mtDNA Sequencing using the MiSeq FGx

1 Purpose

1.1 Libraries undergo sequencing-by-synthesis on the MiSeq FGx Sequencing System to develop mitochondrial DNA profiles for the unknown samples.

2 System Check

2.1 A System Check should be run prior to every MiSeq sequencing run.

2.2 Prior to initiating a system check, power cycle the instrument by selecting *Shut Down* on the *Manage Instrument* screen, and then turn off the instrument using the power switch. Wait at least 60 seconds, and then turn on the instrument and start the software.

2.3 From the Home screen, select *Manage Instrument*.

2.4 Select System Check.

2.5 Check all boxes EXCEPT those listed under “Optics” (Optics tests are performed by the vendor.) Then select *Next*.

2.6 Retrieve and rinse a wash tray and a wash bottle. A designated wash tray and wash bottle is available in the laboratory for the purposes of a system check.

2.7 Fill each well of the wash tray with approximately 6 mL of laboratory-grade water.

2.8 Fill the 500-mL wash bottle with ~350 mL of laboratory-grade water.

2.9 Load the wash tray and wash bottle onto the instrument.

2.9.1 Open the reagent compartment door and reagent chiller door and slide the wash tray into the reagent chiller until it stops. Close the reagent chiller door.

2.9.2 Remove the waste bottle and discard the contents appropriately. Return the waste bottle to the reagent compartment.

2.9.3 Slowly lower the sipper handle, making sure that the sippers lower into the wash bottle and waste bottle.

2.10 Select *Next*.

2.11 The System Check takes approximately 1.5 hrs to complete.

2.12 Ensure that each test of the system check has passed before proceeding with a run. If any test of the system check fails, contact a supervisor for further instruction.

3 LIMS Processing

3.1 Refer to the LIMS Process Manual for the general test batch processing protocol.

3.2 This sequencing batch requires the samples to be loaded onto a plate in LIMS.

3.2.1 Within the test batch, select all the output samples and click *Load Plate*.

3.2.2 Fill in the "Plate Name" field with your run name.

3.2.3 Add samples to the plate according to their unique sample suffix.

3.2.4 Click *Download Instrument.* This will create a spreadsheet containing the sample information for your run.

3.3 Navigate to MiSeqSequencing within the LIMS to Instrument folder on the network and open the spreadsheet for your run.

3.4 Within the spreadsheet, review your sample names for any spaces or parentheses and remove them if present.

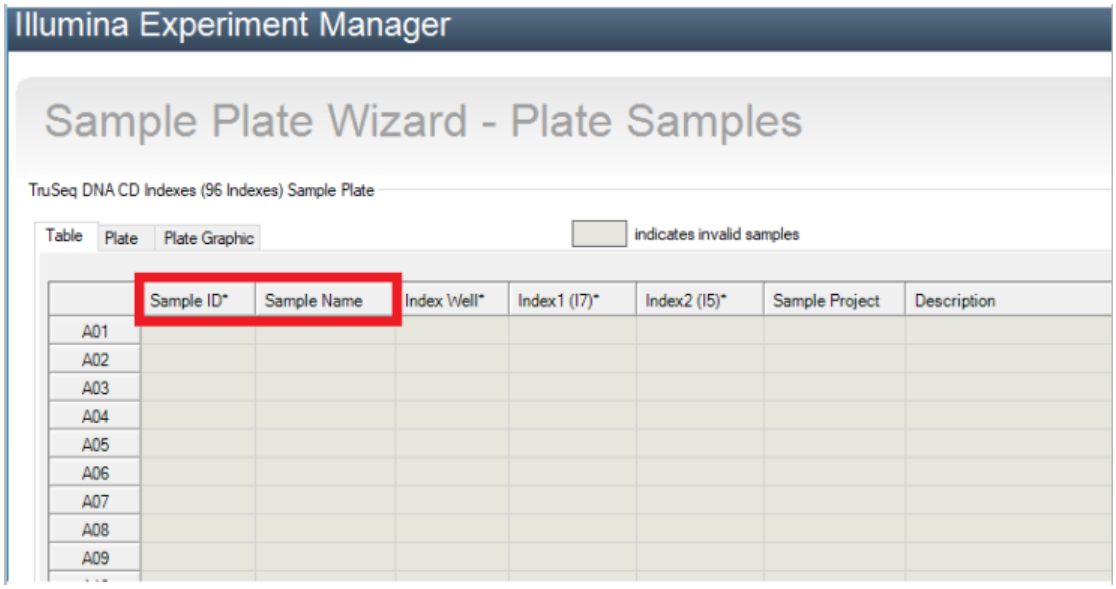
3.5 Copy the entire OCME ID column from the spreadsheet (including blank cells).

4 Creating Sample Sheet

4.1 Open the Illumina Experiment Manager software.

4.2 Follow instructions in the Illumina Experiment Manager Guide to create sample sheet. A sample plate must be created as an intermediate step in creating a sample sheet.

4.3 In the sample plate wizard, paste the previously copied sample names into the Sample ID and Sample Name columns shown below.



4.4 Witness Step: Navigate to the plate tab in the sample plate wizard. Have a witness compare the sample layout in the sample plate wizard to the load plate screen in LIMS. The samples should be loaded in the same position in both LIMS and the sample plate wizard. Sample suffixes should match the position in which they are loaded.

4.5 On the sample sheet wizard – sample selection page ensure that every sample name has been transferred. A blank sample name at this step indicates that a special character (space, parenthesis, etc.) is present that the software will not accept. Return to step 3.4 and edit sample names.

4.6 Once the sample sheet is complete, save it to the MiSeqSequencing folder within the LIMS to Instrument folder.

4.7 Transfer a copy of the sample sheet to the instrument.

5 Creating the Library pool

5.1 Retrieve a single 1.5 mL microcentrifuge tube and label it with the run name followed by the word “pool.”

5.2 Add 4 µL of each library to the microcentrifuge tube, including positive and negative controls. Pooled libraries may include both normalized/re-normalized libraries and purified libraries with concentrations less than 4 nM.

5.3 The tube containing the pooled libraries may be stored at -20 °C freezer. Seal the plate containing the normalized libraries and store at -20 °C freezer.

6 Preparation

6.1 Retrieve the library pool for your run.

6.2 Retrieve the following reagents and allow them to equilibrate to room temperature.

MiSeq Reagent Cartridge
HT1 Buffer
Incorporation Buffer (PR2)
Flow Cell
PhiX Sequencing Control (20pM or 10nM)
0.2N NaOH
10mM Tris-HCl + 0.1% Tween™ 20 (pH 8.5)

6.3 Log all reagent lot numbers in LIMS as appropriate.

6.4 Note the flow cell ID and the MiSeq Reagent Cartridge barcode number in LIMS.

6.5 Write the run name on the lid of the flow cell canister.

6.6 When handling the MiSeq Reagent Cartridge, do not put pressure on the bottom of the cartridge. Always handle the cartridge by its sides.

6.7 Remove the HT1 buffer from its packaging and place it in the freezer.

6.8 Fill a small container with DI water. Place the MiSeq Reagent Cartridge into the container to thaw. The water line should not rise above the marked “Max Water Line” on the cartridge. Thawing should take ~1hr to complete.

Note: Before thawing the MiSeq Reagent Cartridge confirm that a passing system check was performed. Once thawed, the MiSeq Reagent Cartridge may be kept at 4 °C for a MAXIMUM of 6 hours prior to loading.

6.9 Retrieve two (2) 1.5 mL microcentrifuge tubes and label one for denaturation and the other for the library loading solution on the instrument.

6.10 Determine whether 20pM PhiX Sequencing Control is available. If not, prepare 20pM PhiX Sequencing Control following the instructions below. This may be performed concurrently with the denaturation of the library pool.

6.10.1 Retrieve and label a 1.5 mL microcentrifuge tube. Include your initials and the date of creation.

6.10.2 Retrieve and thaw HT1 buffer. It should be thawed, but VERY cold. Keep at 4°C, or -20°C until ready for use.

6.10.3 Into the labeled tube, add the appropriate volumes of the following:

Preparing 20 pM PhiX Control	
10nM PhiX Control	2.0 µL
10mM Tris-HCl + 0.1% Tween™ 20 (pH 8.5)	3.0 µL
0.2N NaOH	5.0 µL

6.10.4 Mix the PhiX thoroughly.

6.10.5 Incubate the PhiX mix for 5 minutes at room temperature.

6.10.6 Add 990 µL of chilled HT1 buffer to the PhiX mix.

6.10.7 The 20 pM PhiX Control solution may be stored at –20°C for up to 2 weeks.

7 Procedure

7.1 Denature the library pool:

7.1.1 Retrieve the HT1 buffer and thaw. It should be thawed, but VERY cold. Keep at 4 °C until ready for use.

7.1.2 Retrieve the previously prepared denaturation 1.5 mL microcentrifuge tube and add the following to denature the library pool.

Denaturing Library Pool	
Library Pool	5.0 µL
0.2N NaOH	5.0 µL

7.1.3 Mix thoroughly.

7.1.4 Incubate the libraries for 5 minutes at room temperature.

7.1.5 Add 990 µL of chilled HT1 buffer to the denatured library pool.

7.2 Prepare Final Sequencing Solution:

7.2.1 Retrieve the previously prepared 1.5 mL microcentrifuge tube for the library loading solution and add the following to denature the library pool.

Preparing Final Sequencing Solution	
HT1 buffer	225 µL
Denatured library pool	337.5 µL
20 pM PhiX control	37.5 µL

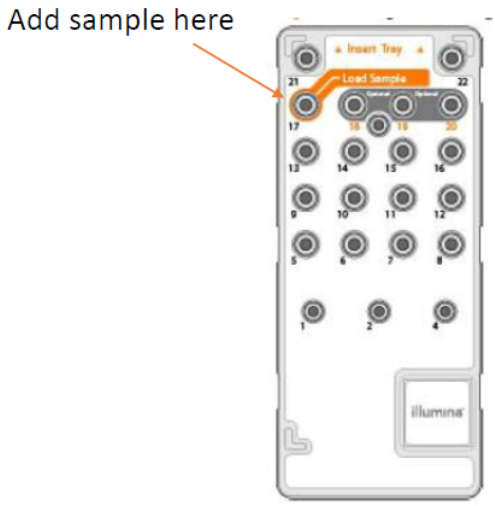
7.3 Prepare MiSeq Reagent Cartridge:

7.3.1 Dry the outside of the MiSeq Reagent Cartridge by wiping with lint free wipes.

7.3.2 Mix cartridge by inverting 10 times.

7.3.3 Wipe the foil over the well labeled "Load Sample" (position 17). Pierce with a clean 1000 µL pipette tip.

7.4 Load 600 µL of the Final Sequencing Solution to the well labeled "Load Sample" (position 17) on the cartridge. Be sure to avoid touching the edges of the well with the pipet tip.



8 MiSeq/MiSeq FGx Operation for Sequencing

8.1 Log in, if necessary.

8.2 On the home screen, select *Sequence*.

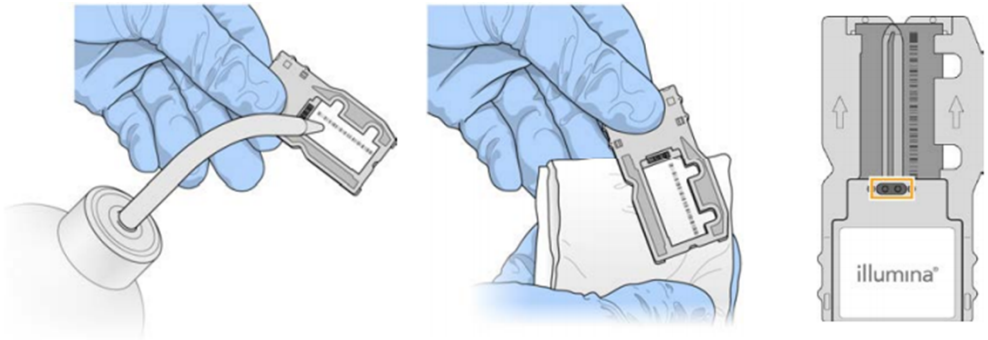
8.3 When prompted to select run type, click *Research Use Only*.

8.4 Ensure 'Use BaseSpace for storage and analysis' is **unchecked**. Click *Next*.

8.5 Follow the on-screen instructions to place the required components on the instrument in accordance with the steps below.

8.6 Retrieve the flow cell. Remove it from its container and rinse with distilled water.

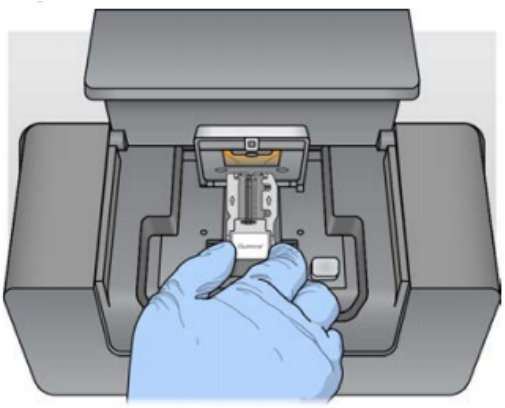
8.7 Dry the flow cell with a lint-free wipe. Avoid the gaskets when drying the flow cell.



8.8 Open the flow cell compartment lid and the flow cell compartment door. Hold the compartment door as it opens to prevent the door from hitting against the instrument.

8.9 If a flow cell is present on the instrument, remove the old flow cell and store in the newly opened container.

8.10 Place the flow cell on the flow cell stage and close the flow cell door and lid.



8.11 Ensure the software identifies the flow cell RFID. Click *Next*.

8.12 Retrieve the PR2 bottle and invert the bottle to mix.

8.13 Open the reagent door, raise the sippers, and carefully remove the wash bottle from the instrument, and pour any remaining wash buffer down the drain.

8.14 Open the PR2 bottle and place it on the instrument replacing the wash bottle.

8.15 Empty the waste bottle into the MiSeq Reagent Waste Container near the instrument.

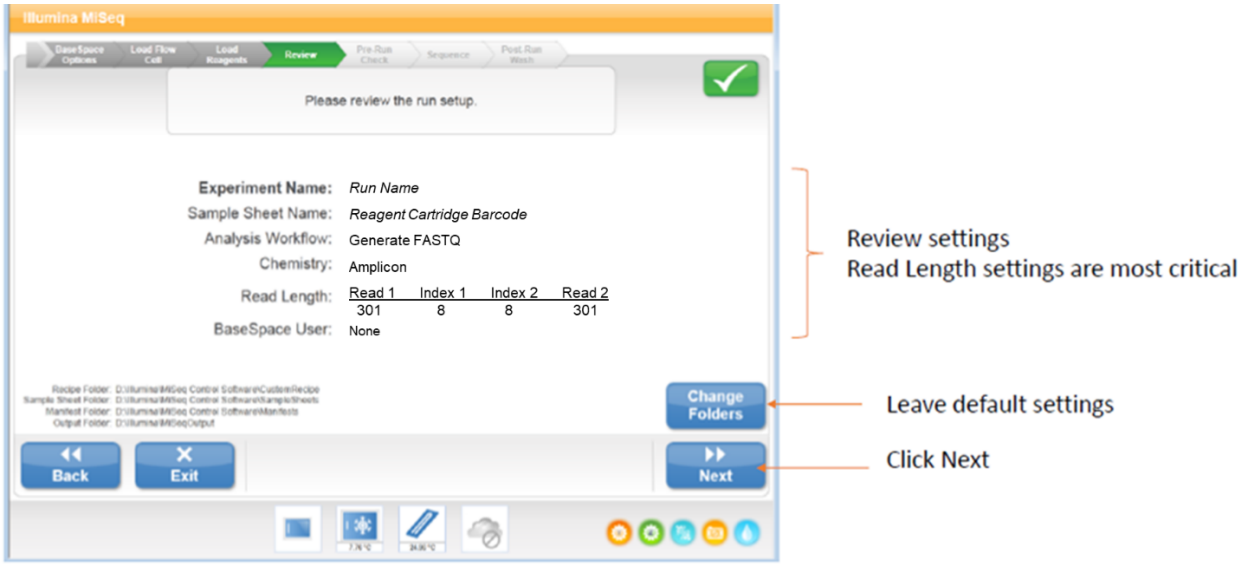
8.16 Replace the waste bottle and lower the sippers. Click *Next*.

8.17 Carefully open the chiller door and remove the wash tray. Empty any remaining wash buffer down the drain. Using a lint free wipe, soak up any wash buffer that may have spilled into the chiller compartment.

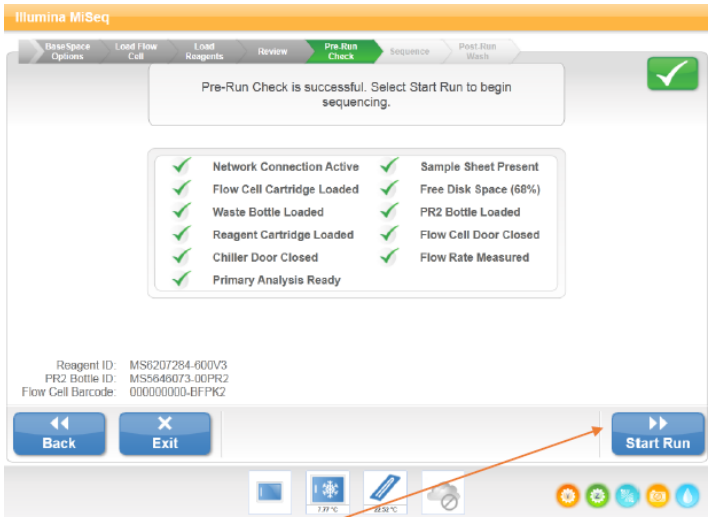
8.18 Carefully place the MiSeq Reagent Cartridge into the chiller with the barcode facing outwards. Close the chiller door.

8.19 Make sure to click *Change Sample Sheet* and select the sample sheet for the experiment. The file name should match the barcode of the cartridge. Click *Next*.

8.20 Review the run setup to ensure all entered information is correct, especially read length settings. Click *Next*.

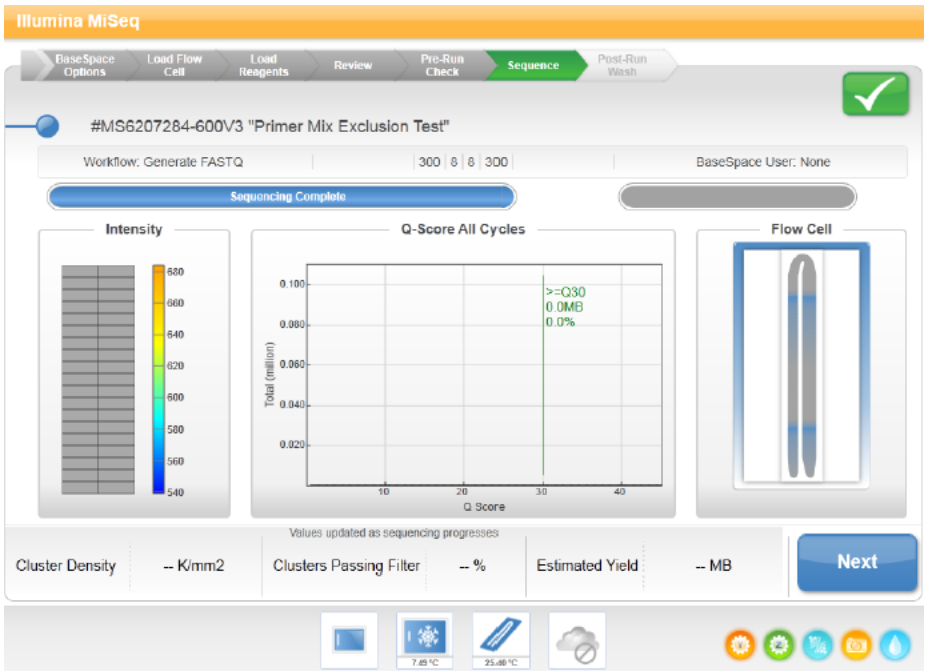


8.21 Once the pre-run is complete, click “Start Run”. Each experimental run takes approximately 56 hours.



8.22 The MiSeq is sensitive to vibration. Touching the instrument after starting a run could adversely affect sequencing results. After selecting Start Run, do not open the flow cell compartment or the reagent compartment doors, or touch the instrument monitor except to pause the run. Make sure to close all files on the MiSeq before starting a run, and do not open files during a run.

9 Collecting Data and Evaluation Run Quality



9.1 Once it is indicated that the sequencing run is complete, evaluate the quality metrics for the run reported by the MiSeq Control software.

9.1.1 Cluster Density is a value that reflects the average number of clusters found per mm2 of the flow cell surface. Cluster density for the run should ideally be between 659-1370 k/mm2.

9.1.1.1 Runs that exceed this range should be interpreted with caution, as high cluster density may lead to misassignment of calls during sequencing.

9.1.1.2 Runs that yield a cluster density below 659 should also be interpreted with caution, and this may be caused by degradation or otherwise poor-quality template.

9.1.2 The Cluster Passing filter indicates the percentage of clusters that pass Illumina's "chastity" filter, defined as the ratio of the brightest base intensity divided by the sum of the brightest and second-brightest base intensities. Passing threshold is no more than 1 base call having a "chastity" value below 0.6 in the first 25 cycles, removing the least-reliable clusters from the results. (See required readings for Illumina (2015).)

9.1.2.1 For runs reporting a cluster passing filter value below 43.6%, data should be interpreted with care, as this may be an indication of poor-quality template.

9.1.3 Error probability (Q30) is the percentage of bases that have a quality score >30, (i.e., 1 base call predicted to be incorrect out of every 1000). This probability is calculated after cycle 25.

9.1.3.1 In practice, robust data may yield a Q30 value as high as 68.1%. For runs yielding Q30 values lower than 34.4%, data should be interpreted with care, as this may be an indication of poor-quality template.

9.2 Once the run quality metrics have been evaluated and recorded, click *Next* to proceed to the post-run wash.

9.3 Locate the data files from your run on the instrument and transfer them so they are available for analysis.

10 Post-Run Wash

10.1 A Post-Run wash must be performed to complete a sequencing run.

10.2 Discard all waste in the MiSeq waste container into a hazardous waste container designated for formamide containing waste.

10.3 The used flow cell should remain on the instrument.

10.4 The used cartridge should be discarded. The MiSeq sample tube in position 8 of the cartridge should be placed into a hazardous waste container designated for formamide containing waste.

10.5 Retrieve the following reagents.

Laboratory-grade water
Tween™ 20
5.25% NaOCl

10.6 Retrieve the wash tray and wash bottle.

10.7 Prepare 0.01% NaOCl solution:

10.7.1 Retrieve and label two (2) 1.5-mL microcentrifuge tubes. Label one for 5% bleach and the other for 1:25 bleach.

10.7.2 Add 40 µL of bleach to the 5% tube.

10.7.3 Add 2 µL of laboratory-grade water to the 5% tube to form a 5% bleach solution.

10.7.4 Add 36 µL of the 5% bleach to 864 µL of laboratory-grade water to form a 1:25 bleach solution.

10.7.5 Retrieve a MiSeq sample tube.

10.7.6 Add 50 µL of the 1:25 bleach solution to 950 µL of laboratory-grade water in the MiSeq sample tube.

10.8 Place the MiSeq sample tube in position 17 of the wash tray.

10.9 Prepare MiSeq Wash Solution according to the QA reagent sheet MiSeq/MiSeq FGx Wash Solution.

10.10 Add wash solution to each well of the wash tray (except for position 17) to fill completely. Approximately 6 mL must be added to fill.

10.11 Add 350 ml of wash solution to the 500 mL MiSeq wash bottle.

10.12 Place the wash tray and wash bottle on the instrument as shown on the instrument screen.

10.13 Follow the instructions on the MiSeq screen to perform the post-run wash by clicking *NEXT*.

10.14 The post-run wash takes ~20 minutes to run. The flow cell, wash bottle, and wash tray must remain on the instrument until the next run.