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TruSight™ Cystic Fibrosis

Answers to frequently asked questions about the TruSight Cystic Fibrosis *in vitro* diagnostic next-generation sequencing solution for comprehensive profiling of cystic fibrosis variants.

Introduction

Cystic fibrosis (CF) affects approximately 70,000 children and adults worldwide.[1] The disease appears when an individual inherits two disease-causing variants in trans of the cystic fibrosis transmembrane receptor (*CFTR*) gene. CF affects a diverse population, with the highest recognized incidence observed in individuals of Caucasian descent.[2,3] Early diagnosis and treatment of CF can improve both survival and quality of life.[4] However, current CF testing methods focus on *CFTR* variants most commonly found in Caucasians, potentially missing CF causative variants in other demographics that may have clinical relevance.

To address this challenge, Illumina offered the MiSeq™Dx Cystic Fibrosis 139-Variant Assay and the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay. These assays were the first Food and Drug Administration (FDA)–cleared next-generation sequencing (NGS)-based *in vitro* diagnostic (IVD) tests for cystic fibrosis. These preexisting assays have been consolidated into a single NGS solution for cystic fibrosis testing: TruSight Cystic Fibrosis.

General information

What are the differences between MiSeqDx Cystic Fibrosis and TruSight Cystic Fibrosis Assays?

MiSeqDx Cystic Fibrosis assays consist of two different FDA-cleared, CE-IVD marked CF assay testing kits: the MiSeqDx Cystic Fibrosis 139-Variant Assay (Catalog no. DX-102-1004) and the MiSeqDx Cystic Fibrosis Clinical Sequencing Assay (Catalog no. DX-102-1001). These products were launched in 2013 and use the MiSeqDx Sequencing System with the MiSeqDx Reagent Kit v1. Both these assays use a coupled kit solution that provides library prep and sequencing reagents.

TruSight Cystic Fibrosis combines the MiSeqDx Cystic Fibrosis assays into one FDA-regulated assay. TruSight Cystic Fibrosis (Catalog no. 20036925) combines the TruSight Cystic Fibrosis 139-Variant Assay and the TruSight Cystic Fibrosis Clinical Sequencing Assay into one library prep product providing both testing options. TruSight Cystic Fibrosis requires the use of the MiSeqDx Sequencing System and MiSeqDx Reagent Kit v3 (Catalog no. 20012552). MiSeqDx Reagent Kit v3 sequencing reagents are not provided with the library prep kit and must be purchased separately.

For more detailed information, refer to the TruSight Cystic Fibrosis Package Insert (Document no. 1000000097720) and data sheet. These documents can be found on the product support site.

Are there any regional restrictions in obtaining TruSight Cystic Fibrosis?

In April 2020, TruSight Cystic Fibrosis will be available for purchase in the United States and most European countries. Additional regions will be added as regulatory approvals are obtained.

What are the differences between the TruSight Cystic Fibrosis 139-Variant Assay and TruSight Cystic Fibrosis Clinical Sequencing Assay?

The TruSight Cystic Fibrosis 139-Variant Assay is a qualitative *in vitro* diagnostic assay that detects 139 clinically relevant CF-causing mutations and variants in the *CFTR* gene simultaneously in genomic DNA isolated from human peripheral whole blood specimens. The test is intended for:

- Carrier screening in adults of reproductive age
- Confirmatory diagnostic testing of newborns and children
- Initial test to aid in the diagnosis of individuals with suspected CF

The TruSight Cystic Fibrosis Clinical Sequencing Assay is a targeted sequencing *in vitro* diagnostic assay that resequences the protein coding region and intron/exon boundaries of the *CFTR* gene in genomic DNA isolated from human peripheral whole blood. The test is intended for:

- Aid in the diagnosis of individuals with suspected CF
- Use when the patient has an atypical or nonclassical presentation of CF
- Use in situations where other mutation panels have failed to identify both causative mutations

Sample and library preparation

What is the required sample type?

DNA extracted from whole blood is the required sample type.

What type of sample extraction can be used?

Any validated sample extraction method may be used.

How do you quantify the DNA?

A spectrophotometer is recommended for quantifying extracted DNA.

How long can samples by stored, and under what conditions?

Whole blood specimens can be stored no longer than the following:

- 7 days at room temperature
- 30 days at 2°C to 8°C
- 30 days at -25°C to -15°C

Can dried blood spots be used as input?

No, the assay is not validated for use with dried blood spots. Dried blood spots contain less than the recommended amount of DNA and are likely to result in lower call rates than whole blood.

What is the recommended DNA input? Is the input amount recommended or required?

The required sample input is 250 ng DNA extracted from whole blood. DNA inputs ranging from 25 ng to 1250 ng have been used to produce accurate results.

Can you automate TruSight Cystic Fibrosis library preparation?

No, library preparation is only offered in a manual format.

Are the target probes used in TruSight Cystic Fibrosis the same as the target probes used in the MiSeqDx Cystic Fibrosis kit?

Yes, the probes are identical for the TruSight Cystic Fibrosis and MiSeqDx Cystic Fibrosis kits.

Can a single library prep be used for the TruSight Cystic Fibrosis 139-Variant Assay and TruSight Cystic Fibrosis Clinical Sequencing Assay?

Yes, prepared libraries stored as diluted amplicon libraries (DALs) can be used up to 28 days after preparation. There is no difference between libraries prepared for the TruSight Cystic Fibrosis 139-Variant Assay and TruSight Cystic Fibrosis Clinical Sequencing Assay.

What are the sample throughput expectations?

The TruSight Cystic Fibrosis workflow provides flexibility in determining how many samples are prepared in each library prep event and pooled for each sequencing event. Some possible combinations are suggested below:

- One 96-sample library preparation can be pooled for sequencing with 24 samples for the TruSight Cystic Fibrosis Clinical Sequencing Assay and separately 72 samples for the TruSight Cystic Fibrosis 139-Variant Assay
- One 96-sample library preparation can be pooled for sequencing with four separate 24-sample library preparations; with three sets of 24 samples for the TruSight Cystic Fibrosis 139-Variant Assay and one pooled set of 24 samples for the TruSight Cystic Fibrosis Clinical Sequencing Assay
- Two independent library preparation events, one with 66 samples and a later prep with 30 samples, both sequenced for the TruSight Cystic Fibrosis 139-Variant Assay

Can I run fewer than 24 samples?

At least 24 libraries must be prepared and pooled for sequencing. Users may prepare a sample sheet that does not include some of those samples if reports are not desired for all libraries sequenced.

How long can I store the pooled library (ie, DAL)?

DALs can be stored for up to 28 days.

What consumables and equipment do I need to run TruSight Cystic Fibrosis?

Required materials and equipment are listed in the TruSight Cystic Fibrosis Package Insert (Document no. 1000000097720). Important equipment:

- Passively cooled heat block
- Refrigerated centrifuge

Sequencing

What sequencing system is Trusight Cystic Fibrosis designed for? Can it be used with the standard MiSeq System?

TruSight Cystic Fibrosis has been designed for use with the MiSeqDx Sequencing System and MiSeqDx Reagent Kit v3. It is not compatible for use the standard research use only MiSeq System.

How many TruSight Cystic Fibrosis 139-Variant Assay or TruSight CF Clinical Sequencing Assay tests can I run per flow cell? Is there enough coverage on a MiSeqDx v3 flow cell to have a throughput of 96 samples?

Both TruSight Cystic Fibrosis assays have been designed to run with the MiSeqDx Reagent Kit v3. The MiSeqDx v3 sequencing flow cell has been validated to support 24-96 tests of each assay per sequencing run. TruSight Cystic Fibrosis has been be validated to support a minimum of 24 samples per flow cell run.

Can I do reflex testing from the TruSight Cystic Fibrosis 139-Variant Assay to the TruSight Cystic Fibrosis Clinical Sequencing Assay?

Yes, the TruSight Cystic Fibrosis Clinical Sequencing Assay can be used when a patient has atypical or nonclassical presentation of CF or when other mutation panels have failed to identify both causative mutations.

How do you perform reflex testing using existing library pool? Can I have a report that provides fewer than 24 samples?

If you plan to do reflex testing, we recommend preparing 3-5 DALs at the end of the library prep. After the TruSight Cystic Fibrosis 139-Variant Assay sequencing run is complete, prepare a new sample sheet for a TruSight Cystic Fibrosis Clinical Sequencing Assay run that only includes the samples for which you want results. Use the DAL that contains all of the libraries of interest with the TruSight Cystic Fibrosis Clinical Sequencing Assay run; only the samples entered on the sample sheet will be reported.

How do I troubleshoot sample failures?

Leftover PCR cleanup product from the clean-up plate (CLP) can be run on a 2–4% agarose gel to confirm the success of PCR. If no band is detected for a given sample, library preparation for that sample should be repeated. For more information, read the TruSight Cystic Fibrosis Package Insert (Document no. 1000000097720).

Which MiSeqDx reagents do I need to order for TruSight Cystic Fibrosis?

The MiSeqDx Reagent Kit v3 (Catalog no. 20012552) is required for sequencing of TruSight Cystic Fibrosis libraries.

Can I use the MiSeqDx CF library prep kits (Catalog no. DX-102-1004 or DX-102-1001) with MiSeq v3 reagents?

No, the instructions for MiSeqDx Cystic Fibrosis library preparation require users to use sequencing reagents provided with the MiSeqDx Cystic Fibrosis Kit.

Do I need to run a minimum number of samples for index diversity? What restrictions are there regarding index combinations?

At least 24 libraries must be prepared and pooled for sequencing, but a subset can be selected for requeue analysis. Each sample must have a unique index combination, but there are no restrictions on which index combinations are used.

What is the typical expected cluster density range for a successful run? Is there a required cluster density?

Sequencing with MiSeqDx Reagent Kit v3 (Catalog no. 20012552) provides robust tolerance of a wide range of cluster densities without sequencing failure. Approximately 800-1500 is typical range of cluster density for TruSight CF Assays.

What software is needed to run TruSight Cystic Fibrosis?

All software required to run TruSight Cystic Fibrosis, including Local Run Manager, is preinstalled on the MiSeqDx instrument (for instuments installed after January 2019). The specifc analysis modules to be installed on an instrument is based on customer needs and will be installed on an as needed basis. Local Run Manager can also be used off-instrument. The instructions for off-instrument installation of Local Run Manager can be found in the Local Run Manager Software Guide (Document no. 1000000002702). For instruments purchased prior to January 2019, you may need to work with your local services teams to obtain the appropriate upgrades for Trusight Cystic Fibrosis compatibility.

Data analysis

Where is TruSight Cystic Fibrosis data analysis performed?

Data analysis for the TruSight Cystic Fibrosis Assays is conducted on-instrument.

Can data be stored externally? Where is the data from a run stored?

Data for each sequencing run is stored on the MiSeqDx instrument at MiSeqAnalysis\<Run Folder Name>. Data can also be stored externally by changing the default output folder within the MiSeq Operating System User Interface. For instructions on how to set default output folder location, see MiSeqDx Instrument Reference Guide for MOS v2 (Document no. 1000000021961).

What raw data files are included for long-term storage?

BCL, FASTQ, and VCF files are included for long-term storage.

Where I can find the raw sequencing data from a run (such as FASTQ or VCF files)?

When a sequencing run is complete, there will be an output folder containing all of the sequencing files. The path for this can be found in the "Sequencing Information" tab of Local Run Manager. In that path, there is an "Alignment_#" folder that contains a zipped file. After unzipping the file, the VCF and BAM files will be available. FASTQ files are available on the local drive (on the MiSeqDx computer). The default path for this is D:\Illumina\MiSeqAnalysis\{RunID}\Data\Intensities\BaseCalls.

What are the differences in data storage requirements between MiSeqDx Cystic Fibrosis and TruSight Cystic Fibrosis?

If storing files per sample (ie VCF or FASTQ files), there is not much difference per sample. If storing whole run files, TruSight Cystic Fibrosis sequencing runs with v3 reagents can be up to 2× larger size than files from the MiSeqDx Cystic Fibrosis Assay. Run Folder sizes can be minimized by not saving image files.

Troubleshooting

Why is a template line wash recommended after the completion of each sequencing run? What impact will there be if I forget to wash the line?

Template line washes are essential to limit run-to-run carryover. If a wash is not conducted, elevated call rates are possible in the negative control sample. DNA sample results are not impacted if a wash is not conducted.

If my run fails (ie, a run suitability failure) do I need to do a new library prep? Can I use my existing library prep?

If a sequencing run failure can be attributed to an instrument issue, existing libraries can be resequenced. If the negative control call rate is > 10% and a template line wash was not conducted in the previous run, it is recommended that the operator complete a post-run wash with a template line wash and repeat the sequencing run. Other failures, such as contamination during library preparation causing a negative control call rate that is > 10%, or incorrect variant calls in positive or wild-type controls will require new library preparation.

What is the typical concentration of a library? What is the typical size of my final library?

Due to bead normalization, measurement of pooled library concentrations is not needed. Cleaned-up PCR products can be used for troubleshooting efforts in the event of sample failures by performing gel electrophoresis for each sample of interest with a 2-4% TBE agarose gel and 100 bp ladder to confirm presence of an 300-400 bp library product. If needed to correct any pooling errors, the storage plate (SGP) can be used up to three days later for repooling and resequencing.

References

1. Cystic Fibrosis Foundation. www.cff.org/What-is-CF/About-Cystic-Fibrosis/. Accessed November 10, 2019.
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4. Rock MJ, Levy H, Zaleski C, Farrell PM. Factors accounting for a missed diagnosis after newborn screening. *Pediatr Pulmonol*. 2011;46(12):1166–1174.

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