

MiSeqDx[®] Cystic Fibrosis 139- Variant Assay Reference Guide

FOR IN VITRO DIAGNOSTIC USE

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Revision History

Document #	Revision	Date	Description of Change
15038349	03	August 2017	<ul style="list-style-type: none">• In the Specify Samples for the Run section, added information that set 1 and 2 of the 48 sample configuration can be used for 96 samples and two sequencing runs.• Revised step 2 in the Enter Samples Manually section to indicate that positive and negative control samples are required.• Moved the Edit Run Information section to the Local Run Manager Software Reference Guide for MiSeqDx (document # 1000000011880).• In the example table of the Import Samples section, added zeros in front of the numbers in the Sample_Well column. Although you can create an import sample file without the leading zeros in the column, the table is now consistent with the template that comes with the software.• Changed the title of the Enter Run Information for the MiSeqDx to Enter Run Information.• Hybridization of Oligonucleotide Pool - Procedure: To ensure the inclusion of NTC sample, removed prior language ("all wells containing genomic DNA") and replaced with "all sample wells."• PCR Amplification - Preparation: Added a centrifuge step for the PCR Polymerase tube to ensure proper volumes of PCR Polymerase are used.• Library Normalization and Pooling - Procedure: Removed "Seal SGP with an adhesive plate seal and set aside" because storage of the SGP plate is no longer recommended.

Document #	Revision	Date	Description of Change
15038349	02	June 2017	<ul style="list-style-type: none"> • Updated the EC Rep labeling from Emergo Europe to Illumina Cambridge Limited. • Added information for Local Run Manager software users throughout the guide. • Changed the title of the section Create a Sample Sheet to Prepare Sample Information and made minor wording changes to the section so that it applies to both MiSeq Reporter and Local Run Manager users. • In the section Automated Sequencing and Data Analysis section, added information on Real Time Analysis (RTA) and the role of RTA in primary analysis. • Added a new section called MiSeqDx Instrument Interface Methods to explain the software function differences between MiSeq Reporter and Local Run Manager, and how to determine which software is installed with your MiSeqDx. • Added a new section called Instrument and Software Guides. The section provides information on what instrument and software guides to use, based on the instrument configuration of your MiSeqDx and the analysis software installed with it. • Added a section called Enter Run Information for the MiSeqDx to introduce the differences between using IWM and the Local Run Manager CF 139 Assay module to enter run and sample information. • Index primer information was revised to include headers consistent with Local Run Manager. • Revised Acronym List: <ul style="list-style-type: none"> • Redefined NTC from Negative Template Control to No Template Control. • Deleted Positive Control (POS).

Document #	Revision	Date	Description of Change
15038349	01	March 2017	<ul style="list-style-type: none"> • Pre-Amp Equipment and Materials - Updated requirements for the tabletop centrifuge. • Enter Sample Information: Added a note to only edit Sample Sheets in Illumina Worklist Manager. • Removal of Unbound Oligonucleotides - Preparation: Added a cautionary statement regarding controlling the centrifuge temperature. • Changed library storage from SGP/PAL to DAL stage: <ul style="list-style-type: none"> • The safe stopping points after SGP preparation and PAL preparation have been removed and replaced with a safe stopping point after DAL preparation. Two (2) to 3 tubes of DAL are prepared instead of a single DAL tube. • The Library Normalization section is now called Library Normalization and Pooling. • Library dilution buffer is also used in the Library Normalization and Pooling section. • The Library Pooling section is now called Library Sequencing. • The Prepare for Library Pooling section is now called Prepare for Library Sequencing. • Denature and Dilute PhiX Internal Control: Revised instructions to make a smaller volume (100 µl) of 20 pM PhiX Internal Control Library. • Library Normalization and Pooling - Procedure: Added a note to explain when to thaw the reagent cartridge. • Denature and Dilute PhiX Internal Control: Added a note detailing NaOH preparation. • Throughout: Added Australia sponsor information to the back cover.
15038349	B	March 2015	<ul style="list-style-type: none"> • Enter Sample Information: Added second sentence to step 5. • Sample Throughput and Index Representation: Added sentence to first note. • Hybridization of Oligo Pool - Preparation: Added sentence to beginning of last note after step 5.
15038349	A	February 2014	<ul style="list-style-type: none"> • Initial Release

Introduction

Intended Use

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

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

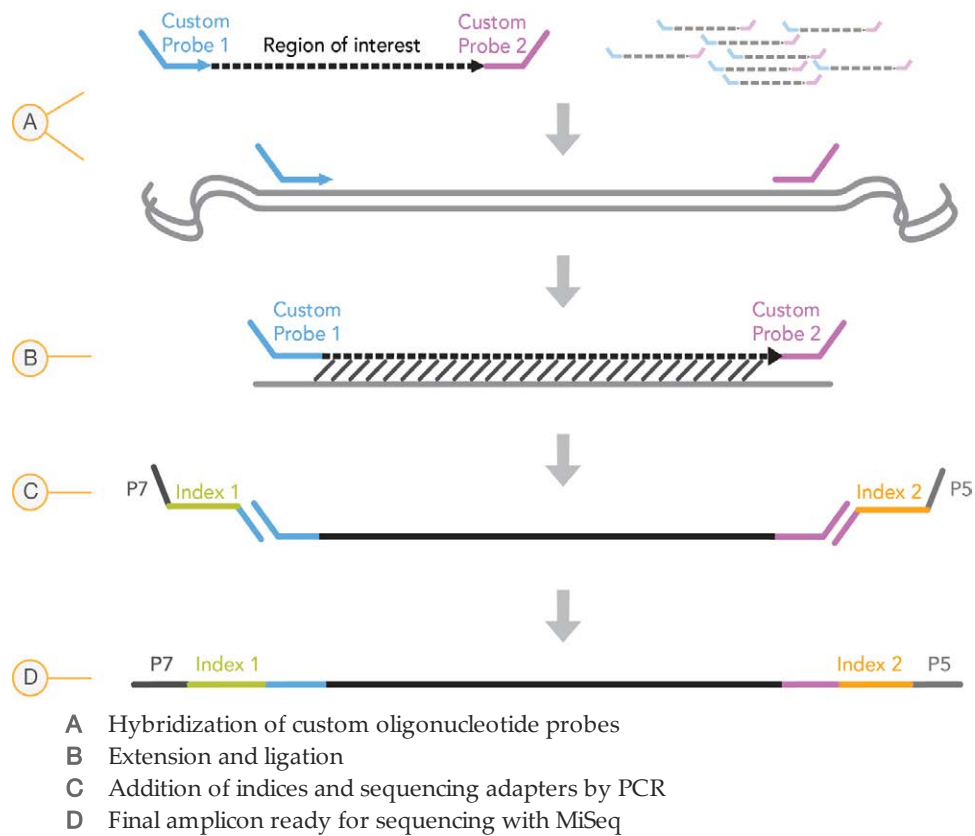
The test is intended to be used on the Illumina MiSeqDx instrument.

About this Guide

This reference guide provides more detailed instructions, technique tips, and helpful hints to newly trained users to help guide proper execution of the MiSeqDx Cystic Fibrosis 139-Variant Assay protocol. This is intended as a supplement and is not meant to replace the package insert.

How Does the Assay Work?

One pair of CF oligonucleotides is designed for each *CFTR* amplicon. Hybridization of these oligonucleotides to genomic DNA occurs in a 96-well plate, followed by extension and ligation to form DNA templates consisting of the regions of interest flanked by universal primer sequences. Using indexed primers supplied with the kit, DNA templates are then amplified by PCR, pooled into a single tube, and sequenced on the MiSeqDx instrument.



Process Overview

The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay process can be summarized into the following steps:

Prepare Sample Information

First, prepare sample information that will be used by the MiSeqDx to identify each sample and its corresponding index. Enter the sample ID, indices, and other parameters applicable to the 96-well plate. For more information, see *Enter Run Information* on page 21.

The sample information you enter for the MiSeqDx can also be used as a guide for setting up the plate during the assay workflow.

Prepare Libraries

Prepare the libraries using the protocol detailed in this user guide.

Sequence Samples on the MiSeqDx

The MiSeqDx Cystic Fibrosis 139-Variant Assay must be sequenced on a MiSeqDx instrument using a paired-end 150 cycle run with dual indexing. For instructions for performing a sequencing run on the MiSeqDx, see the MiSeqDx instrument reference guide for your configuration. See *Instrument and Software Guides* on page 18.

Automated Sequencing and 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 8.

For more information on the analysis workflow, see the guides for the analysis software that is installed with your MiSeqDx. See *Instrument and Software Guides* on page 18.

MiSeqDx Instrument Interface Methods

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

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

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

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

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



NOTE

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

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

- ▶ The **Lab Tracking Form** can be used to record information such as operator name, sample and index information, start and stop times, reagent lot numbers, and barcodes.
- ▶ For MiSeq Reporter software, the **Illumina Worklist Manager** is used to create the sample sheet using a wizard-based application. The Illumina Worklist Manager provides a feature for recording parameters for the sample plate, such as sample ID,

dual indices, and other features applicable to the run. Local Run Manager provides the same features. However there is no separate sample sheet. Enter Sample setup information directly into the **Local Run Manager CF 139 Variant analysis module**.



NOTE

You can download the above Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay documents from the Illumina website. Go to the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay support page and click the **Documentation & Literature** tab.

Getting Started

This section describes the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay kit contents, consumables and equipment used, DNA input recommendations, and best practices to apply during the protocol.

MiSeqDx Cystic Fibrosis 139-Variant Assay Kit Contents

The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay Kit contains the following components. Store the kit components at the specified temperature and in designated pre- and post-amplification areas.

Because the pre- and post-amp reagents are shipped together, it is important to unpack the reagents in the pre-amp lab area, and then move the post-amp reagents to the proper post-amp storage area.

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 1

Table 1 Box 1A Pre-Amp Reagents

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

Table 2 Box 1B Post-Amp Reagents

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

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

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 2

Table 3 Box 2 Post-Amp Reagents

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

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 3

Table 4 Box 3A Pre-Amp Reagents

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

Table 5 Box 3B Post-Amp Reagents

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

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 4

Table 6 Box 4 Post-Amp Reagents

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

MiSeqDx Cystic Fibrosis 139-Variant Assay, Box 5

Table 7 Box 5 Pre-Amp Reagents

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

Table 8 Box 5 Post-Amp Reagents

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

Reagents Required, Not Provided

Pre-Amp Reagents

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

Post-Amp Reagents

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

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

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- ▶ **Physically Separate Pre- and Post-Amp Areas**
 - Physically separate laboratory space where pre-amp processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where post-amp processes are performed.
 - Never use the same sink to wash pre-amp and post-amp troughs.
 - Never share the same water purification system for pre-amp and post-amp processes.
 - Store all supplies used in the protocols in the pre-amp area, and transfer to the post-amp area as needed.
- ▶ **Use Dedicated Equipment and Supplies**
 - Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-amp and post-amp lab processes, and never share between processes.
 - Dedicate separate storage areas (freezers and refrigerators) to pre-amp and post-amp consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-amp lab area, and then move the post-amp reagents to the proper post-amp storage area.

Pre- and Post-Amp Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using 0.5% Sodium Hypochlorite (10% Bleach).



CAUTION

To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

Daily Cleaning of Pre-Amp Area

A daily cleaning of the pre-amp area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-amp area.

Identify pre-amp areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-amp processes. High-risk areas might include, but are not limited to, the following items:

- ▶ Bench tops
- ▶ Door handles
- ▶ Refrigerator/freezer door handles
- ▶ Computer mouse
- ▶ Keyboards

Daily Cleaning of Post-Amp Area

Reducing the amount of PCR product in the post-amp area helps reduce the risk of contamination in the pre-amp area. Daily cleaning of the post-amp area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps achieve this.

Identify post-amp areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:

- ▶ Thermal cyclers
- ▶ Bench space used to process amplified DNA
- ▶ Door handles
- ▶ Refrigerator/freezer door handles
- ▶ Computer mouse
- ▶ Keyboards

Weekly Cleaning of All Lab Areas

Once a week, perform a thorough cleaning of the pre-amp and post-amp areas using 0.5% Sodium Hypochlorite (10% Bleach).

- ▶ Clean all bench tops and laboratory surfaces.
- ▶ Clean all instruments that are not cleaned daily.
- ▶ Thoroughly mop lab floors.
- ▶ Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-amp area; therefore, anything falling to the floor must be treated as contaminated.

- ▶ Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.
- ▶ Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned with a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
- ▶ Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

Precautions

Adhere to the following recommendations when preparing libraries for sequencing using this protocol.

Ensuring Consistency

- ▶ **Use multi-channel pipettes**—To ensure consistency across samples, use a multi-channel pipette where possible. Calibrate pipettes regularly.
- ▶ **Consistency for smaller sample preparations**—Each reagent tube supplied with the kit contains sufficient volume to generate results using manual pipettes and reagent troughs following standard laboratory techniques. To ensure accurate reagent volume delivery, single pipette reagent into each well or multi-channel pipette into a PCR 8-tube strip.

Handling Magnetic Beads

- ▶ **Use at room temperature**—Allow the beads to reach room temperature prior to use.
- ▶ **Vortex until well-suspended**—Immediately prior to use, vortex the beads until they are well-suspended and the color appears homogeneous.
- ▶ **Mix samples thoroughly**—After adding the beads to the samples, mix thoroughly by pipetting up and down ten times. Illumina also recommends using a shaker to thoroughly mix samples.
- ▶ **Allow maximum binding**—For best results, incubate the bead/sample mixtures at room temperature for the entire duration indicated in the protocol.
- ▶ **Slowly aspirate cleared solution**—After placing the plate on the magnetic stand, wait for the solution to clear before proceeding. Keep the plate on the magnetic stand when slowly aspirating cleared solution, taking care not to disturb the separated beads.

Avoiding Cross-Contamination

- ▶ **Change tips between dispensing reagents and samples**—Always use fresh pipette tips between dispensing reagents and samples.
- ▶ **Mix plates as directed**—Mix samples with a multi-channel pipette and centrifuge the plate when indicated. Do not vortex the plates.
- ▶ **Use aerosol-resistant tips**—Using aerosol-resistant pipette tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.

Washing with 80% Ethanol During the PCR Clean-Up Step

- ▶ **Prepare fresh 80% ethanol**—Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air and impact results.
- ▶ **Remove all ethanol from wells**—Make sure that all ethanol is removed from the bottom of the wells, as it may contain residual contaminants. Use a P20 multi-channel pipette to remove residual ethanol and accelerate drying.
- ▶ **Allow complete evaporation**—Allow at least five minutes of drying time off the magnetic stand at room temperature for complete evaporation. Residual ethanol can impact the performance of subsequent reactions.

DNA Input Requirements

- ▶ The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay protocol requires 250 ng of genomic DNA. Illumina strongly recommends quantifying the starting genomic material.
- ▶ **Input DNA Quantitation**—Quantify the starting genomic material using UV spectrophotometer methods based on A260/A280 OD readings.
- ▶ **Assessing DNA Quality**—Absorbance measurements at 260 nm are commonly used to quantify DNA. The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. This protocol is optimized for DNA with absorbance ratio values greater than 1.5.

Quality Controls

- ▶ Good laboratory practices mandate that a positive control DNA sample and a negative (no-template) control sample are included in every run.
- ▶ The positive control DNA sample should be a well-characterized sample with a known CFTR mutation.
- ▶ Illumina also recommends that a wild type control is included on every run.

Acronyms

Table 9 Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay Acronyms

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

Instrument and Software Guides

The instrument and software guides that you use with the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay depend on the drive configuration of the MiSeqDx instrument and the analysis software installed with it.

If you are not sure which software is installed with the MiSeqDx, see *MiSeqDx Instrument Interface Methods* on page 8. If you are not sure of the drive configuration of your MiSeqDx, contact Illumina Technical Support.

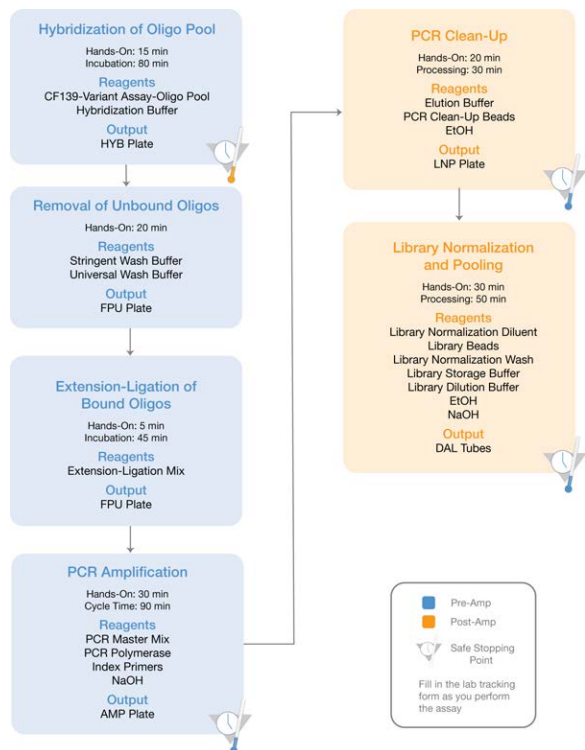
Guides	Dual Boot Drive with Local Run Manager Software	Dual Boot Drive with MiSeq Reporter Software	Single Drive with MiSeq Reporter Software
<i>MiSeqDx Site Prep Guide for Instruments with Dual Boot Configuration</i> (document # 15070066) Site Prep Guide	x	x	
<i>MiSeqDx Instrument Reference Guide for MOS v2</i> (document # 100000021961) Instrument Reference Guide	x		
<i>Local Run Manager Software Reference Guide for MiSeqDx</i> (document # 100000011880) Analysis Software Guide	x		
<i>Local Run Manager CF 139-Variant Analysis Module Workflow Guide</i> (document # 100000012184) Analysis Software Guide	x		
<i>MiSeqDx Reference Guide for Instruments with Dual Boot Configuration</i> (document # 15070067) Instrument Reference Guide		x	
<i>MiSeqDx Instrument Site Prep Guide</i> (document # 15038351) Site Prep Guide			x
<i>MiSeqDx Instrument Reference Guide</i> (document # 15038353) Instrument Reference Guide			x

Guides	Dual Boot Drive with Local Run Manager Software	Dual Boot Drive with MiSeq Reporter Software	Single Drive with MiSeq Reporter Software
<i>MiSeq Reporter Software Guide</i> (document # 15038356) Analysis Software Guide		x	x
<i>MiSeqDx Instrument Safety and Compliance Guide</i> (document # 15034477) Instrument Safety and Compliance Guide	x	x	x

Assay Workflow

The following diagram illustrates the Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay workflow. Safe stopping points are marked between steps.

Figure 1 Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay Workflow



Enter Run Information

MiSeq Reporter or Local Run Manager are the two software options available to set up a Cystic Fibrosis 139-Variant Assay run. For complete information, see the analysis software guides for your configuration listed in *Instrument and Software Guides* on page 18. If you use MiSeq Reporter software, use Illumina Worklist Manager to generate a sample sheet.

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

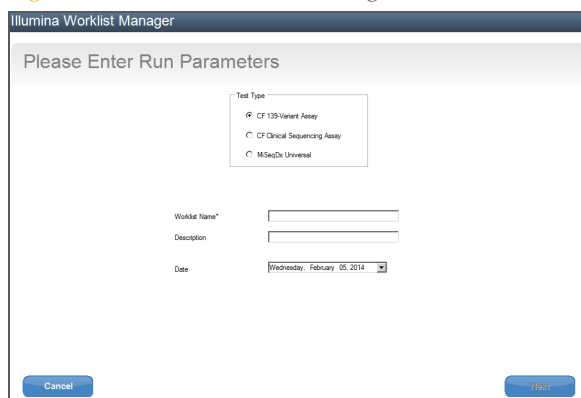
For more information on the differences between MiSeq Reporter and Local Run Manager, see *MiSeqDx Instrument Interface Methods* on page 1.

Using Illumina Worklist Manager (IWM)

MiSeqDx Sample Sheet Preparation

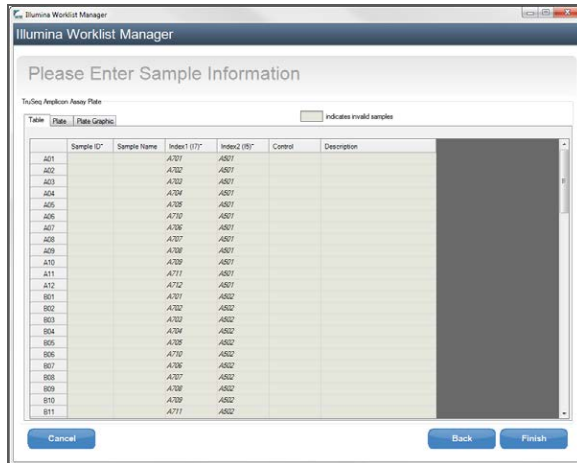
- 1 From the Illumina Worklist Manager Welcome screen, select **Create Worklist**. The Enter Run Parameters screen opens.

Figure 2 Illumina Worklist Manager, Enter Run Parameters screen



- 2 In the Test Type field, select **CFTR 139-Variant Assay**.
- 3 In the Worklist Name field, enter a name for the sample sheet. This is a required field.
 - 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. (The barcode ID is located on the reagent cartridge label directly below the barcode.)
 - 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. The current date appears by default.
- 6 Select **Next**. The Enter Sample Information screen opens.

Figure 3 Illumina Worklist Manager, Enter Sample Information screen



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. The sample ID is used to track the sample from preparation through sequencing and analysis. The ID is usually a barcode; however, any value is acceptable.
 - b **Index 1 and Index 2**—Specify the index adapter that will be used for each Index Read. Illumina recommends using combinations that result in at least one A or C base (red) and at least one G or T base (green) at every cycle.

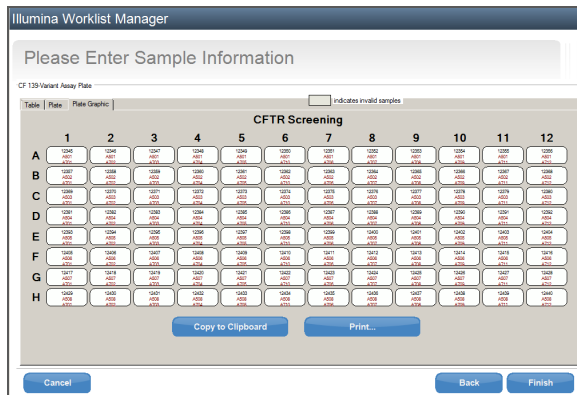


NOTE

Please refer to *Sample Throughput and Index Representation* on page 24 for assistance in choosing the appropriate indexes.

- 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.

Figure 4 Illumina Worklist Manager, Plate Graphic tab



- 5 Select **Finish**. When saving the sample sheet, the software creates both a .csv and .png file of the Plate Graphic automatically and saves them in the same location for use with experiment setup.



NOTE

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

Using the Local Run Manager CF 139 Variant Analysis Module

Set Parameters

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

Specify Samples for the Run

Specify samples for the run using the following options:

▶ **Enter samples manually**


- 1 Select the number of sample from the Number of Samples drop-down box. Consider the following information when you make a selection.
 - ▶ Choose the number of samples that is nearest to the number of samples you are testing. The drop-down list contains samples in multiples of 8. If the exact number of samples is not in the list, select the number nearest, but less than the number you are testing, so that index diversity requirements are met. For example, if you want to test 18 samples, select 16 samples. Then add 2 additional samples. Make sure to select index adapters for extra wells.
 - ▶ For 48 samples, there are two different configurations available. One configuration is 48-Set 1, and the other is 48-Set 2. The configuration sets contain different i7 index primers and well positions. These sets can be used to set up two sequencing runs for a total of 96 samples.
 - 2 Use the blank table on the Create Run screen. Suggested sample wells are highlighted.
- ▶ **Import samples**—Navigate to an external file in a comma-separated values (*.csv) format. A template is available for download on the Create Run screen.

After you have populated the samples table, you can export the sample information to an external file, and use the file as a reference when preparing libraries or import the file for another run.

Enter Samples Manually

- 1 Enter a unique sample name in the Sample Name field. Use alphanumeric characters, dashes, or underscores.
- 2 Right-click and select positive and negative control samples.
- 3 [Optional] Enter a sample description in the Sample Description tab.


Use alphanumeric characters, dashes, underscores, or spaces.

- 4 [Optional] Select an Index 1 adapter from the Index 1 (i7) drop-down list. This step is optional because the i7 and i5 index combinations that autopopulate the wells highlighted already meet index diversity requirements.
- 5 [Optional] Select an Index 2 adapter from the Index 2 (i5) drop-down list. This step is optional because the i7 and i5 index combinations that autopopulate the wells highlighted already meet index diversity requirements.
- 6 Click the  **Print** icon to display the plate layout.
- 7 Select **Print** to print the plate layout as a reference for preparing libraries.
- 8 [Optional] Click **Export** to export sample information to an external file.
- 9 Click **Save Run**.

Import Samples

- 1 Click **Import Samples** and browse to the location of the sample information file. There are two types of files you can import.
 - ▶ Click **Template** to create a new plate layout. The template file contains the correct column headings for import. Enter sample information in each column for the samples in the run. Delete example information in unused cells, and then save the file.

Sample_Name	Description	I7_Index_ID	I5_Index_ID	Sample_Well	Control
Sample1		A701	A503	A01	
Sample2		A701	A503	A02	
Sample3		A701	A504	A03	
Sample4		A702	A504	A04	
Sample5		A702	A505	A05	
Sample6		A702	A505	A06	
Sample7		A710	A503	A07	Positive
Sample8		A710	A504	A08	Negative

- ▶ Use a file of sample information that was exported from the CF 139 Variant analysis module using the Export feature.
- 2 Click the  **Print** icon to display the plate layout.
- 3 Select **Print** to print the plate layout as a reference for preparing libraries.
- 4 [Optional] Click **Export** to export sample information to an external file.
- 5 Click **Save Run**.

Sample Throughput and Index Representation

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

**NOTE**

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

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

See Table 10 for choosing index primer combinations for 48 or 96 sample library preparations.

Table 10 Index Primer Combinations for 48-Sample or 96-Sample Sequencing Runs

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

If sequencing fewer than the 48 samples in a sequencing run, select the appropriate indexes based on their sequences to maintain color balance in the green and red channels (see Table 12 and Table 13). At a minimum, runs with 8 to 48 samples must include the indexing primer combinations identified in Table 11.

To accurately process smaller runs, at least eight samples must be present. If six unique samples (excluding the positive and negative controls) are not available, it is acceptable to fill the run with sample replicates or any human genomic DNA sample. See Table 11 for the minimal set of color-balanced indexes to use for 8-sample sequencing runs.

Table 11 Index Primer Combinations for 8-Sample Sequencing Runs

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

Index Primer Sequences

Table 12 Index 1 (I7) Set 1 & Set 2 Primers

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

Table 13 Index 2 (I5) Primers

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

Hybridization of Oligonucleotide Pool

During this step, the cystic fibrosis oligonucleotide pool containing upstream and downstream oligonucleotides specific to the cystic fibrosis transmembrane conductance regulator (CFTR) gene is hybridized to genomic DNA samples.



WARNING

This set of reagents contains 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 additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Estimated Time

- ▶ Total duration: 1 hour 35 minutes
- ▶ Hands-on: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
CF 139-Variant Assay-Oligo Pool	1 tube	-25°C to -15°C	Illumina
Hybridization Buffer	1 tube	-25°C to -15°C	Illumina
Genomic DNA (50 ng/μl recommended)	5 μl	-25°C to -15°C	User
96-well skirted PCR plate	1 plate	15°C to 30°C	User
Adhesive aluminum foil seal	2 seals	15°C to 30°C	User
Sterile troughs	As needed	15°C to 30°C	User

Preparation

- 1 Remove the CF 139-Variant Assay-Oligo Pool, Hybridization Buffer, genomic DNA samples, and positive control sample from -25°C to -15°C storage and thaw at room temperature.
- 2 Vortex the CF 139-Variant Assay-Oligo Pool and Hybridization Buffer vigorously to make sure that all precipitates have completely dissolved, then briefly centrifuge the tubes to collect liquid.



NOTE

Before using Hybridization Buffer, hold the tube in front of a light and visually inspect to make sure that all precipitates have completely dissolved.

- 3 Set a 96-well heat block to 95°C.
- 4 Pre-heat an incubator to 37°C to prepare for the extension-ligation step.

- 5 Create the sample plate according to the plate graphic printed from Illumina Worklist Manager or Local Run Manager. Verify the location of positive and negative controls match. Illumina recommends processing samples in batches no smaller than eight.



NOTE

For workflow efficiency, if a run is being set up with 96 samples, generate a separate plate graphic printout for each half plate (set of 48 samples) from Illumina Worklist Manager or Local Run Manager and use this to set up the run.

For Illumina Worklist Manager, generate 2 separate sample sheets. For Local Run Manager, enter sample information for the set of 48 samples for each run directly into the CF 139 Variant analysis module.

Use of controls enables Illumina Technical Support to provide effective troubleshooting assistance. Illumina Technical Support will not provide assistance unless these control reactions were included in the run.

Procedure

- 1 Label a new 96-well PCR plate "**HYB_Plate_ID**".
- 2 Add 5 μ l of sample or control at 50 ng/ μ l (250 ng total) to the appropriate wells in the **HYB** plate. Follow the generated plate layout for correct well selection.



NOTE

Verify that DNA sample layout and the positions of positive and negative controls match the plate graphic.

- 3 Using a multi-channel pipette, add 5 μ l of the CF 139-Variant Assay-Oligo Pool to all sample wells. Change tips after each column to avoid cross-contamination.
- 4 Using a multi-channel pipette, add 40 μ l of Hybridization Buffer to each sample in the **HYB** plate. Gently pipette up and down 3–5 times to mix. Change tips after each column to avoid cross-contamination.



NOTE

Ensure any crystals or precipitate in Hybridization Buffer have dissolved.



NOTE

Do not mix the CF 139-Variant Assay-Oligo Pool and Hybridization Buffer for storage. If combined, the CF 139-Variant Assay-Oligo Pool becomes unstable, even when stored frozen.

- 5 Seal the **HYB** plate with adhesive aluminum foil and secure the seal with a rubber roller or sealing wedge.
- 6 Centrifuge at $1000 \times g$ at 20°C for 1 minute.
- 7 Place the **HYB** plate in the pre-heated block at 95°C and incubate for 1 minute.
- 8 Reduce the temperature of the pre-heated block to 40°C and continue incubating until the heat block reaches 40°C. Ramp down time takes approximately 80 minutes.



NOTE

During incubation, the heat block temperature gradually decreases from 95°C to 40°C. This process typically takes 80 minutes. This gradual cooling is critical for proper hybridization; therefore, PCR thermal cyclers with active cooling (e.g., Peltier, thermoelectric cooled) are not recommended for this process.



SAFE STOPPING POINT

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

Removal of Unbound Oligonucleotides

This process removes unbound oligonucleotides from genomic DNA using a filter capable of size selection. Two wash steps using Stringent Wash Buffer ensure complete removal of unbound oligonucleotides. A third wash step using Universal Wash Buffer removes residual Stringent Wash Buffer and prepares samples for the extension-ligation step.



WARNING

This set of reagents contains 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 additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Estimated Time

- ▶ Total duration: 20 minutes
- ▶ Hands-on: 20 minutes

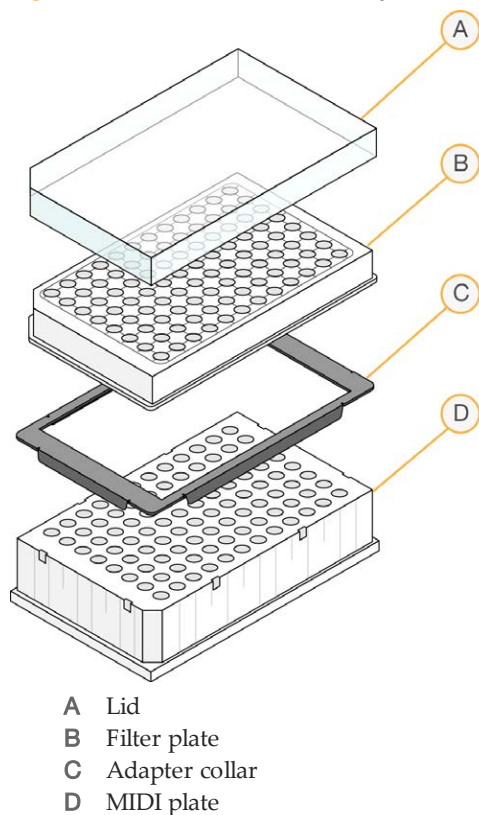
Consumables

Item	Quantity	Storage	Supplied By
Extension-Ligation Mix	1 tube	-25°C to -15°C	Illumina
Stringent Wash Buffer	1 bottle	2°C to 8°C	Illumina
Universal Wash Buffer	1 tube	2°C to 8°C	Illumina
Filter Plate	1 plate	15°C to 30°C	Illumina
Adapter collar	1 plate	15°C to 30°C	Illumina
MIDI plate	1 plate	15°C to 30°C	User
Troughs	As needed	15°C to 30°C	User

Preparation

- 1 Remove Extension-Ligation Mix from -25°C to -15°C storage and thaw at room temperature.
Extension-Ligation Mix is used in the Extension-Ligation step and takes approximately 20 minutes to thaw.
- 2 Remove Stringent Wash Buffer and Universal Wash Buffer from 2°C to 8°C storage and set aside at room temperature.
- 3 Assemble the filter plate assembly unit (FPU) in the following order (from top to bottom):

Figure 5 Filter Plate Unit Assembly



- 4 Label the filter plate "**FPU_Plate_ID**". Plate ID should match the ID used for the **HYB** plate.
- 5 Pre-wash the filter plate membrane as follows:
 - a Using a multi-channel pipette, add 45 μ l of Stringent Wash Buffer to each well.
 - b Cover the **FPU** plate with the filter plate lid and keep it covered during each centrifugation step.
 - c Centrifuge the **FPU** 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 After the hybridization is complete, confirm the heat block has cooled to 40°C . While the **HYB** plate is still in the heat block, reinforce the seal using a rubber roller or sealing wedge. If 40°C is not reached within 80 minutes, continue incubating until the heat block has cooled to 40°C .
- 2 Remove the **HYB** plate from the heat block and centrifuge at $1000 \times g$ at 20°C for 1 minute to collect condensation.

- 3 Using a multi-channel pipette set to 60 μl , transfer the entire volume of each sample onto the center of the corresponding pre-washed wells of the filter plate. Change tips after each column to avoid cross-contamination.
- 4 Cover the filter plate with the lid and centrifuge at $2400 \times g$ at 20°C for 5 minutes.
- 5 Wash the filter plate as follows:
 - a Using a multi-channel pipette, add 45 μl of Stringent Wash Buffer to each sample well.
Changing tips between columns is not required if you use care to avoid cross-contamination.
 - b Cover the filter plate with the lid and centrifuge at $2400 \times g$ at 20°C for 5 minutes.



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 Repeat the wash as follows:
 - a Using a multi-channel pipette, add 45 μl of Stringent Wash Buffer to each sample well.
Changing tips between columns is not required if you use care to avoid cross-contamination.
 - b Cover the filter plate with the lid and centrifuge at $2400 \times g$ at 20°C for 5 minutes.
 - c If the wash buffer does not drain completely, centrifuge the filter plate again at $2400 \times g$ at 20°C for 5 minutes.
- 7 Discard all the flow-through (containing formamide) collected up to this point in an appropriate hazardous waste container, then reassemble the **FPU**. The same **MIDI** plate can be re-used for the rest of the pre-amplification process.
- 8 Using a multi-channel pipette, add 45 μl of Universal Wash Buffer to each sample well.
Changing tips between columns is not required if you use care to avoid cross-contamination.
- 9 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. Residual wash buffer may inhibit subsequent enzymatic reactions.

Extension-Ligation of Bound Oligonucleotides

This process connects the hybridized upstream and downstream oligonucleotides. A DNA polymerase extends from the upstream oligonucleotide through the targeted region, followed by ligation to the 5' end of the downstream oligonucleotide using a DNA ligase. This results in the formation of products containing the targeted regions of interest flanked by sequences required for amplification.

Estimated Time

- ▶ Total duration: 50 minutes
- ▶ Hands-on: 5 minutes

Consumables

Item	Quantity	Storage	Supplied By
Extension-Ligation Mix	1 tube	-25°C to -15°C	Illumina
Adhesive aluminum foil seal	1 seal	15°C to 30°C	User
Troughs	As needed	15°C to 30°C	User

Procedure

- 1 Using a multi-channel pipette, add 45 µl of Extension-Ligation Mix to each sample well of the filter plate. The Extension-Ligation reaction takes place on the filter plate membrane.
Changing tips between columns is not required if you use care to avoid cross-contamination.
- 2 Seal the filter plate with adhesive aluminum foil, and then cover with the lid to secure the foil during incubation.
- 3 Incubate the entire **FPU** assembly 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

In this step, the extension-ligation products are amplified using primers that add index sequences for sample multiplexing, as well as common adapters required for cluster generation.

Estimated Time

- ▶ Total duration: ~90 minutes
- ▶ Hands-on: 30 minutes

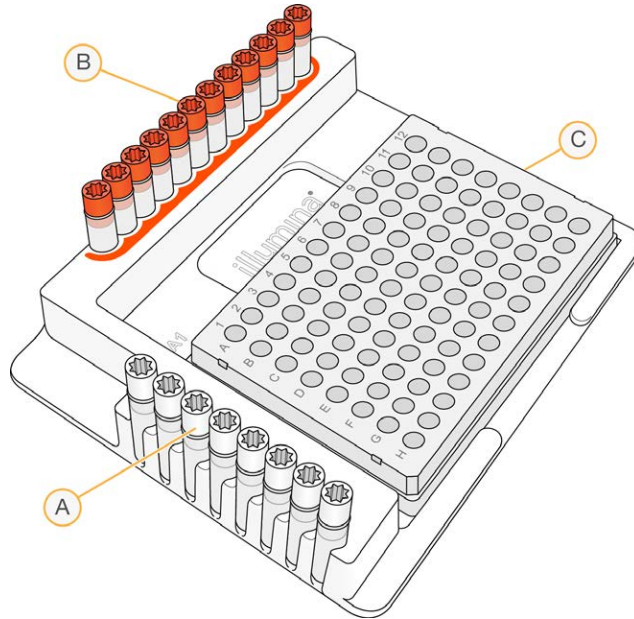
Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix	1 tube	-25°C to -15°C	Illumina
Index Primers A (A501) - H (A508)	1 tube per primer	-25°C to -15°C	Illumina
Index Primers 1 (A701) - 12 (A712)	1 tube per primer	-25°C to -15°C	Illumina
PCR Polymerase	1 tube	-25°C to -15°C	Illumina
Appropriate PCR plate seal	1	15°C to 30°C	User
0.05 N NaOH, freshly prepared	As needed	15°C to 30°C	User
96-well skirted PCR plate	1 plate	15°C to 30°C	User
Troughs	As needed	15°C to 30°C	User

Preparation

- 1 Prepare fresh 0.05 N NaOH by adding 25 μ l of 10 N NaOH to 4975 μ l of RNase/DNase-free water.
- 2 Determine the index primers to be used according to the plate graphic printout from Illumina Worklist Manager or Local Run Manager.
- 3 Remove PCR Master Mix and the appropriate index primers from -25°C to -15°C storage and thaw on a bench at room temperature. Allow approximately 20 minutes to thaw the reagents.
- 4 After the index primers are completely thawed, vortex each tube to mix and briefly centrifuge the tubes in a microcentrifuge. Use 1.7 ml Eppendorf tubes as adapters for the microcentrifuge.
- 5 Arrange the primers in a rack using the following arrangements:
 - a Arrange Index Primers A (A501) - H (A508) primer tubes (white caps, clear solution) vertically, aligned with rows A through H.
 - b Arrange Index Primers 1 (A701) - 12 (A712) primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.

Figure 6 Index Plate Fixture



- A Index Primers A (A501) - H (A508) (white caps)
- B Index Primers 1 (A701) - 12 (A712) (orange caps)
- C AMP plate

- 6 Label a new 96-well PCR plate "AMP" (Amplification Plate).
- 7 Add index primers to the AMP plate as follows:
 - a Using a multi-channel pipette, add 4 μ l of the selected index primers [A (A501) – H (A508)] (clear solution) to the appropriate well in a column of the AMP plate. Changing tips between columns is not required.
 - b To avoid index cross-contamination, discard the original *white* caps and apply new *white* caps.
 - c Using a multi-channel pipette, add 4 μ l of the selected index primers [1 (A701) – 12 (A712)] (yellow solution) to the appropriate row of the AMP plate. ***Tips must be changed after each row to avoid index cross-contamination.***
 - d To avoid index cross-contamination, discard the original *orange* caps and apply new *orange* caps. Remove all the index primer tubes from the working area.
- 8 Prepare the PCR Master Mix/PCR Polymerase PCR working solution as follows:
 - a Briefly centrifuge the PCR Polymerase tube before use to remove air bubbles.
 - b For 96 samples, add 56 μ l of PCR Polymerase to 2.8 ml of PCR Master Mix.
 - c Invert the prepared PCR working solution 20 times to mix.

You will add this working solution to the AMP plate in the next section. The PCR working solution is stable at room temperature for 10 minutes.



NOTE

Always add PCR Polymerase to PCR Master Mix just prior to use. Never store the combined PCR working solution.

Procedure

- 1 When the 45-minute extension-ligation reaction is complete, remove the FPU from the incubator. Remove the aluminum foil seal and replace with the filter plate lid.

Removing the aluminum foil seal before centrifugation is recommended to ensure the reaction supernatant will drain into the waste plate effectively.

- 2 Centrifuge the **FPU** at $2400 \times g$ at 20°C for 2 minutes.
- 3 Using a multi-channel pipette, add $25 \mu\text{l}$ of 0.05 N NaOH to each sample well on the filter plate. Ensuring that pipette tips come in contact with the membrane, pipette the NaOH up and down 5–6 times. Tips must be changed after each column.
- 4 Cover and incubate the filter plate at room temperature for 5 minutes.
- 5 While the filter plate is incubating, use a multi-channel pipette to transfer $22 \mu\text{l}$ of the PCR working solution to each well of the AMP plate containing index primers. Change tips between samples.
- 6 Transfer samples eluted from the filter to the AMP plate as follows:
 - a Set a multi-channel P20 pipette to $20 \mu\text{l}$.
 - b Pipette the samples in the first column of the filter plate up and down 5–6 times.
 - c Transfer $20 \mu\text{l}$ from the filter plate to the corresponding column of the **AMP** plate.
 - d Gently pipette up and down 5–6 times to thoroughly combine the DNA with the PCR working solution.



NOTE

Slightly tilt the **FPU** plate to ensure complete aspiration and to avoid air bubbles.

- e 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.*
 - f After all the samples have been transferred, the waste collection MIDI plate of the **FPU** can be discarded. The metal adapter collar should be cleaned and put away for future use.
- 7 Cover the **AMP** plate with the appropriate plate seal 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



SAFESTOPPING 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

This process uses PCR Clean-Up Beads to purify the PCR products from the other reaction components.

Estimated Time

- ▶ Total duration: 50 minutes
- ▶ Hands-on: 20 minutes

Consumables

Item	Quantity	Storage	Supplied By
Elution Buffer	1 tube	15°C to 30°C	Illumina
PCR Clean-Up Beads	400 µl per 8 samples	2°C to 8°C	Illumina
80% Ethanol, freshly-prepared	5 ml per 8 samples	15°C to 30°C	User
96-well MIDI plates	2	15°C to 30°C	User
Adhesive plate seal	As needed	15°C to 30°C	User
Troughs	As needed	15°C to 30°C	User

Preparation



NOTE

Please review the **Precautions** section at the beginning of this protocol regarding the handling of magnetic beads and washing with 80% ethanol during the PCR clean-up.

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



NOTE

Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air impacting the results.

Procedure

- 1 Centrifuge the AMP plate at 1000 × g at 20°C for 1 minute to collect condensation.
- 2 Label a new MIDI plate "**CLP_Plate_ID**" (Clean-up Plate).
- 3 Invert PCR Clean-Up Beads 10 times. Vortex vigorously and then invert again 10 times.
- 4 Visually inspect solution to ensure that beads are resuspended well.
- 5 Using a multi-channel pipette, add 45 µl of PCR Clean-Up Beads to each well of the **CLP** plate.
- 6 Using a multi-channel pipette set to 60 µl, transfer the entire PCR product from the AMP plate to the **CLP** plate. Change tips between samples.
- 7 Seal the **CLP** plate with an adhesive plate seal.
- 8 Shake the **CLP** plate on a microplate shaker at 1800 rpm for 2 minutes.

- 9 Incubate at room temperature (15°C to 30°C) without shaking for 10 minutes.
- 10 Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 11 With the **CLP** plate on the magnetic stand and a multi-channel pipette set to 100 µl, carefully remove and discard the supernatant. Change tips between samples.



NOTE

If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes and confirm that the supernatant has cleared.

- 12 With the **CLP** plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
 - a Using a multi-channel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well. Changing tips is not required if you use care to avoid cross-contamination. You do not need to resuspend the beads at this time.
 - 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.
- 13 With the **CLP** plate on the magnetic stand, perform a second ethanol wash as follows:
 - a Using a multi-channel pipette, 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 appears clear.
 - c Carefully remove and discard the supernatant.
- 14 Use a P20 multi-channel pipette set to 20 µl to remove excess ethanol.
- 15 Remove the **CLP** plate from the magnetic stand and allow the beads to air-dry for 10 minutes.
- 16 Using a multi-channel pipette, add 30 µl of Elution Buffer to each sample. Changing tips is not required if you use care to avoid cross-contamination.
- 17 Seal the plate with an adhesive plate seal.
- 18 Shake the **CLP** plate on a microplate shaker at 1800 rpm for 2 minutes.



NOTE

Ensure all samples are completely resuspended. If there are samples in which the beads are not completely resuspended, gently pipette up and down to resuspend the beads and repeat the previous two steps.

- 19 Incubate at room temperature (15°C to 30°C) for 2 minutes.
- 20 Place the **CLP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 21 Label a new MIDI plate "**LNP_Plate_ID**" (Library Normalization Plate).
- 22 Using a P20 multi-channel pipette and fine tips, carefully transfer 20 µl of the supernatant from the **CLP** plate to the **LNP** plate. Change tips between samples to avoid cross-contamination.



NOTE

If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes and confirm that the supernatant has cleared.

- 23 [Optional] Transfer the remaining 10 μ l 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.
- 24 If stopping at this point, seal the LNP plate with an adhesive plate seal, and then centrifuge at $1000 \times g$ at 20°C for 1 minute to ensure all the supernatant is at the bottom of the well.

**SAFE STOPPING POINT**

After PCR Clean-Up, the plate is stable for up to 3 hours at 2°C to 8°C .

Library Normalization and Pooling

This process normalizes the quantity of each library to ensure equal library representation in the pooled sample. Equal volumes of normalized libraries are then combined and diluted in preparation for sequencing.

Estimated Time

- ▶ Total duration: 1 hour 20 minutes
- ▶ Hands-on: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
Library Normalization Diluent	1 tube	-25°C to -15°C	Illumina
Library Beads	1 tube	2°C to 8°C	Illumina
Library Normalization Wash	2 tubes	2°C to 8°C	Illumina
Library Storage Buffer	1 tube	15°C to 30°C	Illumina
Library Dilution Buffer	1 tube	-25°C to -15°C	Illumina
0.1 N NaOH, freshly prepared	2 ml per 48 samples	15°C to 30°C	User
96-well skirted PCR plate	1 plate	15°C to 30°C	User
15 ml conical tube	1 tube	15°C to 30°C	User
Adhesive plate seal	As needed	15°C to 30°C	User



WARNING

This set of reagents contains 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 additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

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 Remove Library Normalization Diluent and Library Dilution Buffer from -25°C to -15°C storage and bring to room temperature. Use a 20°C to 25°C water bath as needed.



NOTE

Library Normalization Diluent might form visible precipitates or crystals. Before use, vortex vigorously, and then hold the tube in front of a light and visually inspect to make sure that all precipitate has completely dissolved.

- 3 Remove Library Beads and Library Normalization Wash from 2°C to 8°C storage and bring to room temperature. Use a 20°C to 25°C water bath as needed.

- 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

- For 96 samples, add 4.4 ml of Library Normalization Diluent to a fresh 15 ml conical tube. If processing < 24 samples, use a fresh 1.5 ml tube.
- Use a P1000 pipette set to 1000 μ l to resuspend Library Beads thoroughly by pipetting up and down 10 times.



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.

- For 96 samples, pipette 800 μ l of Library Beads to the tube containing Library Normalization Diluent. Mix well by inverting the tube 15–20 times.



NOTE

A P1000 set to 1000 μ l is required to resuspend the beads completely in step 2. Mix only the specified amounts of Library Normalization Diluent and Library Beads. You must store the remaining Library Normalization Diluent and Library Beads separately at their respective recommended temperatures. To preserve stability, Library Beads should never be frozen or mixed with Library Normalization Diluent if not used immediately.

- Using a multi-channel pipette, add 45 μ l of the combined Library Normalization Diluent/Library Beads working solution to each well of the LNP plate containing libraries. Changing tips between columns is not required if you use care to avoid cross-contamination.
- Seal the LNP plate with an adhesive plate seal.
- Shake the LNP plate on a microplate shaker at 1,800 rpm for 30 minutes.



NOTE

This 30-minute incubation is critical for proper library normalization. Incubation times of greater or less than 30 minutes may affect library representation and cluster density.



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 43.


- Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- With the LNP plate on the magnetic stand, using a multi-channel pipette set to 80 μ l, carefully remove and discard the supernatant in an appropriate hazardous waste container.



NOTE

If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest for 2 minutes or until the supernatant has cleared.

- Remove the LNP plate from the magnetic stand and wash the beads with Library Normalization Wash as follows:

- a Using a multi-channel pipette, add 45 μ l of Library Normalization Wash to each sample well.
Changing tips between columns is not required if you use care to avoid cross-contamination.
 - b Seal the **LNP** plate with an adhesive plate seal.
 - c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
 - d Place the plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
 - e Carefully remove and discard the supernatant in an appropriate hazardous waste container.
- 10 Remove the **LNP** plate from the magnetic stand and repeat the wash with Library Normalization Wash as follows:
- a Using a multi-channel pipette, add 45 μ l of Library Normalization Wash to each well.
Changing tips between columns is not required if you use care to avoid cross-contamination.
 - b Seal the **LNP** plate with an adhesive plate seal.
 - c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
 - d Place the plate on the magnetic stand for a minimum of 2 minutes.
 - e Carefully remove and discard the supernatant in an appropriate hazardous waste container.
- 11 Use a P20 multi-channel pipette set to 20 μ l to remove excess Library Normalization Wash.
- 12 Remove the **LNP** plate from the magnetic stand and add 30 μ l of 0.1 N NaOH to each well to elute the sample.
- 13 Seal the **LNP** plate with an adhesive plate seal.
- 14 Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
- 15 During the 5 minute elution, label a new 96-well PCR plate "**SGP_Plate_ID**" (StoraGe Plate).
- 16 Add 30 μ l Library Storage Buffer to each well to be used in the **SGP** plate.
- 17 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.
- 18 Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes.
- 19 Using a multi-channel pipette set to 30 μ l, transfer the supernatant from the **LNP** plate to the **SGP** plate. Gently pipette up and down 5 times to mix.
-  **NOTE**
If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes and confirm that the supernatant has cleared.
- 20 Seal the **SGP** plate with an adhesive plate seal and then centrifuge at 1,000 \times g at 20°C for 1 minute.
- 21 Vortex the Library Dilution Buffer and make sure that all the precipitates have dissolved completely.
- 22 Briefly centrifuge to collect contents.

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

**SAFESTOPPING POINT**

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

Library Sequencing

In preparation for cluster generation and sequencing, the diluted library is heat denatured prior to sequencing on the MiSeqDx. PhiX is used as an internal control for sequencing.

Estimated Time

- ▶ Total duration: 10 minutes
- ▶ Hands-on: 10 minutes

Consumables

Item	Quantity	Storage	Supplied By
Library Dilution Buffer	1 tube	-25°C to -15°C	Illumina
10 nM PhiX Internal Control	2 µl	-25°C to -15°C	Illumina
0.1 N NaOH, freshly prepared	1 ml	15°C to 30°C	User
1X TE Buffer	8 µl	15°C to 30°C	User
Eppendorf tubes (screwcap recommended)	2 tubes	15°C to 30°C	User
PCR eight-tube strip	1	15°C to 30°C	User
2.5 L Ice bucket	1	15°C to 30°C	User

Prepare for Library Sequencing

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

Prepare the Reagent Cartridge

The following instructions describe how to thaw the reagent cartridge using a room temperature water bath. This method requires approximately 1 hour.

- 1 Remove the reagent cartridge from -25°C to -15°C storage.
- 2 Place the reagent cartridge 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.

Figure 7 Maximum Water Line



- 3 Allow the reagent cartridge to thaw in the room temperature water bath for approximately 1 hour or until thawed.
- 4 Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge. Make sure that no water has splashed on the top of the reagent cartridge.

Inspect the Reagent Cartridge

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



NOTE

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

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



NOTE

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

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

Denature and Dilute PhiX Internal Control

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

- 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 μ l)
 - 1X TE Buffer (8 μ l)
- 4 Combine the following volumes to result in a 1 nM PhiX Internal Control library:
 - 2 nM PhiX Internal Control library (10 μ l)
 - 0.1 N NaOH (10 μ l)
- 5 Vortex briefly to mix the 1 nM PhiX Internal Control library solution.
- 6 Centrifuge the 1nM PhiX Internal Control at $280 \times g$ 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. After 3 weeks, cluster numbers tend to decrease.

Prepare Samples for Sequencing

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



NOTE

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

What's Next

After the amplicon library has been pooled with the diluted and denatured PhiX, the libraries are ready to be loaded onto the MiSeqDx Reagent Cartridge - CF 139-Variant Assay in the designated reservoir labeled **Load Samples**. The sequencing run is then set up using the MiSeq Operating Software (MOS) interface. See the MiSeqDx instrument reference guide for your configuration. See *Instrument and Software Guides* on page 18.

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 14 Illumina General Contact Information

Website	www.illumina.com
Email	techsupport@illumina.com

Table 15 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety Data Sheets

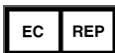
Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.html.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to support.illumina.com, select a product, then click **Documentation & Literature**.



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