Illumina MiSeq Loading and Operation

Materials

Equipment:
→ Illumina MiSeq sequencer
→ P20 Pipette
→ P200 Pipette
→ P1000 Pipette
→ Vortex
→ Mini centrifuge
→ Water bath

Consumables:
→ Sterile filter pipette tips: P20, P200, P1000
→ 1.5 mL microcentrifuge tubes
→ RNase-free screw-cap cryotubes
→ Lint-free tissue wipes

Reagents:
→ Illumina MiSeq Reagent Kit
→ Nuclease-free water
→ Laboratory-grade water
→ Tween 20
→ NaOH (pellets or aqueous solution)

Sample Sheet Creation

A sample sheet is used to tell the Illumina MiSeq which barcodes were added to which samples. This information will be used in the demultiplexing process that happens on the Miseq machine. Sample sheets can be created on the instrument itself using the Illumina Experiment Manager software, or using the Illumina MiSeq Sample Sheet Template. If you use the sample sheet template to create your sample sheet, edit it in a spreadsheet software such as Excel and transfer the completed sample sheet to the MiSeq using a USB drive.

The fields you will have to complete in your sample sheet template are explained below:

[Header]

- **Experiment Name**: Enter a unique experiment name for your run (e.g., “20220530_SC2_batch1”)
- **Date**: Enter the run date in the format MM/DD/YY
- **Workflow**: There are several different workflows you can choose from. If you are sequencing a pool of multiple indexed samples and want to perform demultiplexing on the machine, enter “GenerateFASTQ”.
- **Chemistry**: For any workflows that use dual indexing, such as Nextera and TruSeq Custom Amplicon, the chemistry field is required and should be set to “amplicon”. Otherwise, leave this field blank.

[Reads]:

- Enter the forward and reverse read lengths in the rows below “[Reads]”. For paired end sequencing, the read length in each direction is the number of cycles indicated on the MiSeq kit you used, divided by two, plus one. In other words, the forward and reverse read lengths are 151 for a 300-cycle kit.

[Data]

- **Sample_ID**: *Required.* List the sample IDs for all sequences included on this sequencing run. These can be as simple as 1, 2, 3, etc. Every sample must have a unique sample ID.
- **Sample_Name**: *Required.* List the same names for all sequences included on this sequencing run. These will be used to name output files, so they must be unique.
- **Index**: *Required.* Enter the name of the i7 index used on the corresponding sample.
- **I7_index_ID**: *Optional.* Enter the name of the i7 index used on the corresponding sample. If you do not know the name of the index, you can enter the index sequence again instead.
- **index2**: *Required.* Enter the name of the i5 index used on the corresponding sample.
- **I5_index_ID**: *Optional.* Enter the name of the i5 index used on the corresponding sample. If you do not know the name of the index, you can enter the index sequence again instead.
- **Sample_Project**: *Optional.* Field where you can indicate the name of the sample project. Can be left blank.
- **Description**: *Optional.* Field where you can provide additional information. Can be left blank.

Reagent Preparation

Equipment:
→ Water bath
→ Ice bucket

Reagents:
→ MiSeq Reagent Kit

Protocol:

1. Miseq reagents need to be thawed before sequencing. Remove the reagent kit from the -20°C freezer and thaw it in a water bath using room temperature water for approximately 30 minutes. If you do not have a water bath, an ice box or other water-tight container with room temperature water works just as well. Ensure that the water level does not exceed the “Fill Line” indicated on the cartridge.

   Note: Remove the hybridization buffer (HT1 buffer) from the reagent box before discarding and thaw on the bench. Place the buffer on ice after thawing.

2. Once the reagent cartridge is thawed, place it on ice or in the 4°C refrigerator until use. Use the cartridge within 24 hours of thawing.

3. Keep the hybridization buffer on ice. This will be used to dilute the sample at a later step.

Sodium Hydroxide Preparation

Please note that NaOH is a caustic reagent, which can cause chemical burns. Use proper protective equipment when handling it in both pellet and solution form.

Equipment and Consumables:
→ P20 pipette, P200 pipette
→ 50 mL conical tube
→ 1.5 mL Eppendorf tube
→ Pipette tips: P20, P200

Reagents:
→ NaOH (sodium hydroxide) in pellet or liquid form
→ Nuclease-free water
→ Laboratory-grade water

OPTION A: Prepare NaOH from Pellets

1. Prepare 4M (=4N) sodium hydroxide (NaOH) solution. The 4M solution can be used for up to 3 months after initial preparation. If a fresh dilution is required, prepare it at least 1 day prior to sequencing.
    a. Weigh 8g of NaOH pellets and transfer into a 50 mL conical tube.
    b. Slowly add 35 mL of laboratory-grade water to the tube. Please note that this reaction is exothermic and the tube will become warm.
    c. Add an additional 15 mL of water and invert the tube to mix.
2. Let the 4M solution rest overnight before using. Write the date prepared on the tube.
3. Prepare a fresh 0.2M (=0.2N) NaOH solution that will be used to denature the DNA library pool:

Component	Volume
4 M NaOH	5 µL
Nuclease-free water	95 µL
Total Volume	**100 µL**

OPTION B: Prepare NaOH from 2N Stock Solution

1. Prepare fresh 0.2M (=0.2N) NaOH solution that will be used to denature the DNA library pool:

Component	Volume
2 M NaOH	10 µL
Nuclease-free water	90 µL
Total Volume	**100 µL**

Sample Preparation

Follow the appropriate sample preparation protocol depending on the final concentration of the final library pool. Use the MiSeq Loading Quicksheet (download as .xlsx file) for ease of preparing sample dilutions.

Equipment and Consumables:
→ P20 pipette, P200 pipette
→ Vortex
→ 1.5 mL Eppendorf tube
→ Pipette tips: P20, P200

Reagents:
→ Final pooled DNA library
→ Illumina MiSeq Hybridization Buffer (from MiSeq Reagent Kit)
→ Nuclease-free water
→ 0.2N NaOH
→ Elution buffer (e.g., from a Qiagen extraction kit)

If your final library pool is 4nM or greater:

1. Dilute the library pool to 4 nM with Elution Buffer using the (C1)*(V1)=(C2)*(V2) formula.
2. Aliquot 5 µL of the 4 nM dilution into a new 1.5 mL tube.
3. Add 5 µL of 0.2 N NaOH to the 5 µL of diluted library pool. Vortex, spin, and let stand at room temperature for 5 minutes to denature the DNA in the sample.

   Note: Denatured library pool concentration is now at 2 nM.
4. Add 990 µL of Hybridization Buffer to the 2 nM denatured library pool. Mix by inversion and spin.

   Note: DNA in the pooled library is now single-stranded and at concentration of 20 pM.
5. Dilute to the final loading concentration of 10 pM (adjust as needed) as follows:

Component	Volume
Hybridization buffer	300 µL
Library at 20 pM	300 µL
Total Volume	**600 µL**

   Note: Mix the sample by inversion. Spin down and place on ice until use.

If your final library pool is 1.2 – 3.9nM:

1. Add 5 µL of your pooled library to a 1.5 mL tube.

2. Add 5 µL of 0.2 N NaOH to the 5 µL of the library pool. Vortex, spin, and let stand at room temperature for 5 minutes to denature the DNA in the sample.

3. Determine the amount of hybridization buffer to add to achieve a 10pM final loading concentration using the (C1)*(V1)=(C2)*(V2) formula.

   Worked example:

   After Step 2, a 1.2 nM library will be at 0.6 nM or 600 pM.

   (600 pM pool) * (10 µL of library) = (10 pM final concentration) * (X µL of hybridization buffer)

   (6000) = (10 pM final concentration) * (X µL of hybridization buffer)

   (X µL of hybridization buffer) = (6000) / (10 pM final concentration)

   X µL of hybridization buffer = 600 µL

   In this example, 10 µL of 600 pM DNA Library should be added to 590 µL of hybridization buffer.

   This will make the 10 pM final concentration (total volume: 600 µL).

4. Add the calculated amount of hybridization buffer to the denatured library pool to achieve the 10pM loading concentration used above:

Component	**Volume**
Hybridization buffer	Use formula
Denatured library	10 µL
Total Volume	**600 µL**

Note: Mix the sample by inversion. Spin down and place on ice until use.

If your final library pool is less than 1.2 nM:

1. This library pool may be too low in concentration to produce good sequencing results. If you wish to proceed, use the MiSeq Loading Quicksheet (download as .xlsx file) to calculate the appropriate dilution.

Machine Washing and Preparation

It is important to make sure that the fluidics lines of the MiSeq are thoroughly flushed between sequencing runs to prevent any precipitate accumulation. It is also important that the fluidics systems are well maintained when the instrument is idle. The MiSeq must be washed with 0.5% Tween 20 in laboratory-grade water before and after **every** use. The software will prompt you to perform the post-run wash after the run is complete and not allow another run to start unless a wash has been performed. You will have to initiate a wash manually before every run.

Equipment:
→ Illumina MiSeq sequencer
→ MiSeq wash tray
→ MiSeq wash bottle

Reagents:
→ Laboratory-grade water
→ Tween 20

Protocol:

1. To prepare a 10% wash stock, add 5 mL 100% Tween 20 to 45 mL laboratory-grade water. This results in 10% Tween 20. Save this stock solution for future use.
2. To prepare 0.5% Tween 20, add 25 mL of the 10% Tween 20 to 475 mL laboratory-grade water. Invert several times to mix.
3. Open the MiSeq Control Software and select the *Wash Instrument* icon. Then select *Post-Run Wash.*
4. Verify that a flow cell is in place.

   Note: DO NOT RUN ANY WASH WITHOUT A FLOW CELL. The flow cell ensures that there is a vacuum present inside of the reagent lines. This vacuum keeps air bubbles out of the machine fluidics.
5. The machine will prompt you to fill the MiSeq wash tray with the MiSeq Wash Solution (0.5% Tween Solution).
   a. Open the reagent chiller door and check the volume of the wash bottle. Fill the bottle at least half way if it is at a low level. **Note:** Lift the sipper all the way up when removing and replacing the bottle.
   b. Dispose liquid in the waste bottle.
   c. Open the chiller door to remove the wash cartridge.
   d. Fill each well of the wash tray with wash solution and place it back into the instrument. There is no need to completely fill every well, 1-5 mm of headroom is recommended.
   e. Close both chiller doors ensuring that the PR2 sipper has been placed in the lowered position.

6. Select *Start Wash* to begin the wash.
7. Leave all wash components in place until the next wash or sequencing run is started.
8. Once a month, perform a Maintenance wash on the machine. This wash is a more thorough wash, which performs multiple rinses of the fluidics and takes around 90 minutes. The set-up for this wash is the same as for a post-run wash. You will need to return to the machine twice during this wash to refill the wash tray and wash bottle and start the next step. If the Maintenance wash is overdue, the MiSeq will not start a new run until this is performed.
9. If you have no plans to use the MiSeq for a significant period of time, perform a standby wash. This will put the MiSeq into a standby state. Repeat the standby wash for every 30 days the machine is idle. You will need to perform a Maintenance wash before launching the next sequencing run.

Note: If you notice evidence of contamination between runs you can consider performing a template line wash as part of the post-run wash. For full details on this see page 30 of the MiSeq System Guide.

Cleaning the Flow Cell and Loading the Reagent Cartridge

Once the final library is diluted to loading concentration, clean flow cell and load pool into MiSeq cartridge.

Equipment:
→ Sink or other receptacle to collect water
→ P1000 pipette

Consumables:
→ P1000 pipette tips
→ Squirt bottle filled with distilled water or 1 mL pipette and bottle of laboratory-grade water
→ Kim wipes or other lint free tissue

Reagents:
→ MiSeq reagent cartridge

Protocol:

1. Invert the MiSeq reagent cartridge and visually verify that all reagent wells have completely thawed.
2. Tap the cartridge on the counter to settle all reagents to the bottom of the kit.
3. Using a 1 mL pipette tip, pierce the foil for position 17 (surrounded by an orange circle). Discard the pipette tip after use.
4. Add 600 µL of the 10 pM library pool to the hole created in the previous step. Avoid touching the pierced foil with the tip of the pipette, and avoid dispensing onto the walls of the well.
5. Remove the flow cell from the 4°C refrigerator and carefully take it out of the container.

   Note: Save the flow cell container and remaining hybridization buffer in case there is a delay in run start and the flow cell needs to be stored again.
6. Over a sink or paper towels, rinse the flow cell thoroughly with laboratory-grade water.
7. Blot the flow cell dry with lint-free tissue wipes verifying that the flow cell is completely dry and free of smudges or blemishes. Avoiding touching the two black port holes at the top of the flow cell.
8. *Optional.* Wipe flow cell glass with alcohol wipe. Then blot dry with lint-free tissue wipes verifying that the flow cell is completely dry and free of smudges or blemishes.
9. Immediately proceed with loading the MiSeq. Do not let the kit sit for long periods with the sample inside.

Starting a Sequencing Run

Equipment:
→ Illumina MiSeq sequencer

Reagents:
→ MiSeq reagent cartridge with library added
→ Clean and dry flow cell
→ PR2 buffer

Protocol:

1. Before the run, power cycle the MiSeq. From the Home screen, select Manage Instrument then Shut Down. Toggle the power switch to the OFF positions and wait at least 60 seconds. Toggle the power switch back to the ON position, and wait for the machine to boot up.
2. Additionally, before the run, confirm that a sample sheet has been created for the pool.
3. Transport the loaded MiSeq cartridge, flow cell, and PR2 buffer to the MiSeq.
4. On the home screen, select *Sequence*.
5. The MiSeq will prompt for the flow cell to be loaded.
    a. Clean the flow cell as described in **Cleaning the Flow Cell and Loading the Reagent Cartridge** above.
    b. Open the flow cell holder door, and press the white button to release the used flow cell that is being held in place.
    c. Place the new, cleaned flow cell into the flow cell holder. The black gaskets will be facing upwards and the notch on the flow cell should be on the right-hand side.
    d. Gently push the flow cell to the back of its slot while you close the flow cell holder and the flow cell holder door.
    e. Select *Next* once the flow cell RFID is identified.
6. The MiSeq will now prompt that the reagents be put into place and a sample sheet be selected.
    a. Open the large chiller door and the small reagent chiller door.
    b. Lift the PR2 sipper and remove the bottle of wash buffer.
    c. Empty the MiSeq waste container. **Note:** MiSeq waste contains trace amounts of formamide and should be disposed of properly using your institution’s hazardous waste procedure though usually it's safe to dispose of in the sink.

d. Uncap the new bottle of PR2 buffer and place it into the MiSeq. **Note:** Place the cap from the new bottle of buffer onto the wash bottle to keep it clean between washes.

e. Lower the PR2 sipper.

f. Remove the wash cartridge from the small reagent chiller and replace it with the loaded MiSeq reagent cartridge. **Note:** Dump the remaining wash solution in the sink and let the wash cartridge dry before reusing.

g. Close the small reagent chiller and the larger chiller door.

7. Select the Sample Sheet from the location that it was saved in.

8. Select *Load Sample Sheet* and confirm that the run and cycle parameters match what is desired.

9. Select *Start Flow Check* to perform the pre-run fluidics check. This will take approximately five minutes.

10. Select *Start* when the flow check has completed.

    a. If the flow check fails, remove the flow cell and clean it again.

    b. If the flow check fails a second time, perform a volume test.

    Note: A MiSeq 2 x 150 V2 run will take approximately 24 hours to complete. Important run statistics will be available after approximately 1 hour. Clusters passing filter should be less than 1000 k/mm^2 for V2 chemistry. If higher, consider stopping the run and starting another run with the loading library concentration decreased by 2 pM.

11. After completion, perform a post-wash run.