510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY

A. 510(k) Number:

K132750

B. Purpose for Submission:

New device

C. Measurand:

Protein coding regions and intron/exon boundaries of the cystic fibrosis transmembrane conductance gene regulator (CFTR) gene

D. Type of Test:

High-throughput, Targeted DNA Sequencing

E. Applicant:

Illumina, Inc.

F. Proprietary and Established Names:

Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay

G. Regulatory Information:

1. Regulation section:

   21 CFR 866.5900 CFTR (cystic fibrosis transmembrane conductance regulatory) gene mutation detection system

2. Classification:

   Class II

3. Product code:

   PFS, System, Cystic Fibrosis Transmembrane Conductance Regulator Gene, Variant Gene Sequence Detection

4. Panel:

   Immunology (82)

H. Intended Use:

1. Intended use(s):
   The Illumina MiSeqDx™ Cystic Fibrosis Clinical Sequencing Assay is a targeted sequencing in vitro diagnostic system that re-sequences 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 K_2EDTA. 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). The test 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.

2. Indication(s) for use:

   Same as intended use

3. Special conditions for use statement(s):

   For prescription use only

4. Special instrument requirements:

   Illumina MiSeqDx Instrument using the following software versions: MOS v1.0.27; RTA v1.16.18; MSR v2.2.30; IWM v1.0.14; UMS v1.0.0.5; MTS v1.0.7; Reference Genome File v1.1; Recipe Fragments File v1.0.0; and Manifest File Revision B.

I. Device Description:

The Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay consists of library preparation and sample indexing reagents, sequencing reagents and consumables, MiSeqDx instrument and data analysis software. A total of 5203 base pairs are sequenced in this assay. The assay additionally reports on the PolyT/PolyTG region in intron 9 and 2 large deletions (CFTRdele2,3 and CFTRdele22,23), bringing the total number of callable positions to 5206.

The following is a description of the assay coverage using human genome build 19 (hg19) as the reference sequence.

Table 1. Regions of the CFTR gene sequenced by the assay

CFTR Exon	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
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. This is 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 separately.
*For the deep intronic mutations, 5 nucleotides flanking the SNV on either side are also included.

The assay is designed in a single configuration for 6 runs of 48 samples per kit. The reagent cartridge, flow cell, SBS solution, and filter plates are designed for single use. The assay components are divided into 8 separate boxes, based on amplification stage (pre- or post-amplification) and storage conditions, and include the following components:

	Quantity per kit	Volume
Box 1A Pre-Amplification Reagents		
CF Clinical Sequencing Assay Oligo Pool	1 tube	600 µL
Hybridization Buffer	1 tube	4.32 mL
Extension-Ligation Mix	1 tube	4.8 mL
Index Primers C (A503), D (A504), and E (A505)	1 tube per primer	192 µL
Index Primers 1 (A701), 2 (A702), and 10 (A710)	1 tube per primer	128 µL
PCR Polymerase	1 tube	56 µL
PCR Master Mix	1 tube	2.8 mL
Box 1B Post-Amp Reagents		
Library Normalization Diluent	1 tube	4.6 mL
Library Dilution Buffer	1 tube	4.5 mL
PhiX Internal Control	1 tube	10 µL
Box 2 Post-Amp Reagents		
MiSeqDx Reagent Cartridge – CF Clinical Sequencing Assay	6 cartridges	1 cartridge
Box 3A Pre-Amp Reagents		
Stringent Wash Buffer	1 bottle	24 mL
Universal Wash Buffer	1 tube	4.8 mL
Box 3B Post-Amp Reagents		
PCR Clean-Up Beads	1 tube	5 mL
Library Normalization Wash	6 tubes	4.8 mL
Library Beads	1 tube	1.2 mL
MiSeqDx Flow Cell – CF Clinical Sequencing Assay	6 containers	1 flow cell
Box 4 Post-Amp Reagents		
MiSeqDx SBS Solution (PR2) – CF Clinical Sequencing Assay	6 bottles	353.1 mL
Box 5 Pre-Amp Reagents		
Filter Plate	6 plates	N/A
Box 5 Post-Amp Reagents		
Elution Buffer	1 tube	4.8 mL
Library Storage Buffer	1 tube	3.5 mL

A brief description of some of the primary components is listed below:

- CF Clinical Sequencing Oligo Pools: Oligonucleotides specific for the genomic

regions targeted by the test. For each region, there is an upstream locus specific oligonucleotide and a downstream locus specific oligonucleotide

- Extension-Ligation Mix: Buffer containing DNA polymerase and DNA ligase, which is applied to the sample on the filter plate and catalyzes the connection of the upstream locus specific oligonucleotide to the downstream locus specific oligonucleotide
- PCR Master Mix: Contains all of the components required for PCR amplification except for PCR primers and DNA polymerase
- Index PCR Primers: three (3) i7 and three (3) i5 index PCR primers for universal amplification of the ligated products. These primers incorporate P5 and P7 sequences, which are complementary to the sequences of the capture oligonucleotides attached to the flow cell. These primers also incorporate a sample specific sequence tag that is required to pool 8 samples into a single flow cell/MiSeq run.
- AMPure XP beads: Streptavidin coated magnetic beads used to capture the PCR product for removal of unincorporated primers and nucleotides.
- Library Normalization Diluent/Library Breads: Allow for bead-based normalization of the amount of PCR product produced across different samples.
- MiSeq Reagent Cartridge: Pre-filled, single use reagent cartridge which contains the reagents required for cluster generation and SBS sequencing. The pooled libraries are added to the cartridge which is then inserted into the MiSeq instrument. The components of the Reagent Cartridge are as follows:
    - Incorporation Mix: Contains DNA polymerase, fluorescently labeled nucleotides and buffer used for incorporation of reversible terminator nucleotide during SBS reaction
    - Scan Mix: Contains buffers to flush out unincorporated fluorescently labeled nucleotides in order to facilitate scanning of the clusters during the SBS reaction
    - Cleavage Mix: Contains buffers and enzyme that removes the terminator and fluorescent signal from incorporated fluorescently labeled nucleotide, which allows the incorporation of additional nucleotides in later rounds of the SBS reaction
    - Amplification Mix: Contains buffer, DNA polymerase, and unlabeled nucleotides that are used to bridge amplify the prepared library during the cluster generation process
    - Amplification Mix for Read 2
    - Linearization Pre-mix
    - Formamide
    - Linearization Mix 1: Contains enzyme and buffer required to linearize the first read clusters in preparation for their use in the SBS reaction
    - Linearization Mix 2
    - Resynthesis Mix: Contains enzyme and buffer for the synthesis of reads during the cluster generation process
    - SBS Primer for Read 1
    - SBS Primer for Indexing Read
    - SBS Primer for Read 2
    - Water

- Flow Cell: Single-use glass substrate with covalently bound oligonucleotides for capture and solid phase amplification and SBS sequencing of the targets created during library preparation

J. Substantial Equivalence Information:

1. Predicate device name(s):

   Luminex xTAG Cystic Fibrosis 60 Kit v2

2. Predicate 510(k) number(s):

   k083845

3. Comparison with predicate:

Similarities		
Item	New Device MiSeqDx CF Clinical Sequencing Assay	Predicate Luminex xTAG Cystic Fibrosis 60 Kit v2
Indication for Use	The test is intended to be used as an aid in the diagnosis of individuals with suspected cystic fibrosis (CF).	Same
Specimen Type	Genomic DNA isolated from peripheral whole blood	Same
Anticoagulant	EDTA	EDTA or citrate
Nucleic Acid Extraction	DNA extraction using validated laboratory method	Same

Differences		
Item	New Device MiSeqDx CF Clinical Sequencing Assay	Predicate Luminex xTAG Cystic Fibrosis 60 Kit v2
Intended Use	The Illumina MiSeqDx(TM) Cystic Fibrosis Clinical Sequencing Assay is a targeted sequencing in vitro diagnostic system that re-sequences the protein coding regions and intron/exon boundaries of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene in genomic DNA isolated	The xTAG® Cystic Fibrosis 60 kit v2 is a device used to simultaneously detect and identify a panel of mutations and variants in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in human blood specimens. The

Differences		
Item	New Device MiSeqDx CF Clinical Sequencing Assay	Predicate Luminex xTAG Cystic Fibrosis 60 Kit v2
	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). The test 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.	panel includes mutations and variants currently recommended by the American College of Medical Genetics and American College of Obstetricians and Gynecologists (ACMG/ACOG) plus some of the world's most common and North American prevalent mutations. The xTAG Cystic Fibrosis 60 kit v2 is a qualitative genotyping test which provides information intended to be used for carrier testing in adults of reproductive age, as an aid in newborn screening, and in confirmatory diagnostic testing in newborns and children.
Uses for which the Test is Not Indicated	Not indicated for stand-alone diagnostic purposes, fetal diagnostic testing, for pre-implantation testing, carrier screening, newborn screening, or population screening	Not indicated for fetal diagnostic testing, pre-implantation testing, or for stand-alone diagnostic purposes
Interpretation of Results	Intended to be interpreted by a board-certified clinical molecular geneticist or equivalent	Specialized interpretation not required
Assay Type	High-throughput targeted amplicon-based sequencing	Qualitative nucleic acid multiplex test
Variants Detected	Single nucleotide variants and small indels (<3 bp) from the protein coding regions and	60 CFTR mutations and 4 variants (benign polymorphisms)

Differences		
Item	New Device MiSeqDx CF Clinical Sequencing Assay	Predicate Luminex xTAG Cystic Fibrosis 60 Kit v2
	intron/exon boundaries of the CFTR gene, including two deep intronic mutations and two large deletions	
Technology	High-throughput, Targeted DNA Sequencing, Sequencing by Synthesis (SBS). Reversible terminator-based method to detect single bases as they are incorporated into growing DNA strands. Fluorescently-labeled terminators are detected using a dual-color laser. Base calls are made directly from signal intensity measurements during each sequencing cycle.	Multiplex PCR followed by multiplex allele specific primer extension for genotyping, hybridized to multiplex fluorescent microparticles, and run on fluidic microbead reader which includes a dual-color laser detection system that enables optical scanning by flow cytometry.
Instrument System	MiSeqDx	Luminex 100 or 200 IS

K. Standard/Guidance Document Referenced (if applicable):

Guidance for Industry and FDA Staff: Class II Special Controls Guidance Document: CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) Gene Mutation Detection Systems; October 26, 2005

Guidance for Industry and FDA Staff: Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices; May 11, 2005

General Principles of Software Validation; Final Guidance for Industry and FDA Staff; January 11, 2002

Guidance for Industry - Cybersecurity for Networked Medical Devices Containing Off-the-Shelf (OTS) Software; January 14, 2005

Guidance for Industry, FDA Reviewers and Compliance on Off-the-Shelf Software Use in Medical Devices; September 9, 1999

CLSI Standard EP05-A2, Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline – Second Edition.

CLSI Standard EP07-A3, Interference Testing in Clinical Chemistry, Approved Guideline – Third Edition.

CLSI Standard EP09-A2, Method Comparison and Bias Estimation Using Patient Samples, Approved Guideline – Second Edition.

CLSI Standard EP12-A2, User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline – Second Edition.

CLSI Standard EP14-A2, Evaluation of Matrix Effects, Approved Guideline – Second Edition.

CLSI Standard EP17-A, Protocols for the Determination of Limits of Detection and Limits of Quantification, Approved Guideline.

CLSI Standard EP25-A, Evaluation of Stability on In Vitro Diagnostic Reagents, Approved Guideline.

L. Test Principle:

The Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay encompasses two main procedures. The first is to manually 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 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. 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. The third step, PCR Amplification, amplifies the extension-ligated 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). 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 by Sequencing by Synthesis (SBS) chemistry.

The second procedure is to sequence the prepared samples using SBS chemistry on the MiSeqDx. 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 end result is base-by-base sequencing.

The MiSeq Reporter software processes base calls generated during primary analysis and produces information about each sample based on information specified in the samples sheet, called secondary analysis. As described below, secondary analysis includes de-multiplexing, FASTQ file generation, alignment, variant calling, and generation of VCF files containing information about CFTR variants found at specific positions. De-multiplexing separates data from pooled samples based upon the unique sequence indexes that were added during the PCR amplification step. The FASTQ format 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 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. MiSeq Reporter software uses a banded Smith-Waterman algorithm that performs local sequence alignments to determine similar regions between two sequences. Variant calling then records insertion and deletions (indels), and other structural variants in a standardized and parsable test file.

M. Performance Characteristics (if/when applicable):

1. Analytical performance:

   a. Precision/Reproducibility:

   Reproducibility:

   The reproducibility of the MiSeqDx assay was evaluated at 3 external sites, by two operators at each site on 3 nonconsecutive days. The study included 2 sample panels consisting of mock blood and genomic DNA (gDNA) samples. The mock blood samples were created from leukocyte-depleted whole blood spiked with cell lines known to harbor a CFTR mutation. The gDNA samples were derived from cell lines with known CFTR variants. Panel A was comprised of 8 mock blood samples and 38 gDNA samples. Panel B was comprised of 38 gDNA samples. A new sample library was prepared for each run. A total of 18 runs per panel were performed. There were 16 duplicate samples between the 2 panels, giving a total of 76 unique samples of the total 92. Positive and negative control samples were included in each run.

   A positive percent agreement (PPA), negative percent agreement (NPA), and overall agreement (OA) were assessed. Sanger sequencing was used to identify the variants with the exception of the 2 large deletions that were verified using a validated PCR assay followed by sequencing of the resulting amplicons.

   There were 7 runs identified as invalid due to failed QC metrics and were re-run. Additionally, 5 samples failed QC metrics. These samples were not run again and results from these samples were considered non-matches at every position. Two of the samples had a 0% call rate, which upon further investigation, it was determined that the samples were not added when making up the library preparation. For the remaining samples, there were 63 discordant results between the assay and Sanger

sequencing along the 5206 callable positions. Of these 39 were due to a no call by the assay. Of the remaining 24 discordant calls, 18 occurred in sample 80 and were for the PolyTG/PolyT region. The remaining 6 discordant results were miscalls occurring in a single run (i.e., disagreed with calls reported by the other 17 runs).

Overall, the study achieved a genotype-level PPA of 99.2%, NPA of 99.7%, and OA of 99.7% which met all of the acceptance criteria for the study.

Table 2 below represents the summary reproducibility results for observed variants, excluding the PolyTG/PolyT region, while the Table 3 summarizes the PolyTG/PolyT results. PPA, NPA, and OA were determined for all 5206 callable positions. However the variants observed are shown below for simplicity and because the majority of discordant results were in the context of variant detection, most notably in the PolyTG/PolyT region.

Table 2: Reproducibility for Variants Observed in Sample Panel

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls[¤]	Miscalls	
1	c.1408G>A	V470M	6	18	6	6	6	0	0	100
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
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

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls[¤]	Miscalls	
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
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
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

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls¤	Miscalls	
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
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	117120145	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
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

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls¤	Miscalls	
26	117120141	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
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
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
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

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls¤	Miscalls	
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
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.2012delT	2143delT	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_263delTT	394delTT	12	36	12	12	12	0	0	100
55, 92	c.1408G>A	V470M	12	36	12	12	12	0	0	100
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

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls¤	Miscalls	
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	117120141	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
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
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

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls[¤]	Miscalls	
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
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
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

Sample	HGVS Name (or Location if no HGVS)	Variant Name	Total Results		Agreeing Calls			Total # (All Sites)		OA (%)
			Per Site	All Sites	Site 1	Site 2	Site 3	No Calls¤	Miscalls	
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
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	117120141	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	11720145	11720145	6	18	6	6	6	0	0	100
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 (PPA)*			2580	7740	2562	2553	2565	37	23	99.22
Total All WT (NPA)			2871132	8613396	2865930	2855526	2865932	26006	2	99.70
Total All WT and variants (OA)			2873712	8621136	2868492	2858079	2868497	26043	25	99.70

¤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%
a Covers all 5206 callable positions interrogated by the assay

The panels used for the reproducibility studies included a number of PolyTG/PolyT tract variations that allowed for examination of assay performance over the majority of permutations in tract size. With the exception of (TG)11(T)7/(TG)11(T)9 samples,

the assay performed similarly regardless of PolyTG/PolyT tract size. The table below outlines reproducibility results by PolyTG/PolyT tract size.

Table 3: Reproducibility for PolyTG/PolyT Variants Observed in Sample Panel

Panel	Sample	Genotype	Test 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%
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%
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%
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%
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.

Lot-to-Lot Reproducibility:

A single operator performed a lot-to-lot reproducibility study using the three kit lots and one instrument. Each kit lot included all the reagents and materials intended to be packaged as part of the Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay. A panel of 47 unique gDNA samples tested in duplicate was tested on each lot. The samples were selected to represent a wide array of potential variants. Samples were extracted from immortalized cell lines and included the ACMG 23 mutations, 21 deletion/insertion variants, 5 homozygous samples, 28 compound heterozygotes, 1 sample containing 1 of the large deletions, samples which represent the commonly observed sequence possibilities at the PolyTG/Poly T region, and single nucleotide polymorphisms (SNPs) in 15

different exons and 9 different intronic regions. There were no miscalls. There were 5 no calls for lot 1, 1 no call for lot 2 and 0 no calls for lot 3. Results showed that all reagent lots met their acceptance criteria and performed equivalently.

Instrument-to-Instrument Reproducibility:

To establish instrument comparability, assay performance (call rate, reproducibility, sample first pass rate, and accuracy) was assessed on 3 different MiSeqDx instruments. Three operators each performed a single run on 3 different days on each of 3 different MiSeqDx instruments using a single lot of reagents. Instrument performance metrics were defined by sequencing output (in gigabases), reads (in millions), read length (2x150), and quality base calling score (%Q) > 30.

The 48 sample panel used in the lot-to-lot reproducibility study (47 samples plus a no-template control [NTC]) which represented different types of sequence variations was used in this study. If a NTC generated a call rate of <2%, then contamination would be suspected and the entire sequencing run would be considered failed and must be repeated starting from library preparation.

The Clinical Sequencing Assay sequences 5203 bases within the exonic regions and flanking intronic sequence and additionally reports on 2 large deletions and the PolyTG/PolyT sequence within intron 9, making the total number of positions sequences 5,206. With the exception of a single sample which had a no-call at a PolyTG/PolyT site, all 423 tests (47 samples x 9runs [3 operators x 3 instruments]) had a 100% call rate and reproducibility was 100% concordant. Accuracy examination by bi-directional sequencing also showed all samples had the expected sequence/genotype.

Thermal Cycler Evaluation:

Three different commonly used, commercially available thermal cyclers were compared for use in library preparation for the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay. Three unique sample sets were processed through the 3 thermal cyclers across 3 days. Each sample set was processed in triplicate each day (one replicate per thermal cycler). The sample set consisted of 15 extracted gDNAs from cell lines and 1 NTC. The sample panel included variants for every allele of the ACMG 23, 21 deletion/insertion variants, 5 homozygous samples, 27 compound heterozygotes, 1 sample containing one of the targeted large deletions, samples with commonly observed variants in the Poly TG/PolyT region and SNPs in 15 different exons and 9 different intronic regions. The data for each thermal cycler met specifications for call rate, accuracy and sample first pass rate.

DNA Extraction Study:

To evaluate the effect of the extraction step on sample reproducibility, an additional, separate extraction study was conducted. Three different commonly used DNA extraction methods from K_2EDTA anticoagulated whole blood were tested on 14 blood samples (2 samples were wild-type, while the other 12 represented 8 unique genotypes). The genomic DNA was isolated using three commonly used commercially available kits representing different methodologies (*i.e.*, magnetic bead separation, alcohol precipitation, and silica filter column).

The 3 DNA extraction methods were tested by 2 different operators who performed 3 runs per extraction method giving 9 replicate extractions per sample performed on separate days. All extracted gDNA samples were run in duplicate. All 3 extraction methods met acceptance criteria for call rate, reproducibility, and pass rate with an accuracy of 100%.

Repeatability:

To examine assay repeatability, 47 replicates of a single sample (with a NTC) were tested. The same experiment was repeated for a second sample by the same operator on the same instrument. The percent coefficient of variation (% CV) for coverage was determined for each of the 80 amplicons examined in this assay across 47 replicates. Results ranged from 7-18% CV with an average of 12% for sample 1 and 12% for sample 2.

b. Linearity/Assay Reportable range:

Not applicable.

c. Traceability, Stability, Expected values (controls, calibrators, or methods):

Real-Time Stability:

In order to determine the expiration dating of the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay reagents and consumables, the kit was evaluated at six time points after manufacturing: 5 months, 6.25 months, 7.25 months, 9 months, 9.25 months, and 12 months with three separately manufactured lots of reagents and consumables. Each lot of reagents was run in duplicate for the first 3 time points and singlicate at the 9 month time point. A panel of 45 specimens was used to assess performance. The results from each lot at each time point was assessed for call rate (acceptance criteria ≥99%), and accuracy (acceptance criteria ≥99.9%). No miscalls were observed for any of the time points. The largest number of no calls (N = 5) was observed with one lot at 7.25 months; however the call rate exceeded the acceptance criteria. All No Calls observed at any time point were for the PolyTG/PolyT variant. Based on the data provided, the initial shelf-life for the assay is 9.25 months.

Open Tube Stability (Freeze/Thaw):

Three independently manufactured lots of frozen library preparation/normalization reagents were used for testing. The assay reagents are single use and not stored frozen, and so were not tested in this protocol. All reagents were subjected to 6 freeze/thaw cycles. At each cycle (1 per day for 6 days), reagents were thawed at room temperature, the reagent volume required for testing was withdrawn, and the tubes were returned to -15 to -25°C for overnight storage. Thirteen gDNA samples were tested, including 1 NTC for a total of 14 samples. The 14 samples were pooled into a single library at each freeze/thaw time point and sequencing was run in the assay mode. Each freeze/thaw cycle was assessed for first pass rate (acceptance criteria ≥95% samples need to meet sample call rate specification of ≥99%), call rate (acceptance criteria ≥99%), and accuracy (acceptance criteria ≥99.9%). The data provided supports a maximum of 6 rounds of freeze/thaw.

Specimen Sampling and Handling:

The specimen handling study included the following conditions:

- DNA extracted from K_2EDTA anti-coagulated blood stored at room temperature (20-25°C) for 7 days (claimed in package insert)
- DNA extracted from K_2EDTA anti-coagulated blood stored at 2°C to 4°C for 30 days (claimed in package insert)
- DNA extracted from K_2EDTA anti-coagulated blood stored at -15°C to -25°C for 30 days (claimed in package insert)
- Genomic DNA subjected to 6 freeze/thaw cycles

Additionally, 6 blood samples were divided into 6 aliquots and stored under the following conditions:

- 2°C to 8°C for 1 day
- -15°C to -25°C for 1 day
- 2°C to 8°C for 30 days
- -15°C to -25°C for 30 days
- Room temperature for 7 days
- 30°C for 7 days

Extracted blood was then stored at -15°C to -25°C until the study commenced.

Fifteen samples aliquoted into 6 tubes were used to assess sample storage claims and 2 aliquots of each sample were used to assess freeze/thaw claims (one sample stored and the other undergoing freeze/thaw once a day for 6 days). Samples included SNVs and DIVs as well as homozygotes, heterozygotes, and compound heterozygotes. Acceptance criteria were based on call rate, sample first-pass rate and reproducibility, and all studies met their acceptance criteria.

Reagent Integrity and Shipping:

Shipping studies were designed to evaluate product performance after exposure to simulated shipping conditions. Study elements included atmospheric thermal cycling with both summer and winter profiles depending on the reagent being tested. Reagents believed to be sensitive to heat were subjected to summer profiles (enzymes, oligos, etc), while reagents believed to be sensitive to freeze/thaw were subjected to winter profiles. Distribution testing was also performed to include the following:

- Atmospheric pre-conditioning
- Shock-drop (8 and 9 drops)
- Vibration – Random dynamic load
- Random Vibration – Pick-up and Delivery Vehicle Spectrum
- Vibration – Random under low pressure vacuum

Shipping validation testing was conducted with 8 samples that included deletion/insertion variants (including in a homopolymeric region), SNVs, and 1 large deletion. After shipping testing was complete, all kit components were stored at recommended conditions until functional testing could be performed. Acceptance criteria included the assay requirements (call rate and accuracy ≥ 99%), payload and shipping testing had internal temperature acceptance criteria, and container integrity testing only required no major visual defects (minor defects including punctures, tears, and cracks in the container were acceptable). All testing passed by meeting all acceptance criteria.

d. Detection Limit (Analytical sensitivity):

Analytical Sensitivity at High DNA Input:

The recommended DNA input for this assay is 250 ng. An initial study was conducted to estimate the performance of the assay when the gDNA input is greater or less than the recommended DNA input concentration. Four different DNA samples were tested at input levels of 1250, 250, 100, and 25 ng. The 4 DNA samples covered 7 mutations and included heterozygous single nucleotide variants (SNVs), heterozygous and homozygous deletion/insertion variants (DIVs), one large deletion, and a deletion cum insertion. Either 20 (1250 ng) or 24 (all other concentrations) replicates were tested for each sample. Samples were processed by 2 operators over 4 days using a single lot of reagents and a single thermal cycler. The same lot of gDNA samples was used throughout the study. Genotype calls were compared to bidirectional Sanger sequencing except for the 2 large deletions which were confirmed using validated duplex PCR followed by sequencing of resulting amplicons. Both assays met the pre-determined acceptance criteria for all concentrations tested.

One sample at 25 ng had a call rate of 98% which was deemed unacceptable. There was a single no-call at 1250 ng (both no-calls at 1250 ng were at the PolyTG/PolyT

region). At 25 ng there were 140 no-calls with 136 arising from 6 replicates of the same sample.

Analytical Sensitivity at Low DNA Input:

Fourteen DNA specimens were tested at 9 different DNA input levels; 1250, 500, 250, 100, 50 25, 10, 5, and 1 ng. Variation types tested included SNVs, small insertion/deletions (including F508del), one large deletion, and compound insertion/deletions. A single lot of reagents were used and the same lot of DNA samples and a single thermal cycler were used. This study passed acceptance criteria at all concentrations except 1, 5, and 10 ng (25-fold below recommended DNA input). Based on the results of both studies, the upper and lower bounds for DNA input into the assay are 1250 and 25 ng, respectively.

e. Analytical Specificity (Interfering Substances):

Bilirubin, Hemoglobin, Cholesterol, and Buffer Component Interference:

The effect of potential interferents on the performance of the assay was examined using 8 samples carrying 8 unique genotypes, and blood from the same individual was tested across all 4 interfering substances. Sample types included 1 PolyTG/PolyT variant, 1 indel (F508del), and 6 SNVs. Bilirubin (684 and 137 µmol/L), hemoglobin (2 and 0.4 g/L), and cholesterol (13 and 2.6 mmol/L) were spiked into blood aliquots prior to DNA extraction. Wash buffer from DNA extraction (15%) was spiked into genomic DNA samples prior to library preparation. For the assessment of each inhibiting substance, data for each spiked sample was compared to an untreated aliquot of the same blood/DNA sample. Impact on call rate, reproducibility, and sample first pass rate were determined. All 88 samples met the acceptance criteria of the test.

Lipid and Short Draw Interference:

A study to assess the potential interference of triglycerides (37 mmol/L and 7.4 mmol/L) and high and low concentrations of K_2EDTA (7 mg/mL and 2.8 mg/mL) to mimic short blood draws was conducted. Eight whole blood samples were used for this study. Two samples were WT, 2 were F508del, and the remaining 4 were SNVs. With the exception of R75Q, all SNVs were replicates of those tested in Interference Study A (above), but were different blood samples. All tested conditions gave 100% correct calls, and no samples required a re-concentration to obtain the appropriate amount of DNA for the assay.

f. Assay cut-off:

Not applicable

g. Sample carryover:

The goal of this study was to ascertain that sample carryover between samples within an instrument run and between successive sequencing runs met design requirements. Two genomic DNA samples with unique CFTR genotypes were assessed. One sample had 2 variants and one sample had 3 variants. In the intra-run test, 1 library composed of 2 samples with unique variants was set up in a checkerboard matrix pattern at alternating high (500 ng) and low (100 ng) concentrations along with 4 NTCs. For the inter-run test, 2 libraries were prepared; the library contained either a single distinct genomic DNA sample or one NTC. For both the inter and intra-run tests, sample carryover was assessed by measuring the error rate at the position of variant calls for all samples used in the study. The design input requirement was carryover ≤ 2%.The highest carryover rate seen in this test was 0.31% for intra-run testing and 0.3% for inter-run testing.

2. Comparison studies:

a. Method comparison to reference method:

Accuracy:

Accuracy of the Illumina MiSeqDx Clinical Sequencing Assay was assessed by evaluating 500 samples representing a wide variety of CFTR variants from four separate sources; 369 were clinical gDNA specimens, 79 were cell line gDNA, and 52 were synthetic samples derived from plasmid DNA. Accuracy was based on comparison with Sanger sequencing of all 500 samples over all 5,206 positions interrogated by the MiSeqDx assay. A total of 203 variants from reference were detected. These variants covered most of the 27 exons (with the exception of exons 16 and 26) and an intronic region within CFTR including the PolyTG/PolyT homopolymeric region. Variant types analyzed in the accuracy study included SNVs, small indels, and 2 large deletions. All 23 mutations recommended by the ACMG were included in the study, and all were examined with clinical samples in addition to other sample types. As noted below there were 6 miscalls and 4 no calls in the study. One of the no calls was in the context of wild-type sequence, while the rest were in the context of variants, most notably in the PolyTG/PolyT region. Overall this study met its acceptance criteria.

Table 4: Accuracy by Variant

Genotype (Common Name/cDNA name/coordinate)	Variant Type	CFTR gene region (hg19)	Positive calls (Variants)			No Calls¤	Miscalls	PPA (%)
			Clinical Samples	Cell Line Samples	Synthetic Samples			
117120141	SNV	Exon1	25	3	0	0	0	100
117120145	SNV	Exon1	3	2	0	0	0	100
M1V	SNV	Exon1	0	0	1	0	0	100

CFTR dele2, 3	Del	Intron1	4	1	0	0	0	100
R31C	SNV	Exon2	3	1	0	0	0	100
Q39X	SNV	Exon2	0	0	1	0	0	100
E60X	SNV	Exon3	6	1	0	0	0	100
P67L	SNV	Exon3	1	0	1	0	0	100
R74W	SNV	Exon3	0	2	0	0	0	100
R74Q	SNV	Exon3	2	0	0	0	0	100
R75X	SNV	Exon3	3	1	0	0	0	100
R75Q	SNV	Exon3	20	1	0	0	0	100
G85E	SNV	Exon3	6	2	0	0	0	100
394delTT	DIV	Exon3	3	1	0	0	0	100
405+1G>A	SNV	Intron3	0	0	1	0	0	100
406-1G>A	SNV	Exon4	4	0	0	0	0	100
E92K	SNV	Exon4	0	0	1	0	0	100
E92X	SNV	Exon4	0	1	1	0	0	100
Q98X	SNV	Exon4	0	0	2	0	0	100
I105SfsX2	DIV	Exon4	0	2	0	0	0	100
457TAT>G	DIV	Exon4	0	0	1	0	0	100
D110H	SNV	Exon4	1	0	1	0	0	100
R117C	SNV	Exon4	4	0	0	0	0	100
R117H	SNV	Exon4	17	2	0	0	0	100
Y122X	SNV	Exon4	0	1	0	0	0	100
F143LfsX10	DIV	Exon4	0	1	0	0	0	100
574delA	DIV	Exon4	0	0	2	0	0	100
Q151K	SNV	Exon4	1	0	0	0	0	100
621+1G>T	SNV	Intron4	7	5	0	0	0	100
621+3A>G	SNV	Intron4	1	0	0	0	0	100
663delT	DIV	Exon5	1	0	1	0	0	100
G178R	SNV	Exon5	1	1	0	0	0	100
711+1G>T	SNV	Intron5	3	1	0	0	0	100
711+3A>G	SNV	Intron5	0	0	1	0	0	100
711+5 G->A	SNV	Intron5	0	0	1	0	0	100
712-1 G->T	SNV	Exon6	0	0	1	0	0	100
H199Y	SNV	Exon6	0	0	1	0	0	100
P205S	SNV	Exon6	1	0	1	0	0*	100
L206W	SNV	Exon6	8	1	0	0	0	100
A209S	SNV	Exon6	0	1	0	0	0	100
Q220X	SNV	Exon6	0	0	1	0	0	100
L227R	SNV	Exon6	0	0	1	0	0	100
852del22	DIV	Exon6	0	0	1	0	0	100
E279D	SNV	Exon7	1	0	0	0	0	100

R297Q	SNV	Exon8	2	0	0	0	0	100
1078delT	DIV	Exon8	1	1	0	0	0	100
L320V	SNV	Exon8	1	0	0	0	0	100
G330X	SNV	Exon8	1	1	0	0	0	100
R334W	SNV	Exon8	6	1	0	0	0	100
I336K	SNV	Exon8	0	1	0	0	0	100
T338I	SNV	Exon8	0	0	1	0	0	100
1154insTC	DIV	Exon8	0	1	0	0	0	100
S341P	SNV	Exon8	0	0	1	0	0	100
R347H	SNV	Exon8	6	1	1	0	0	100
R347P	SNV	Exon8	3	2	0	0	0	100
R352Q	SNV	Exon8	5	0	0	0	0	100
Q359K/T360K	SNV	Exon8	0	0	1	0	0	100
1213delT	DIV	Exon8	0	0	1	0	0	100
1248+1G>A	SNV	Intron8	0	0	1	0	0	100
1259insA	DIV	Exon9	0	0	2	0	0	100
W401X (c.1202G>A)	SNV	Exon9	0	0	1	0	0	100
W401X (c.1203G>A)	SNV	Exon9	0	0	1	0	0	100
1341+1G->A	SNV	Intron9	0	0	2	0	0	100
PolyTGPolyT	PolyTG/PolyT	Intron9	369	79	52	3	4[#]	98.60
1461ins4	DIV	Exon10	0	0	1	0	0	100
A455E	SNV	Exon10	4	2	0	0	0	100
1525-1G->A	SNV	Exon11	0	0	1	0	0	100
S466X (C->A)	SNV	Exon11	0	0	1	0	0	100
S466X (C->G)	SNV	Exon11	1	0	1	0	0	100
L467P	SNV	Exon11	0	0	1	0	0	100
V470M	SNV	Exon11	311	71	0	0	0	100
1548delG	DIV	Exon11	1	0	1	0	0	100
P477S	SNV	Exon11	0	1	0	0	0	100
S485T	SNV	Exon11	1	0	0	0	0	100
S489X	SNV	Exon11	0	0	2	0	0	100
S492F	SNV	Exon11	0	0	1	0	0	100
Q493X	SNV	Exon11	4	2	0	0	0	100
I506V	SNV	Exon11	7	0	0	0	0	100
I507del	DIV	Exon11	4	2	0	0	0	100
F508del	DIV	Exon11	84	29	0	0	0	100
I507V	SNV	Exon11	0	1	0	0	0	100
F508C	SNV	Exon11	1	1	0	0	0	100
1677delTA	DIV	Exon11	1	0	0	0	0	100

V520F	SNV	Exon11	2	0	0	0	0	100
Q525X	SNV	Exon11	0	0	1	0	0	100
E527E	SNV	Exon11	3	2	0	0	0	100
E528E	SNV	Exon11	6	2	0	0	0	100
1717-8G->A	SNV	Intron11	0	0	1	0	0	100
1717-1G>A	SNV	Exon12	4	1	0	0	0	100
G542X	SNV	Exon12	12	3	0	0	0	100
S549R (c.1645A>C)	SNV	Exon12	0	0	1	0	0	100
S549N	SNV	Exon12	2	2	1	0	0	100
S549R (c.1647T>G)	SNV	Exon12	3	1	0	0	0	100
G551D	SNV	Exon12	8	3	0	0	0	100
Q552X	SNV	Exon12	0	0	1	0	0	100
R553X	SNV	Exon12	8	2	0	0	0	100
I556V	SNV	Exon12	1	0	0	0	0	100
L558S	SNV	Exon12	0	0	1	0	0	100
A559T	SNV	Exon12	4	0	1	0	0	100
R560K	SNV	Exon12	0	0	1	0	0	100
R560T	SNV	Exon12	6	1	0	0	0	100
1811+1.6kb A->G	SNV	Intron12	0	0	1	0	0	100
1812-1 G->A	SNV	Exon13	0	2	0	0	0	100
A561T	SNV	Exon13	1	0	0	0	0	100
V562I	SNV	Exon13	1	0	0	0	0	100
Y569D	SNV	Exon13	0	0	1	0	0	100
P574H	SNV	Exon13	0	1	0	0	0	100
G576A	SNV	Exon13	4	1	0	0	0	100
D579G	SNV	Exon13	0	0	1	0	0	100
E585X	SNV	Exon13	0	0	1	0	0	100
1898+1G>A	SNV	Intron13	2	1	0	0	0	100
1898+3A>G	SNV	Intron13	0	0	1	0	0	100
H609R	SNV	Exon14	0	1	0	0	0	100
D614G	SNV	Exon14	0	0	2	0	0	100
R668C	SNV	Exon14	5	2	0	0	0	100
R668H	SNV	Exon14	1	0	0	0	0	100
2143delT	DIV	Exon14	2	1	0	0	0	100
K684TfsX4	DIV	Exon14	0	0	1	0	0	100
2183AA>G	DIV	Exon14	3	1	0	0	0	100
2184delA	DIV	Exon14	1	1	0	0	0	100
2184insA	DIV	Exon14	3	0	1	0	0	100
S686Y	SNV	Exon14	0	1	0	0	0	100
R709X	SNV	Exon14	1	0	2	0	0	100

K710X	SNV	Exon14	3	0	0	0	0	100
E725K	SNV	Exon14	2	0	0	0	0	100
2307insA	DIV	Exon14	3	0	2	0	0	100
L732X	SNV	Exon14	0	0	2	0	0	100
2347delG	DIV	Exon14	0	0	2	0	0	100
P750L	SNV	Exon14	1	0	0	0	0	100
V754M	SNV	Exon14	2	1	0	0	0	100
R764X	SNV	Exon14	1	0	2	0	0	100
2585delT	DIV	Exon14	0	0	2	0	0	100
E822X	SNV	Exon14	0	0	2	0	0	100
2622+1G>A	SNV	Intron14	0	0	2	0	0	100
E831X	SNV	Exon15	0	0	1	0	0	100
D836Y	SNV	Exon15	0	1	0	0	0	100
W846X	SNV	Exon15	0	1	0	0	0	100
R851X	SNV	Exon15	0	0	1	0	0	100
T854T	SNV	Exon15	212	44	0	0	0	100
2711delT	DIV	Exon15	0	0	1	0	0	100
V868V	SNV	Exon15	2	0	0	0	0	100
c.2657+2_2657+3 insA	DIV	Intron16	0	0	1	0	0	100
2789+5G>A	SNV	Intron16	9	1	0	0	0	100
Q890X	SNV	Exon17	1	0	0	0	0	100
A923A	SNV	Exon17	1	0	0	0	0	100
L927P	SNV	Exon17	0	0	1	0	0	100
S945L	SNV	Exon17	0	0	1	0	0	100
M952T	SNV	Exon17	1	0	0	0	0	100
3007delG	DIV	Exon17	0	0	1	0	0	100
T966T	SNV	Exon17	5	0	0	0	0	100
G970R	SNV	Exon17	0	0	1	0	0	100
S977F	SNV	Exon18	0	0	1	0	0	100
3120G>A	SNV	Exon18	1	0	0	0	0	100
3120+1G>A	SNV	Intron18	7	1	0	0	0	100
3121-1G->A	SNV	CF Syn_Ex19	0	0	1	0	0	100
L997F	SNV	Exon19	2	1	0	0	0	100
I1027T	SNV	Exon19	1	2	0	0	0	100
3272-26A>G	SNV	Intron19	0	1	0	0	0	100
F1052V	SNV	Exon20	0	1	0	0	0	100
L1065P	SNV	Exon20	0	0	1	0	0	100
R1066C	SNV	Exon20	6	0	0	0	0	100
R1066H	SNV	Exon20	1	0	1	0	0	100
G1069R	SNV	Exon20	0	1	0	0	0	100

R1070W	SNV	Exon20	0	2	0	0	0	100
R1070Q	SNV	Exon20	0	1	0	0	0	100
L1077P	SNV	Exon20	0	0	1	0	0^	100
W1089X	SNV	Exon20	4	0	0	0	0	100
Y1092X (C>A)	SNV	Exon20	3	1	0	0	0	100
Y1092X (C>G)	SNV	Exon20	0	0	1	0	0	100
T1095T	SNV	Exon20	7	0	0	0	0	100
M1101K	SNV	Exon20	2	2	0	0	0	100
E1104X	SNV	Exon20	0	0	1	0	0	100
c.3368-2A>T	SNV	Intron20	0	1	0	0	0	100
D1152H	SNV	Exon21	10	1	0	0	0	100
V1153E	SNV	Exon21	1	0	0	0	0	100
R1158X	SNV	Exon22	7	1	0	0	0	100
R1162X	SNV	Exon22	5	1	0	0	0	100
R1162L	SNV	Exon22	0	2	0	0	0	100
3659delC	DIV	Exon22	4	1	0	0	0	100
S1196X	SNV	Exon22	1	0	0	0	0	100
W1204X (c.3611G>A)	SNV	Exon22	0	0	1	0	0	100
W1204X (c.3612G>A)	SNV	Exon22	0	0	1	0	0	100
3791delC	DIV	Exon22	2	0	0	0	0	100
I1234V	SNV	Exon22	1	0	1	0	0	100
S1235R	SNV	Exon22	9	1	0	0	0	100
3849+10kbC>T	SNV	Intron22	11	2	0	0	0	100
G1244E	SNV	Exon23	0	0	1	0	0	100
3876delA	DIV	Exon23	6	1	0	0	0	100
S1251N	SNV	Exon23	1	0	1	0	0	100
3905insT	DIV	Exon23	3	1	0	0	0	100
D1270N	SNV	Exon23	0	2	0	0	0	100
W1282X	SNV	Exon23	9	1	0	0	0	100
P1290P	SNV	Exon23	10	3	0	0	0	100
4005+1G->A	SNV	Intron23	0	0	1	0	0	100
4016insT	DIV	Exon24	0	0	1	0	0	100
T1299T	SNV	Exon24	3	0	0	0	0	100
N1303K	SNV	Exon24	9	1	0	0	0	100
Q1313X	SNV	Exon24	0	0	1	0	0	100
G1349D	SNV	Exon25	0	1	0	0	0	100
4209TGTT>AA	DIV	Exon25	0	0	1	0	0	100
CFTRdele22,23	Del	Intron24	1	0	1	0	0	100
4382delA	DIV	Exon27	0	0	1	0	0	100
Y1424Y	SNV	Exon27	6	2	0	0	0	100

Q1463Q	SNV	Exon27	150	32	0	0	0	100
Total All Variants (PPA)			2072			3	4	99.66
Total All WT (NPA)			2600928[a]			1	2**	100.00
Total All WT and Variants (OA)			2603000			4	6	100.00

Bold = ACMG recommended variants

[¤]Samples were not retested.
[*] 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 re-prepared, using the same plasmid, it would be detected.
[**] 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.
[a] Covers all 5206 callable positions interrogated by the assay

PolyTG/PolyT Accuracy:

Twenty-three different PolyTG/PolyT genotypes were examined across 448 samples. The resulting variant calls for each sample were compared to bi-directional sequencing. Four samples were discordant with bi-directional sequencing results. Each of the discordances was noted to be within ±1 PolyTG relative to the bi-directional sequencing results. The accuracy of the PolyTG/PolyT results were assessed for all the samples included in the study and a demonstrated a miscall rate of 0.89% (4/448).

Table 5: Accuracy for the PolyTG/PolyT Tract Variants

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	50.0
(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	100.0
(TG)10(T)7/(TG)12(T)5	1	0	0	0	0	100
(TG)10(T)7/(TG)12(T)7	10	1	0	0	1	90.9
(TG)10(T)9/(TG)10(T)9	7	6	0	0	0	100

Genotype	Clinical Samples	Cell Line Samples	Synthetic Samples	# Miscalls	# No Calls	% Accuracy
(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	100.0
(TG)10(T)9/(TG)11(T)9	3	0	0	0	0	100
(TG)10(T)9/(TG)12(T)5	3	2	0	0	0	100
(TG)10(T)9/(TG)12(T)7	13	0	0	0	1	92.3
(TG)11(T)5/(TG)11(T)7	6	0	0	1	0	83.3
(TG)11(T)7/(TG)11(T)7	52	8	0	0	0	100
(TG)11(T)7/(TG)11(T)9	2	1	0	3	0	0.0
(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	100.0
(TG)12(T)7/(TG)12(T)7	2	2	0	0	0	100
Total	448			4	3	98.44

b. Matrix comparison:

Not applicable

3. Clinical studies:

a. Clinical Sensitivity and Specificity:

The clinical sensitivity and specificity can be estimated based on the information from the CFTR2 database (as of August 2013) as published in Sosnay PR et al., "Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene" Nat. Genet., published online on 25 August 2013; doi:10.1038/ng.2745.

4. Clinical cut-off**:**

Not applicable

5. Expected values/Reference range:

Cystic fibrosis is the most common autosomal recessive disorder in the Caucasian population with an incidence of approximately 1 in 3,200 live births. The incidence of CF in other ethnic groups varies: approximately 1 in 9,500 in Hispanics, 1 in 15,300 in African Americans, and 1 in 32,100 in Asian Americans. Variants identified by this assay will vary in frequency among different populations.

N. Instrument Name:

MiSeqDx Instrument

O. System Descriptions:

1. Modes of Operation:

   The MiSeqDx instrument requires limited user interaction to operate and commence a sequencing run. The software uses a graphical interface to walk the user step-by-step through loading of the reagents for a run (i.e., flow cell, reagent cartridge, and PR2 buffer) and empty the waste bottle. The system then automatically initializes the fluidics and optics, performs cluster generation, and commences the sequencing by synthesis cycles while acquiring data.

   The MiSeqDx system consists of four software applications that are installed on the instrument [MiSeq Operating Software (MOS); and Real Time Analysis (RTA), MiSeq Reporter (MRS), and MiSeq Test Software (MTS)] and two software applications [Illumina Worklist Manager (IWM) and User Management Software (UMS)] that are installed on user supplied personal computers. The MTS is only utilized by field service personnel and the MRS may also be installed on user supplied personal computers for off instrument analysis. The IWM software is used to create a sample sheet during library preparation and the UMS is used to grant various access level permissions to users. The MiSeq Operating Software (MOS) supports the ability to setup and run the sequencing workflow and save the resulting files for image processing and base calling (primary data analysis) performed by RTA software and MRS which performs de-multiplexing, alignment, variant calling, and report generation (secondary data analyses).

   The MiSeqDx software utilizes assay specific radio-frequency identifier (RFID) tagged reagents and consumables which enables it to automatically select and configure the appropriate run type to match the kit being used and control the in vitro diagnostic workflow. The RFID tag allows the system to ensure that the correct consumable type is being used and this information is stored as part of the instrument run-log for traceability purposes.

2. Software:

   FDA has reviewed applicant's Hazard Analysis and software development processes for this line of product types:

   Yes ___x_____ or No _____

3. Specimen Identification:

   The MiSeqDx Cystic Fibrosis Clinical Sequencing Assay kit contains materials sufficient for up to 48 samples per kit. Eight samples, including controls, must be analyzed for each run, and the kit contains sufficient reagents for 6 runs. If 6 patient samples and 2 controls are not available for a run, the remaining samples can be made up with normal genomic DNA. Three unique index i7 reverse PCR primers (named A701, A702, and A710) and three unique index i5 forward PCR primers (named A503-A505) for universal

amplification of the ligated products are included. These are added during the library preparation process. The sample sheet, a file that the end user provides the software, contains the link between each of the sample names and their associated index sequences. After completion of the sequencing run, MiSeqDx CF Reporter software de-multiplexes the samples using the index sequences and creates FASTQ files as the data analysis output for sequence alignment and variant calling.

4. Specimen Sampling and Handling:

   Samples are handled manually prior to being placed on the instrument. The test begins with the isolation of gDNA from a peripheral whole blood sample. The gDNA is then quantified and qualified prior to being processed through the library preparation step. This step specifically amplifies the intended genomic regions of each sample while also adding the indexes (for sample identification). The library is then processed to remove any remaining preparation reagents (e.g., unused primers). It is then normalized, to ensure that each library is equally represented, and pooled. The resulting sample libraries are then transferred into a MiSeq reagent cartridge which contains all of the reagents required for cluster generation and SBS. The MiSeqDx Reagent Cartridge, MiSeqDx Flow Cell, and MiSeqDx SBS Solution are then inserted into the MiSeqDx instrument, which performs cluster generation, sequencing and data analysis.

5. Calibration:

   There is no end-user calibration of the system. During installation of the platform, a company representative (Field Applications Scientist) begins a series of tests to validate the performance of the instrument subsystems, which include optical alignment, fluidic delivery, and thermal calibration, among others. In the case of a test failure, the MiSeqDx company representative uses a set of instrument-specific tools to adjust and/or repair the instrument to meet operational specifications. Re-calibration occurs during the preventive maintenance visit.

6. Quality Control:

   An internal control, bacteriophage PhiX (ΦX174) gDNA is added at the end of the library preparation step, prior to library pooling and placement on the instrument. Successful sequencing of the PhiX genome indicates that the sequencing chemistry worked as expected.

   Illumina requires that one positive control DNA sample and a negative control [No Template Control (NTC)] be 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 and positive controls should be rotated in a manner consistent with the 2008 ACMG Technical Standards and Guidelines for CF mutation testing and the 2013 ACMG clinical laboratory standards for next-generation sequencing. The use of a wild type control, run as a sample, is recommended, but it should not replace the positive or negative control.

P. Other Supportive Instrument Performance Characteristics Data Not Covered In The "Performance Characteristics" Section above:

1. Validation of PCR Method Used for Large Deletion Confirmation in Accuracy Studies:
The assay reports on 2 large deletions within the CFTR gene. Because Sanger sequencing is not designed to detect large deletions, a PCR assay was validated as a confirmatory method for large deletion detection.

For each deletion, a duplex PCR containing 2 primer sets was used. One primer set flanks the deletion breakpoints while the other amplifies a region internal to the deletion. Therefore, homozygotes will have one PCR product while heterozygotes will have 2 which are detected by size separation on an agarose gel.

In order to validate this assay, a panel of 22 samples was tested. The panel consisted of cell line gDNA samples, blood-derived gDNA samples, and synthetic plasmids. No clinical samples were used in the validation studies because these variants are quite rare (~250 for dele2,3 and 9 for dele22,23 in the CFTR2 database). Plasmids were generated such that they contain the gDNA in the context of the deletion and 300bp on either side. The plasmids were either used as is, or were blended with cell line gDNA to give synthetic heterozygote samples.

The following studies were performed to validate the assay:

- Accuracy: PCR products were Sanger sequenced to confirm identity
- Specificity: Based on the presence of only 2 bands of expected size
- Reproducibility: For intra-run 8 replicates each of 6 samples from the 22 sample set were tested in a single run for both assays. For inter-run, the set of 22 samples 3 times
- Robustness: DNA quality robustness was assessed with 6 DNA samples that underwent freeze/thaw 6 times. DNA quantity robustness was assessed with WT, het, and homozygous samples of each deletion tested at 7 different DNA inputs (from 10x to 0.1x).

All testing met acceptance criteria.

2. Library Normalization:

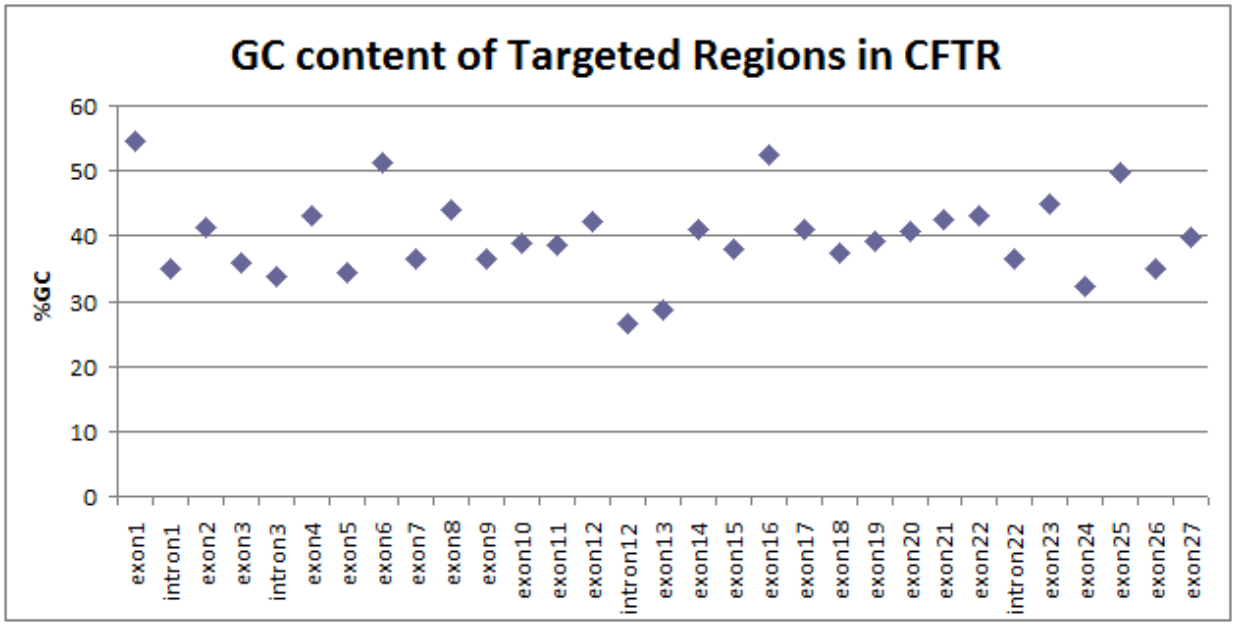
The library normalization step of the assay was validated retrospectively by testing the following parameters.

- Intra and inter-run reproducibility
- Repeatability
- Working DNA input range in which normalization works effectively
- Robustness of library normalization method

All testing met acceptance criteria based on call rate.

3. G/C content:

In order to determine if certain regions of the CFTR gene had unusually high G/C content such that a separate evaluation of assay performance would need to be done in this genomic context, the G/C contend of the targeted regions in CFTR was provided. As can be seen from the chart below, the G/C content of CFTR for the regions analyzed in this assay is generally below 50%.



Q. Proposed Labeling:

The labeling is sufficient and it satisfies the requirements of 21 CFR Part 809.10.

R. Conclusion:

The submitted information in this premarket notification is complete and supports a substantial equivalence decision.