the PolyTG/PolyT variant, and 2 large deletions (CFTRdele2,3, CFTRdele22,23).

- 1 The assay report lists the sample names and genotype for each variant detected for a sample.
 - The genomic coordinate, Human Genome Variation Society (HGVS) cDNA name, and protein name (if available) are reported for each variant.
 - The variant type is identified as single nucleotide variant (SNV), deletion/insertion variant (DIV), PolyTG/PolyT variant (PolyTGPolyT), or large deletion (DEL).
 - The genotype call (whether heterozygous or homozygous) can be inferred from the ‘reference’ base information, which provides the reference sequence at that genomic coordinate, and the ‘result’ description which provides the two alleles at the genomic position in the sample. For example, if the Reference is ‘G’ and the Result is ‘A/G’, this indicates a G>A change at that genomic coordinate and that the genotype is heterozygous for the variant allele. Likewise, if the Reference is ‘G’, and the Result is ‘T/T’, this indicates a G>T change at that genomic coordinate and that the genotype is homozygous for the variant allele.
 - The sequencing depth at the variant position is provided in the “Depth” field and the allelic frequency in the “Frequency” section.
- 2 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.
 - The genomic coordinate for any position or region for which the confidence value is below the threshold is listed separately in the “Coordinates not called” section. Users should evaluate the positions not called against relevant variant information to identify variants that may be missed and their corresponding population frequencies to determine if sample repeat is required.
- 3 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.
- 4 It is recommended that any variants outside of what is validated in the accuracy study (see [Accuracy on page 26](#)) be verified by the user using a validated reference method prior to reporting the first patient result with those variants.



NOTE

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

- 5 All variant interpretations should be made by a certified clinical molecular geneticist or equivalent following local procedures and guidelines¹¹. Potential interpretation references include but are not limited to: CFTR2 database^{12,13}, Sosnay paper¹⁰, ACMG 2004 guidelines¹⁴, and 2011 ACOG committee opinion⁸. 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 the *Local Run Manager CF Clinical Sequencing Analysis Module Workflow Guide (document # 1000000012185)*.
- 6 The geneticist has the option to use the MiSeq Reporter or Local Run Manager software to enter an interpretation value for each variant reported on a sample using a drop down menu. The interpretation value choices are: CF-causing, Mutation of varying clinical consequence, Mutation of unknown significance, or Non-CF causing. The value entered will be appended to the results file and display in the interpretation column on the Clinical Sequencing Assay report.

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 variant¹⁵. Illumina recommends the use of rotating positive controls consistent with the 2008 ACMG Technical Standards and Guidelines for CF mutation testing¹⁶ and the 2013 ACMG clinical laboratory standards for next-generation sequencing¹⁷. 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 Clinical Sequencing 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 rare variants, and contained anywhere from 1 to 10 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.

For the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay, a total of 5,206 positions were compared to the reference methods of Sanger bi directional sequencing and PCR testing. The genotyping results for SNV and 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 2 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 MiSeqDx Cystic Fibrosis Clinical Sequencing Assay had a genotype-level PA of 99.66%, including PolyTG/PolyT variants (100% excluding PolyTG/PolyT variants). The NA for all WT positions was > 99.99% and the OA for all reported positions was > 99.99%.

Table 16 Overall Accuracy for the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
117120141	c.-8G>C^	SNV	Exon1	25	3	0	0	0	100
117120145	c.-4G>C^	SNV	Exon1	3	2	0	0	0	100
M1V	c.1A>G	SNV	Exon1	0	0	1	0	0	100
CFTR dele2, 3	c.54-5940_ 273+10250 del21kb	Del	Intron1	4	1	0	0	0	100
R31C	c.91C>T	SNV	Exon2	3	1	0	0	0	100
Q39X	c.115C>T	SNV	Exon2	0	0	1	0	0	100
E60X	c.178G>T	SNV	Exon3	6	1	0	0	0	100
P67L	c.200C>T	SNV	Exon3	1	0	1	0	0	100
R74W	c.220C>T	SNV	Exon3	0	2	0	0	0	100
R74Q	c.221G>A	SNV	Exon3	2	0	0	0	0	100
R75X	c.223C>T	SNV	Exon3	3	1	0	0	0	100
R75Q	c.224G>A	SNV	Exon3	20	1	0	0	0	100
G85E	c.254G>A	SNV	Exon3	6	2	0	0	0	100
394delTT	c.262_263 delTT	DIV	Exon3	3	1	0	0	0	100
405+1G>A	c.273+1G>A	SNV	Intron3	0	0	1	0	0	100
406-1G>A	c.274-1G>A	SNV	Exon4	4	0	0	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
E92K	c.274G>A	SNV	Exon4	0	0	1	0	0	100
E92X	c.274G>T	SNV	Exon4	0	1	1	0	0	100
Q98X	c.292C>T	SNV	Exon4	0	0	2	0	0	100
444delA	c.312delA	DIV	Exon4	0	2	0	0	0	100
457TAT>G	c.325_327 delTAT insG	DIV	Exon4	0	0	1	0	0	100
D110H	c.328G>C	SNV	Exon4	1	0	1	0	0	100
R117C	c.349C>T	SNV	Exon4	4	0	0	0	0	100
R117H	c.350G>A	SNV	Exon4	17	2	0	0	0	100
Y122X	c.366T>A	SNV	Exon4	0	1	0	0	0	100
F143LfsX10	c.425delT	DIV	Exon4	0	1	0	0	0	100
574delA	c.442delA	DIV	Exon4	0	0	2	0	0	100
Q151K	c.451C>A	SNV	Exon4	1	0	0	0	0	100
621+1G>T	c.489+1G>T	SNV	Intron4	7	5	0	0	0	100
621+3A>G	c.489+3A>G	SNV	Intron4	1	0	0	0	0	100
663delT	c.531delT	DIV	Exon5	1	0	1	0	0	100
G178R	c.532G>A	SNV	Exon5	1	1	0	0	0	100
711+1G>T	c.579+1G>T	SNV	Intron5	3	1	0	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
711+3A>G	c.579+3A>G	SNV	Intron5	0	0	1	0	0	100
711+5 G>A	c.579+5G>A	SNV	Intron5	0	0	1	0	0	100
712-1 G>T	c.580-1G>T	SNV	Exon6	0	0	1	0	0	100
H199Y	c.595C>T	SNV	Exon6	0	0	1	0	0	100
P205S	c.613C>T	SNV	Exon6	1	0	1	0	0**	100
L206W	c.617T>G	SNV	Exon6	8	1	0	0	0	100
A209S	c.625G>T	SNV	Exon6	0	1	0	0	0	100
Q220X	c.658C>T	SNV	Exon6	0	0	1	0	0	100
L227R	c.680T>G	SNV	Exon6	0	0	1	0	0	100
852del22	c.720_741 delAGGG AGAATG ATGATG AAGTAC	DIV	Exon6	0	0	1	0	0	100
E279D	c.837A>T	SNV	Exon7	1	0	0	0	0	100
R297Q	c.890G>A	SNV	Exon8	2	0	0	0	0	100
1078delIT	c.948delIT	DIV	Exon8	1	1	0	0	0	100
L320V	c.958T>G	SNV	Exon8	1	0	0	0	0	100
G330X	c.988G>T	SNV	Exon8	1	1	0	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
R334W	c.1000C>T	SNV	Exon8	6	1	0	0	0	100
I336K	c.1007T>A	SNV	Exon8	0	1	0	0	0	100
T338I	c.1013C>T	SNV	Exon8	0	0	1	0	0	100
1154insTC	c.1022_10 23insTC	DIV	Exon8	0	1	0	0	0	100
S341P	c.1021T>C	SNV	Exon8	0	0	1	0	0	100
R347H	c.1040G>A	SNV	Exon8	6	1	1	0	0	100
R347P	c.1040G>C	SNV	Exon8	3	2	0	0	0	100
R352Q	c.1055G>A	SNV	Exon8	5	0	0	0	0	100
Q359K/ T360K	c.[1075C>A ;1079C>A]	SNV	Exon8	0	0	1	0	0	100
1213delT	c.1081delT	DIV	Exon8	0	0	1	0	0	100
1248+1G>A	c.1116+1G>A	SNV	Intron8	0	0	1	0	0	100
1259insA	c.1127_11 28insA	DIV	Exon9	0	0	2	0	0	100
W401X (c.1202G>A)	c.1202G>A	SNV	Exon9	0	0	1	0	0	100
W401X (c.1203G>A)	c.1203G>A	SNV	Exon9	0	0	1	0	0	100
1341+1G>A	c.1209+1G>A	SNV	Intron9	0	0	2	0	0	100
PolyTGPolyT	N/A	PolyTG PolyT	Intron9	369	79	52	3	4 [†]	98.60

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
1461ins4	c.1329_1330ins AGAT	DIV	Exon10	0	0	1	0	0	100
A455E	c.1364C>A	SNV	Exon10	4	2	0	0	0	100
1525-1G>A	c.1393-1G>A	SNV	Exon11	0	0	1	0	0	100
S466X (C>A)	c.1397C>A	SNV	Exon11	0	0	1	0	0	100
S466X (C>G)	c.1397C>G	SNV	Exon11	1	0	1	0	0	100
L467P	c.1400T>C	SNV	Exon11	0	0	1	0	0	100
V470M	c.1408G>A	SNV	Exon11	311	71	0	0	0	100
1548delG	c.1418delG	DIV	Exon11	1	0	1	0	0	100
P477S	c.1429C>T	SNV	Exon11	0	1	0	0	0	100
S485T	c.1454G>C	SNV	Exon11	1	0	0	0	0	100
S489X	c.1466C>A	SNV	Exon11	0	0	2	0	0	100
S492F	c.1475C>T	SNV	Exon11	0	0	1	0	0	100
Q493X	c.1477C>T	SNV	Exon11	4	2	0	0	0	100
I506V	c.1516A>G	SNV	Exon11	7	0	0	0	0	100
I507del	c.1519_1521 delATC	DIV	Exon11	4	2	0	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
F508del	c.1521_1523 delCTT	DIV	Exon11	84	29	0	0	0	100
I507V	c.1519A>G	SNV	Exon11	0	1	0	0	0	100
F508C	c.1523T>G	SNV	Exon11	1	1	0	0	0	100
1677delTA	c.1545_1546 delTA	DIV	Exon11	1	0	0	0	0	100
V520F	c.1558G>T	SNV	Exon11	2	0	0	0	0	100
Q525X	c.1573C>T	SNV	Exon11	0	0	1	0	0	100
E527E	c.1581A>G	SNV	Exon11	3	2	0	0	0	100
E528E	c.1584G>A	SNV	Exon11	6	2	0	0	0	100
1717-8G>A	c.1585-8G>A	SNV	Intron11	0	0	1	0	0	100
1717-1G>A	c.1585-1G>A	SNV	Exon12	4	1	0	0	0	100
G542X	c.1624G>T	SNV	Exon12	12	3	0	0	0	100
S549R (c.1645A>C)	c.1645A>C	SNV	Exon12	0	0	1	0	0	100
S549N	c.1646G>A	SNV	Exon12	2	2	1	0	0	100
S549R (c.1647T>G)	c.1647T>G	SNV	Exon12	3	1	0	0	0	100
G551D	c.1652G>A	SNV	Exon12	8	3	0	0	0	100
Q552X	c.1654C>T	SNV	Exon12	0	0	1	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
R553X	c.1657C>T	SNV	Exon12	8	2	0	0	0	100
I556V	c.1666A>G	SNV	Exon12	1	0	0	0	0	100
L558S	c.1673T>C	SNV	Exon12	0	0	1	0	0	100
A559T	c.1675G>A	SNV	Exon12	4	0	1	0	0	100
R560K	c.1679G>A	SNV	Exon12	0	0	1	0	0	100
R560T	c.1679G>C	SNV	Exon12	6	1	0	0	0	100
1811+1.6kb A>G	c.1679+1.6 kbA>G	SNV	Intron12	0	0	1	0	0	100
1812-1 G>A	c.1680-1G>A	SNV	Exon13	0	2	0	0	0	100
A561T	c.1681G>A	SNV	Exon13	1	0	0	0	0	100
V562I	c.1684G>A	SNV	Exon13	1	0	0	0	0	100
Y569D	c.1705T>G	SNV	Exon13	0	0	1	0	0	100
P574H	c.1721C>A	SNV	Exon13	0	1	0	0	0	100
G576A	c.1727G>C	SNV	Exon13	4	1	0	0	0	100
D579G	c.1736A>G	SNV	Exon13	0	0	1	0	0	100
E585X	c.1753G>T	SNV	Exon13	0	0	1	0	0	100
1898+1G>A	c.1766+1G>A	SNV	Intron13	2	1	0	0	0	100
1898+3A>G	c.1766+3A>G	SNV	Intron13	0	0	1	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
H609R	c.1826A>G	SNV	Exon14	0	1	0	0	0	100
D614G	c.1841A>G	SNV	Exon14	0	0	2	0	0	100
R668C	c.2002C>T	SNV	Exon14	5	2	0	0	0	100
R668H	c.2003G>A	SNV	Exon14	1	0	0	0	0	100
2143delT	c.2012delT	DIV	Exon14	2	1	0	0	0	100
K684TfsX4	c.2046_2047 delAA	DIV	Exon14	0	0	1	0	0	100
2183AA>G	c.2051_2052 delAAinsG	DIV	Exon14	3	1	0	0	0	100
2184delA	c.2052delA	DIV	Exon14	1	1	0	0	0	100
2184insA	c.2052_2053 insA	DIV	Exon14	3	0	1	0	0	100
S686Y	c.2057C>A	SNV	Exon14	0	1	0	0	0	100
R709X	c.2125C>T	SNV	Exon14	1	0	2	0	0	100
K710X	c.2128A>T	SNV	Exon14	3	0	0	0	0	100
E725K	c.2173G>A	SNV	Exon14	2	0	0	0	0	100
2307insA	c.2175_2176 insA	DIV	Exon14	3	0	2	0	0	100
L732X	c.2195T>G	SNV	Exon14	0	0	2	0	0	100
2347delG	c.2215delG	DIV	Exon14	0	0	2	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
P750L	c.2249C>T	SNV	Exon14	1	0	0	0	0	100
V754M	c.2260G>A	SNV	Exon14	2	1	0	0	0	100
R764X	c.2290C>T	SNV	Exon14	1	0	2	0	0	100
2585delT	c.2453delT	DIV	Exon14	0	0	2	0	0	100
E822X	c.2464G>T	SNV	Exon14	0	0	2	0	0	100
2622+1G>A	c.2490+1G>T	SNV	Intron14	0	0	2	0	0	100
E831X	c.2491G>T	SNV	Exon15	0	0	1	0	0	100
D836Y	c.2506G>T	SNV	Exon15	0	1	0	0	0	100
W846X	c.2537G>A	SNV	Exon15	0	1	0	0	0	100
R851X	c.2551C>T	SNV	Exon15	0	0	1	0	0	100
T854T	c.2562T>G	SNV	Exon15	212	44	0	0	0	100
2711delT	c.2583delT	DIV	Exon15	0	0	1	0	0	100
V868V	c.2604A>G	SNV	Exon15	2	0	0	0	0	100
c.2657+2_ 2657+3insA	c.2657+2_ 2657+3insA	DIV	Intron16	0	0	1	0	0	100
2789+5G>A	c.2657+5G>A	SNV	Intron16	9	1	0	0	0	100
Q890X	c.2668C>T	SNV	Exon17	1	0	0	0	0	100
A923A	c.2769C>T	SNV	Exon17	1	0	0	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
L927P	c.2780T>C	SNV	Exon17	0	0	1	0	0	100
S945L	c.2834C>T	SNV	Exon17	0	0	1	0	0	100
M952T	c.2855T>C	SNV	Exon17	1	0	0	0	0	100
3007delG	c.2875delG	DIV	Exon17	0	0	1	0	0	100
T966T	c.2898G>A	SNV	Exon17	5	0	0	0	0	100
G970R	c.2908G>C	SNV	Exon17	0	0	1	0	0	100
S977F	c.2930C>T	SNV	Exon18	0	0	1	0	0	100
3120G>A	c.2988G>A	SNV	Exon18	1	0	0	0	0	100
3120+1G>A	c.2988+1G>A	SNV	Intron18	7	1	0	0	0	100
3121-1G>A	c.2989-1G>A	SNV	Exon19	0	0	1	0	0	100
L997F	c.2991G>C	SNV	Exon19	2	1	0	0	0	100
I1027T	c.3080T>C	SNV	Exon19	1	2	0	0	0	100
3272-26A>G	c.3140-26A>G	SNV	Intron19	0	1	0	0	0	100
F1052V	c.3154T>G	SNV	Exon20	0	1	0	0	0	100
L1065P	c.3194T>C	SNV	Exon20	0	0	1	0	0	100
R1066C	c.3196C>T	SNV	Exon20	6	0	0	0	0	100
R1066H	c.3197G>A	SNV	Exon20	1	0	1	0	0	100
G1069R	c.3205G>A	SNV	Exon20	0	1	0	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
R1070W	c.3208C>T	SNV	Exon20	0	2	0	0	0	100
R1070Q	c.3209G>A	SNV	Exon20	0	1	0	0	0	100
L1077P	c.3230T>C	SNV	Exon20	0	0	1	0	0 ^y	100
W1089X	c.3266G>A	SNV	Exon20	4	0	0	0	0	100
Y1092X (C>A)	c.3276C>A	SNV	Exon20	3	1	0	0	0	100
Y1092X (C>G)	c.3276C>G	SNV	Exon20	0	0	1	0	0	100
T1095T	c.3285A>T	SNV	Exon20	7	0	0	0	0	100
M1101K	c.3302T>A	SNV	Exon20	2	2	0	0	0	100
E1104X	c.3310G>T	SNV	Exon20	0	0	1	0	0	100
c.3368-2A>T	c.3368-2A>T	SNV	Intron20	0	1	0	0	0	100
D1152H	c.3454G>C	SNV	Exon21	10	1	0	0	0	100
V1153E	c.3458T>A	SNV	Exon21	1	0	0	0	0	100
R1158X	c.3472C>T	SNV	Exon22	7	1	0	0	0	100
R1162X	c.3484C>T	SNV	Exon22	5	1	0	0	0	100
R1162L	c.3485G>T	SNV	Exon22	0	2	0	0	0	100
3659delC	c.3528delC	DIV	Exon22	4	1	0	0	0	100
S1196X	c.3587C>G	SNV	Exon22	1	0	0	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
W1204X (c.3611G>A)	c.3611G>A	SNV	Exon22	0	0	1	0	0	100
W1204X (c.3612G>A)	c.3612G>A	SNV	Exon22	0	0	1	0	0	100
3791delC	c.3659delC	DIV	Exon22	2	0	0	0	0	100
I1234V	c.3700A>G	SNV	Exon22	1	0	1	0	0	100
S1235R	c.3705T>G	SNV	Exon22	9	1	0	0	0	100
3849+10 kbC>T	c.3717+ 12191C>T	SNV	Intron22	11	2	0	0	0	100
G1244E	c.3731G>A	SNV	Exon23	0	0	1	0	0	100
3876delA	c.3744delA	DIV	Exon23	6	1	0	0	0	100
S1251N	c.3752G>A	SNV	Exon23	1	0	1	0	0	100
3905insT	c.3773_3774 insT	DIV	Exon23	3	1	0	0	0	100
D1270N	c.3808G>A	SNV	Exon23	0	2	0	0	0	100
W1282X	c.3846G>A	SNV	Exon23	9	1	0	0	0	100
P1290P	c.3870A>G	SNV	Exon23	10	3	0	0	0	100
4005+1G>A	c.3873+1G>A	SNV	Intron23	0	0	1	0	0	100
4016insT	c.3884_3885 insT	DIV	Exon24	0	0	1	0	0	100

Genotype (Common Name/cDNA Name/Coordinate)	cDNA Name	Variant Type	CFTR Gene Region (hg19)	Positive Calls (Variants)			No Calls*	Miscalls	Positive Agreement
				Clinical Samples	Cell Line Samples	Synthetic Samples			
T1299T	c.3897A>G	SNV	Exon24	3	0	0	0	0	100
N1303K	c.3909C>G	SNV	Exon24	9	1	0	0	0	100
Q1313X	c.3937C>T	SNV	Exon24	0	0	1	0	0	100
G1349D	c.4046G>A	SNV	Exon25	0	1	0	0	0	100
4209TG TT>AA	c.4077_4080 delTGTT insAA	DIV	Exon25	0	0	1	0	0	100
CFTR dele22,23	c.3964-78_ 4242+577del	Del	Intron24	1	0	1	0	0	100
4382delA	c.4251delA	DIV	Exon27	0	0	1	0	0	100
Y1424Y	c.4272C>T	SNV	Exon27	6	2	0	0	0	100
Q1463Q	c.4389G>A	SNV	Exon27	150	32	0	0	0	100
Total All Variants (PA)†				2072			3	4	99.66
Total All WT (NA)				2600928			1	2 [§]	> 99.99
Total All WT and Variants (OA)				2603000			4	6	> 99.99

DIV is an acronym for Deletion/Insertion Variant.

* Samples were not retested.

^ Software does not report cDNA name for this genomic coordinate.

** The Sanger report listed 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. MiSeqDx reported the variant as homozygous.

† 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 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.

† PA excluding PolyTG/PolyT calls was 100%.

[§] 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. Additionally, for a second sample, Sanger primers could not fully detect the variant Q1463Q due to indels both upstream and downstream of the variant site.

Table 17 PolyTG/PolyT variant accuracy for the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay

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	50.00
(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	90.91
(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
(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	92.31
(TG)11(T)5/(TG)11(T)7	6	0	0	1	0	83.33
(TG)11(T)7/(TG)11(T)7	52	8	0	0	0	100

PolyTGPolyT Genotype	# Clinical Samples	# Cell Line Samples	# Synthetic Samples	# Miscalls	# No Calls*	% Accuracy
(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.

Reproducibility

The reproducibility of the MiSeqDx Cystic Fibrosis System was determined through a blinded study using 3 trial sites and 2 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 276 sample results per operator. The panel contained a mix of genomic DNA from lymphoblastoid cell lines with known mutations in the *CFTR* gene, as well as leukocyte-depleted blood spiked with lymphoblastoid cell lines with known mutations 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 99.7%. All results are based on initial testing.

The genotype-level PA for all variants including the PolyTG/PolyT variant was 99.22% and excluding the PolyTG/PolyT variant was 99.60%. The NA for all WT was 99.70% and the OA for all reported positions was 99.70%. The PolyTG/PolyT variant PA was 97.83%.

Table 18 Reproducibility of the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay (excluding PolyTG/PolyT variants)

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
1	c.1408G>A	V470M	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^e	Miscalls	
1	c.1646G>A	S549N	6	18	6	6	6	0	0	100
1	c.2562T>G	T854T	6	18	6	6	6	0	0	100
2	c.1408G>A	V470M	6	18	6	6	6	0	0	100
2	c.1581A>G	E527E	6	18	6	6	6	0	0	100
2	c.1680-1G>A	1812-1 G>A	6	18	6	6	6	0	0	100
2	c.2562T>G	T854T	6	18	6	6	6	0	0	100
2	c.312delA	444delA	6	18	6	6	6	0	0	100
2	c.3870A>G	P1290P	6	18	6	5	6	0	1	94.44
2	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
3	c.1408G>A	V470M	6	18	6	6	6	0	0	100
3	c.1477C>T	Q493X	6	18	6	6	6	0	0	100
3	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
3	c.2562T>G	T854T	6	18	6	6	6	0	0	100
3	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
4	c.1408G>A	V470M	6	18	5	6	6	1	0	94.44
4	c.1521_1523delCTT	F508del	6	18	5	6	6	1	0	94.44
4	c.2052delA	2184delA	6	18	5	6	6	1	0	94.44

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls [€]	Miscalls	
5	c.1408G>A	V470M	6	18	6	5	6	1^	0	94.44
5	c.224G>A	R75Q	6	18	6	5	6	1^	0	94.44
5	c.2562T>G	T854T	6	18	6	5	6	1^	0	94.44
5	c.3472C>T	R1158X	6	18	6	5	6	1^	0	94.44
5	c.366T>A	Y122X	6	18	6	5	6	1^	0	94.44
5	c.625G>T	A209S	6	18	6	5	6	1^	0	94.44
6	c.1408G>A	V470M	6	18	6	6	6	0	0	100
6	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
6	c.2051_2052delAAinsG	2183AA>G	6	18	6	6	6	0	0	100
7	c.1408G>A	V470M	6	18	6	6	6	0	0	100
7	c.223C>T	R75X	6	18	6	6	6	0	0	100
7	c.2562T>G	T854T	6	18	6	6	6	0	0	100
8	c.1408G>A	V470M	6	18	6	6	6	0	0	100
8	c.1519_1521delATC	I507del	6	18	6	6	6	0	0	100
8	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
8	c.2562T>G	T854T	6	18	6	6	6	0	0	100
8	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
9	c.1408G>A	V470M	6	18	6	6	6	0	0	100
9	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
9	c.2562T>G	T854T	6	18	6	6	6	0	0	100
9	c.3846G>A	W1282X	6	18	6	5	6	0	1*	94.44
9	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
10	c.1408G>A	V470M	6	18	6	6	6	0	0	100
10	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
10	c.2562T>G	T854T	6	18	6	6	6	0	0	100
10	c.3140-26A>G	3272-26A>G	6	18	6	5	6	0	1*	94.44
10	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
11, 39	c.1408G>A	V470M	12	36	12	12	12	0	0	100
11, 39	c.1521_1523delCTT	F508del	12	36	12	12	12	0	0	100
11, 39	c.2002C>T	R668C	12	36	12	12	12	0	0	100
11, 39	c.2562T>G	T854T	12	36	12	12	12	0	0	100
11, 39	c.3717+12191C>T	3849+10kbC>T	12	36	12	12	12	0	0	100
11, 39	c.4389G>A	Q1463Q	12	36	12	12	12	0	0	100
12, 40	c.1408G>A	V470M	12	36	12	12	12	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
12, 40	c.2562T>G	T854T	12	36	12	12	12	0	0	100
12, 40	c.2988+1G>A	3120+1G>A	12	36	12	12	12	0	0	100
12, 40	c.4389G>A	Q1463Q	12	36	12	12	12	0	0	100
12, 40	c.489+1G>T	621+1G>T	12	36	12	12	12	0	0	100
13	c.1408G>A	V470M	6	18	6	6	6	0	0	100
13	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
13	c.178G>T	E60X	6	18	6	6	6	0	0	100
13	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
14	c.1408G>A	V470M	6	18	6	6	6	0	0	100
14	c.1584G>A	E528E	6	18	6	6	6	0	0	100
14	c.2562T>G	T854T	6	18	6	6	6	0	0	100
14	c.3302T>A	M1101K	6	18	6	6	6	0	0	100
15	c.1408G>A	V470M	6	18	6	6	6	0	0	100
15	c.1584G>A	E528E	6	18	6	6	6	0	0	100
15	c.2562T>G	T854T	6	18	6	6	6	0	0	100
15	c.3302T>A	M1101K	6	18	6	6	6	0	0	100
16	c.1408G>A	V470M	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls [€]	Miscalls	
16	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
16	c.3080T>C	I1027T	6	18	6	6	6	0	0	100
17, 41	c.1408G>A	V470M	12	36	12	12	12	0	0	100
17, 41	c.1521_1523delCTT	F508del	12	36	12	12	12	0	0	100
17, 41	c.3528delC	3659delC	12	36	12	12	12	0	0	100
18, 42	c.-4G>C	117120145	12	36	12	12	12	0	0	100
18, 42	c.1408G>A	V470M	12	36	12	12	12	0	0	100
18, 42	c.1521_1523delCTT	F508del	12	36	12	12	12	0	0	100
18, 42	c.350G>A	R117H	12	36	12	12	12	0	0	100
19	c.1408G>A	V470M	6	18	6	6	6	0	0	100
19	c.489+1G>T	621+1G>T	6	18	6	6	6	0	0	100
19	c.579+1G>T	711+1G>T	6	18	6	6	6	0	0	100
20, 43	c.1408G>A	V470M	12	36	12	12	12	0	0	100
20, 43	c.254G>A	G85E	12	36	12	12	12	0	0	100
20, 43	c.489+1G>T	621+1G>T	12	36	12	12	12	0	0	100
21, 44	c.1364C>A	A455E	12	36	12	12	12	0	0	100
21, 44	c.1408G>A	V470M	12	36	12	12	12	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
21, 44	c.1521_1523delCTT	F508del	12	36	12	12	12	0	0	100
22	c.1408G>A	V470M	6	18	6	6	6	0	0	100
22	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
22	c.1679G>C	R560T	6	18	6	6	6	0	0	100
22	c.2562T>G	T854T	6	18	6	6	6	0	0	100
22	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
23	c.1408G>A	V470M	6	18	6	6	6	0	0	100
23	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
23	c.3276C>A	Y1092X (C>A)	6	18	6	6	6	0	0	100
24, 45	c.1408G>A	V470M	12	36	12	12	12	0	0	100
24, 45	c.3909C>G	N1303K	12	36	12	12	12	0	0	100
24, 45	c.4046G>A	G1349D	12	36	12	12	12	0	0	100
25	c.1408G>A	V470M	6	18	6	6	6	0	0	100
25	c.1624G>T	G542X	6	18	6	6	6	0	0	100
26	c.-8G>C	117120141	6	18	6	6	6	0	0	100
26	c.1408G>A	V470M	6	18	6	6	6	0	0	100
26	c.1624G>T	G542X	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
27, 46	c.1408G>A	V470M	12	36	12	12	12	0	0	100
27, 46	c.1652G>A	G551D	12	36	12	12	12	0	0	100
27, 46	c.1657C>T	R553X	12	36	12	12	12	0	0	100
27, 46	c.2562T>G	T854T	12	36	12	12	12	0	0	100
27, 46	c.4389G>A	Q1463Q	12	36	12	12	12	0	0	100
28	c.1408G>A	V470M	6	18	6	6	6	0	0	100
28	c.2562T>G	T854T	6	18	6	6	6	0	0	100
28	c.3717+12191C>T	3849+10kbC>T	6	18	6	6	6	0	0	100
28	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
29	c.1408G>A	V470M	6	18	6	6	6	0	0	100
29	c.2562T>G	T854T	6	18	6	6	6	0	0	100
29	c.91C>T	R31C	6	18	6	6	6	0	0	100
30	c.1408G>A	V470M	6	18	6	6	6	0	0	100
30	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
30	c.2562T>G	T854T	6	18	6	6	6	0	0	100
30	c.3485G>T	R1162L	6	18	6	6	6	0	0	100
30	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
31	c.1408G>A	V470M	6	18	6	6	6	0	0	100
31	c.1585-1G>A	1717-1G>A	6	18	6	6	6	0	0	100
31	c.2562T>G	T854T	6	18	6	6	6	0	0	100
31	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
32	c.1408G>A	V470M	6	18	6	6	6	0	0	100
32	c.2562T>G	T854T	6	18	6	6	6	0	0	100
32	c.3484C>T	R1162X	6	18	6	6	6	0	0	100
32	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
33	c.1040G>C	R347P	6	18	6	6	6	0	0	100
33	c.1408G>A	V470M	6	18	6	6	6	0	0	100
33	c.1652G>A	G551D	6	18	6	6	6	0	0	100
33	c.2562T>G	T854T	6	18	6	6	6	0	0	100
33	c.4272C>T	Y1424Y	6	18	6	6	6	0	0	100
33	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
34	c.1000C>T	R334W	6	18	6	6	6	0	0	100
34	c.3368-2A>T	c.3368-2A>T	6	18	6	6	6	0	0	100
35	c.1523T>G	F508C	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls [€]	Miscalls	
36	c.254G>A	G85E	6	18	6	6	6	0	0	100
36	c.3454G>C	D1152H	6	18	6	6	6	0	0	100
37	c.1007T>A	I336K	6	18	6	6	6	0	0	100
37	c.1408G>A	V470M	6	18	6	6	6	0	0	100
37	c.2562T>G	T854T	6	18	6	6	6	0	0	100
37	c.3705T>G	S1235R	6	18	6	6	6	0	0	100
38	c.1408G>A	V470M	6	18	6	6	6	0	0	100
38	c.1727G>C	G576A	6	18	6	6	6	0	0	100
38	c.2002C>T	R668C	6	18	6	6	6	0	0	100
38	c.2057C>A	S686Y	6	18	6	6	6	0	0	100
38	c.2562T>G	T854T	6	18	6	6	6	0	0	100
38	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
47, 85	c.1408G>A	V470M	12	36	12	12	12	0	0	100
47, 85	c.2562T>G	T854T	12	36	12	12	12	0	0	100
47, 85	c.2657+5G>A	2789+5G>A	12	36	12	12	12	0	0	100
47, 85	c.4389G>A	Q1463Q	12	36	12	12	12	0	0	100
48, 86	c.54-5940_273+10250del21kb	CFTRdele2,3	12	36	12	11	12	1	0	97.22

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^e	Miscalls	
48, 86	c.1408G>A	V470M	12	36	12	11	12	1	0	97.22
48, 86	c.1521_1523delCTT	F508del	12	36	12	11	12	1	0	97.22
49, 87	c.1408G>A	V470M	12	36	12	12	12	0	0	100
49, 87	c.1521_1523delCTT	F508del	12	36	12	12	12	0	0	100
49, 87	c.1766+1G>A	1898+1G>A	12	36	12	12	12	0	0	100
50, 88	c.1408G>A	V470M	12	36	12	12	12	0	0	100
50, 88	c.220C>T	R74W	12	36	12	12	12	0	0	100
50, 88	c.2562T>G	T854T	12	36	12	12	12	0	0	100
50, 88	c.3808G>A	D1270N	12	36	12	12	12	0	0	100
51, 89	c.1408G>A	V470M	12	36	12	12	12	0	0	100
51, 89	c.1521_1523delCTT	F508del	12	36	12	12	12	0	0	100
51, 89	c.2012delIT	2143delIT	12	36	12	12	12	0	0	100
52	c.3744delA	3876delA	6	18	6	6	6	0	0	100
53, 90	c.3773_3774insT	3905insT	12	36	12	12	12	0	0	100
54, 91	c.1408G>A	V470M	12	36	12	12	12	0	0	100
54, 91	c.262_263delITT	394delITT	12	36	12	12	12	0	0	100
55, 92	c.1408G>A	V470M	12	36	12	12	12	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls [€]	Miscalls	
55, 92	c.1519A>G	I507V	12	36	12	12	12	0	0	100
55, 92	c.1521_1523delCTT	F508del	12	36	12	12	12	0	0	100
55, 92	c.2562T>G	T854T	12	36	12	12	12	0	0	100
55, 92	c.3080T>C	I1027T	12	36	12	12	12	0	0	100
55, 92	c.4389G>A	Q1463Q	12	36	12	12	12	0	0	100
56	c.1408G>A	V470M	6	18	6	6	6	0	0	100
56	c.2562T>G	T854T	6	18	6	6	6	0	0	100
56	c.3154T>G	F1052V	6	18	6	6	6	0	0	100
56	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
57	c.-8G>C	117120141	6	18	6	6	6	0	0	100
57	c.1408G>A	V470M	6	18	6	6	6	0	0	100
57	c.2562T>G	T854T	6	18	6	6	6	0	0	100
57	c.3209G>A	R1070Q	6	18	6	6	6	0	0	100
58	c.1408G>A	V470M	6	18	6	6	6	0	0	100
58	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
58	c.2991G>C	L997F	6	18	6	6	6	0	0	100
59	c.1408G>A	V470M	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^e	Miscalls	
59	c.2562T>G	T854T	6	18	6	6	6	0	0	100
59	c.3205G>A	G1069R	6	18	6	6	6	0	0	100
60	c.1408G>A	V470M	6	18	6	6	6	0	0	100
60	c.2562T>G	T854T	6	18	6	6	6	0	0	100
60	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
60	c.617T>G	L206W	6	18	6	6	6	0	0	100
61	c.1408G>A	V470M	6	18	6	6	6	0	0	100
61	c.2260G>A	V754M	6	18	6	6	6	0	0	100
61	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
62	c.1408G>A	V470M	6	18	6	6	6	0	0	100
62	c.2562T>G	T854T	6	18	6	6	6	0	0	100
62	c.988G>T	G330X	6	18	6	6	6	0	0	100
64	c.1040G>A	R347H	6	18	6	6	6	0	0	100
64	c.1408G>A	V470M	6	18	6	6	6	0	0	100
64	c.2562T>G	T854T	6	18	6	6	6	0	0	100
64	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
65	c.948delT	1078delT	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^e	Miscalls	
66	c.1408G>A	V470M	6	18	6	6	6	0	0	100
66	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
66	c.532G>A	G178R	6	18	6	6	6	0	0	100
67	c.1408G>A	V470M	6	18	6	6	6	0	0	100
67	c.1647T>G	S549R (c.1647T>G)	6	18	6	6	6	0	0	100
68	c.1408G>A	V470M	6	18	6	6	6	0	0	100
68	c.1646G>A	S549N	6	18	6	6	6	0	0	100
68	c.2562T>G	T854T	6	18	6	6	6	0	0	100
68	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
69	c.2506G>T	D836Y	6	18	6	6	6	0	0	100
69	c.2537G>A	W846X	6	18	6	6	6	0	0	100
70	c.1408G>A	V470M	6	18	6	6	6	0	0	100
70	c.2562T>G	T854T	6	18	6	6	6	0	0	100
70	c.3485G>T	R1162L	6	18	6	6	6	0	0	100
70	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
71	c.1408G>A	V470M	6	18	6	6	6	0	0	100
71	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
71	c.2562T>G	T854T	6	18	6	6	6	0	0	100
71	c.274G>T	E92X	6	18	6	6	6	0	0	100
71	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
72	c.1022_1023insTC	1154insTC	6	18	6	6	5	1	0	94.44
72	c.1408G>A	V470M	6	18	6	6	5	1	0	94.44
72	c.2562T>G	T854T	6	18	6	6	5	1	0	94.44
72	c.4389G>A	Q1463Q	6	18	6	6	5	1	0	94.44
72	c.489+1G>T	621+1G>T	6	18	6	6	5	1	0	94.44
73	c.1408G>A	V470M	6	18	6	6	6	0	0	100
73	c.1624G>T	G542X	6	18	6	6	6	0	0	100
73	c.1826A>G	H609R	6	18	6	6	6	0	0	100
74	c.1408G>A	V470M	6	18	6	6	5	0	1	94.44
74	c.1429C>T	P477S	6	18	6	6	6	0	0	100
74	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
75	c.1408G>A	V470M	6	18	6	5	6	1 [^]	0	94.44
75	c.1521_1523delCTT	F508del	6	18	6	5	6	1 [^]	0	94.44
75	c.1721C>A	P574H	6	18	6	5	6	1 [^]	0	94.44

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
76	c.1408G>A	V470M	6	18	6	6	6	0	0	100
76	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
76	c.2562T>G	T854T	6	18	6	6	6	0	0	100
76	c.425delT	F143LfsX10	6	18	6	6	6	0	0	100
76	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
77	c.1364C>A	A455E	6	18	6	6	6	0	0	100
77	c.1408G>A	V470M	6	18	6	6	6	0	0	100
77	c.489+1G>T	621+1G>T	6	18	6	6	6	0	0	100
78	c.1408G>A	V470M	6	18	6	6	6	0	0	100
78	c.1581A>G	E527E	6	18	6	6	6	0	0	100
78	c.1680-1G>A	1812-1 G>A	6	18	6	6	6	0	0	100
78	c.2562T>G	T854T	6	18	6	6	6	0	0	100
78	c.312delA	444delA	6	18	6	6	6	0	0	100
78	c.3870A>G	P1290P	6	18	6	6	6	0	0	100
78	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
79	c.1408G>A	V470M	6	18	6	6	6	0	0	100
79	c.220C>T	R74W	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
79	c.2562T>G	T854T	6	18	6	6	6	0	0	100
79	c.3808G>A	D1270N	6	18	6	6	6	0	0	100
80	c.-8G>C	117120141	6	18	6	6	6	0	0	100
80	c.1408G>A	V470M	6	18	6	6	6	0	0	100
80	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
80	c.1657C>T	R553X	6	18	6	6	6	0	0	100
80	c.2562T>G	T854T	6	18	6	6	6	0	0	100
81	c.1408G>A	V470M	6	18	6	6	6	0	0	100
81	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
81	c.1652G>A	G551D	6	18	6	6	6	0	0	100
81	c.2562T>G	T854T	6	18	6	6	6	0	0	100
81	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
82	c.1040G>C	R347P	6	18	6	6	6	0	0	100
82	c.1408G>A	V470M	6	18	6	6	6	0	0	100
82	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
82	c.4272C>T	Y1424Y	6	18	6	6	6	0	0	100
83	c.-4G>C	11720145	6	18	6	6	6	0	0	100

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total* (All Sites)		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls ^ε	Miscalls	
83	c.1408G>A	V470M	6	18	6	6	6	0	0	100
83	c.1521_1523delCTT	F508del	6	18	6	6	6	0	0	100
83	c.350G>A	R117H	6	18	6	6	6	0	0	100
84	c.1408G>A	V470M	6	18	6	6	6	0	0	100
84	c.1519_1521delATC	I507del	6	18	6	6	6	0	0	100
84	c.2562T>G	T854T	6	18	6	6	6	0	0	100
84	c.4389G>A	Q1463Q	6	18	6	6	6	0	0	100
Total All Variants (PA)** (including PolyTG/PolyT data in Table 19)			2580	7740	2562	2553	2565	37	23	99.22
Total All WT (NA)			2871132	8613396	2865930	2855526	2865932	26006	2	99.70
Total All WT and variants (OA)			2873712	8621136	2868492	2858079	2868497	26043	25	99.70

^ε Samples were not retested.

[^] One replicate each of samples 5 and 75 had a 0% call rate. Further investigation indicated that the samples had likely not been added to the sample plate prior to library preparation.

^{*} Upon review, samples 9 and 10 were likely switched by the operator prior to library preparation.

^{**} Excluding PolyTG/PolyT variants, the PA was 99.60%.

Table 19 PolyTG/PolyT Reproducibility for the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay

Panel	Sample	Genotype	# of Results		Agreeing Calls			Total All Sites		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls	Miscalls	
A	1	(TG)12(T)7/(TG)12(T)7	6	18	6	6	6	0	0	100%
A	2	(TG)10(T)9/(TG)10(T)7	6	18	6	6	6	0	0	100%

Panel	Sample	Genotype	# of Results		Agreeing Calls			Total All Sites		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls	Miscalls	
A	3	(TG)10(T)7/(TG)10(T)9	6	18	6	6	6	0	0	100%
A	4	(TG)10(T)9/(TG)11(T)7	6	18	5	6	6	1	0	94.44%
A	5	(TG)10(T)7/(TG)11(T)7	6	18	6	5	6	1	0	94.44%
A	6	(TG)10(T)9/(TG)10(T)7	6	18	6	6	6	0	0	100%
A	7	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	8	(TG)10(T)7/(TG)10(T)9	6	18	6	6	6	0	0	100%
A	9	(TG)10(T)9/(TG)10(T)7	6	18	6	6	6	0	0	100%
A	10	(TG)10(T)9/(TG)10(T)7	6	18	6	6	6	0	0	100%
A	11, 39	(TG)10(T)9/(TG)10(T)7	12	36	12	12	12	0	0	100%
A	12, 40	(TG)10(T)9/(TG)11(T)7	12	36	12	12	12	0	0	100%
A	13	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	14	(TG)10(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	15	(TG)10(T)7/(TG)11(T)7	6	18	6	5	6	1	0	94.44%
A	16	(TG)10(T)9/(TG)10(T)9	6	18	6	6	6	0	0	100%
A	17, 41	(TG)10(T)9/(TG)11(T)7	12	36	12	12	12	0	0	100%
A	18, 42	(TG)10(T)9/(TG)12(T)5	12	36	12	12	12	0	0	100%
A	19	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	20, 43	(TG)10(T)9/(TG)11(T)7	12	36	12	12	12	0	0	100%
A	21, 44	(TG)10(T)9/(TG)10(T)9	12	36	12	12	12	0	0	100%

Panel	Sample	Genotype	# of Results		Agreeing Calls			Total All Sites		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls	Miscalls	
A	22	(TG)10(T)9/(TG)10(T)7	6	18	6	6	6	0	0	100%
A	23	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	24, 45	(TG)10(T)9/(TG)11(T)7	12	36	12	12	12	0	0	100%
A	25	(TG)10(T)9/(TG)10(T)9	6	18	6	6	6	0	0	100%
A	26	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	27, 46	(TG)10(T)7/(TG)11(T)7	12	36	11	12	12	0	1	97.22%
A	28	(TG)10(T)7/(TG)10(T)7	6	18	6	6	6	0	0	100%
A	29	(TG)10(T)7/(TG)12(T)7	6	18	6	4	4	4	0	77.78%
A	30	(TG)10(T)9/(TG)10(T)7	6	18	6	6	6	0	0	100%
A	31	(TG)10(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	32	(TG)10(T)7/(TG)10(T)7	6	18	6	6	6	0	0	100%
A	33	(TG)10(T)7/(TG)11(T)7	6	18	5	6	6	1	0	94.44%
A	34	(TG)11(T)7/(TG)12(T)7	6	18	6	6	6	0	0	100%
A	35	(TG)11(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	36	(TG)11(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
A	37	(TG)11(T)7/(TG)12(T)7	6	18	6	6	6	0	0	100%
A	38	(TG)10(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	47, 85	(TG)10(T)7/(TG)10(T)7	12	36	12	12	12	0	0	100%
B	48, 86	(TG)10(T)9/(TG)11(T)7	12	36	11	11	12	2	0	94.44%

Panel	Sample	Genotype	# of Results		Agreeing Calls			Total All Sites		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls	Miscalls	
B	49, 87	(TG)10(T)9/(TG)11(T)7	12	36	12	12	12	0	0	100%
B	50, 88	(TG)10(T)9/(TG)11(T)7	12	36	12	12	12	0	0	100%
B	51, 89	(TG)10(T)9/(TG)10(T)9	12	36	12	12	12	0	0	100%
B	52	(TG)11(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	53, 90	(TG)11(T)7/(TG)11(T)7	12	36	12	12	12	0	0	100%
B	54, 91	(TG)10(T)9/(TG)11(T)7	12	36	12	12	12	0	0	100%
B	55, 92	(TG)10(T)9/(TG)10(T)7	12	36	12	12	12	0	0	100%
B	56	(TG)10(T)7/(TG)10(T)9	6	18	6	6	6	0	0	100%
B	57	(TG)12(T)7/(TG)12(T)7	6	18	6	6	6	0	0	100%
B	58	(TG)10(T)9/(TG)10(T)9	6	18	6	6	6	0	0	100%
B	59	(TG)11(T)7/(TG)12(T)7	6	18	5	6	6	1	0	94.44%
B	60	(TG)9(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	61	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	62	(TG)10(T)7/(TG)11(T)7	6	18	5	6	6	1	0	94.44%
B	63	(TG)11(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	64	(TG)10(T)7/(TG)11(T)7	6	18	5	6	6	1	0	94.44%
B	65	(TG)11(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	66	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	67	(TG)11(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%

Panel	Sample	Genotype	# of Results		Agreeing Calls			Total All Sites		% Agreement
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls	Miscalls	
B	68	(TG)10(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	69	(TG)11(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	70	(TG)10(T)7/(TG)10(T)7	6	18	6	6	6	0	0	100%
B	71	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	72	(TG)10(T)7/(TG)10(T)9	6	18	5	6	5	2	0	88.89%
B	73	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	74	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	75	(TG)10(T)7/(TG)10(T)9	6	18	6	5	6	1	0	94.44%
B	76	(TG)10(T)7/(TG)10(T)9	6	18	6	6	6	0	0	100%
B	77	(TG)10(T)9/(TG)10(T)9	6	18	6	6	6	0	0	100%
B	78	(TG)10(T)7/(TG)10(T)9	6	18	5	6	6	1	0	94.44%
B	79	(TG)10(T)7/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	80	(TG)11(T)7/(TG)11(T)9	6	18	0	0	0	0	18*	0%
B	81	(TG)10(T)7/(TG)10(T)9	6	18	6	6	6	0	0	100%
B	82	(TG)10(T)9/(TG)11(T)7	6	18	6	6	6	0	0	100%
B	83	(TG)10(T)9/(TG)12(T)5	6	18	6	6	6	0	0	100%
B	84	(TG)10(T)7/(TG)10(T)7	6	18	6	6	6	0	0	100%
Total PolyTG/PolyT Variants (PA)			552	1656	537	540	543	17	19	97.83%

* All 18 samples were concordant with each other but discordant with Sanger bi-directional sequencing.

DNA Extraction

Three commonly used, commercially available extraction methods representing magnetic bead extraction, alcohol precipitation and silica filter column isolation methods, were evaluated using K₂EDTA anti-coagulated whole blood. A total of 14 blood samples were used during the study; two were wild type, while the remaining samples carried unique genotypes representing nine different variants, including both common and rare variants. For the polyTG/polyT variation, samples with (T)5-9 and (TG)10-12 were included. 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	> 99.99%	> 99.99%	100%
Silica Filter Column Isolation	168	> 99.99%	> 99.99%	100%
Magnetic Bead Extraction	168	> 99.99%	> 99.99%	100%

* Percent of samples having call rate of >99% in first run.

DNA Input

The DNA input range of the Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing 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 ≥ 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=4x20=80 samples), while the lower bound of 25 ng was tested with 14 samples, 20 replicates for each sample (n=14x20=280 samples). The accuracy and sample first pass rate was 100% at all DNA input levels.

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. Sixteen whole blood specimens with unique CF genotypes were tested in the study. Four endogenous interfering substances (bilirubin, cholesterol, hemoglobin, and triglycerides) were tested by spiking them into blood specimens prior to DNA extraction. The concentration limits for each substance is 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 Clinical Sequencing 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. No interference was observed from any of the endogenous or exogenous interferents.

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 ≥ 40%.

Test Substance	Total Number of Replicates	Concentration Tested in Blood (Upper Limit)	Concentration Tested in Blood (Lower Limit)	Call Rate
Bilirubin	16	684 µmol/L	137 µmol/L	100%
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%

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