EMORY UNIVERSITY | Emory Integrated Genomics Core

EIGC.011_16S Microbiome Collection through Sequencing

Standard Operating Procedure Staff Review Page:

I have read and understand the procedure listed above.

Employee name	Date SOP review complete

Standard Operating Procedure Approval Page:

Date Implemented: 16 June 2016

Annual Review and Approval

Michael Zwick, PhD
Laboratory Director

Changes Made:
110719 – Created “working stock” reagents, Added controls and negatives.

Note: No laboratory personnel should use this protocol without proper training by the laboratory supervisor or other designated person.

Safety: All employees are required to follow universal safety protocols when working with potentially infectious biohazardous materials. Safety procedures are outlined in the Emory University Bloodborne Pathogen Exposure Control Plan (see Employee Safety Notebook).

Notes before Starting:

Include the following controls:

- **Positive Process (plasmid):** Evaluated after extraction and after library prep. Verifies extraction and library prep are working. Positive results are detectable levels of DNA and appropriately sized library. Negative results indicate failure at extraction or amplification.

- **Negative Process (no sample):** Evaluated after extraction and after library prep. Represents background levels of contamination, should be undetectable. If detected at QC, or if library trace is positive, indicates potential contamination or sample mix-up.

- **Positive Control (Zymo):** Evaluated after library prep. Verifies PCR was successful. Positive results are detectable levels of DNA and appropriately sized library. Negative results indicate failure in library prep reagents or workflow.

- **NTC (water):** Evaluated after library prep. Standard water control for PCR. Should be undetectable with no library product visible. Positive result indicates potential contamination or sample mix-up

For computation, each MiSeq run needs to have at least of each control.

Run PhiX at 20% to increase library diversity

Collection:

Please follow EIGC.011_Appendix_A, The Human Microbiome Project Core Microbiome Sampling Protocol A (HMP Protocol #07-001). Please note that the MoBio kit and tubes are now the Qiagen DNeasy Powersoil kit and tubes.

Required Reagents and Consumables:

Qiagen PowerBead Tubes

Catch-All™ Specimen Collection Swab

Labels for tubes

Procedural Steps:

1. **All soft tissue sites are sampled using Catch-All™ Sample Collection Swabs.**

2. **Immediately after swabbing, each swab must be placed in the 750 µL of buffer in the PowerBead tube.**

3. **The swab handle should be snapped in half to allow the lid to be screwed onto the tube.**

4. **The PowerBead tube + swab should be kept frozen until processing.**

5. **When ready to process, thaw the tube at room temperature, then proceed with the protocol as written with the swab still inside the tube.**

6. **All other non-swab samples can be added directly to the PowerBead tube.**

Extraction:

Please follow EIGC.011_Appendix_C, The DNeasy PowerSoil Kit Handbook.

Required Reagents and Consumables:

- Qiagen DNeasy PowerSoil Kit
- Tissue Lyser or a Vortex Adapter
- Centrifuge
- Ice

Notes Before Starting:

- These steps are to be performed in PRE.
- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- 2 ml collection tubes are provided.
- **USE STOCK SOLUTIONS/ WORKING STOCK SOP FOR ALL BUFFERS TO PREVENT CONTAMINATION.**
- **SCAN ALL REAGENTS FROM POWERSOIL KIT INTO PROJECT SUMMARY SHEET.**
- **WHEN USING MULTI-DISPENSE TECHNIQUE, ENSURE SOLUTIONS DO NOT SPLASH CAUSING CONTAMINATION.**

Procedural Steps:

1. **Prep tubes and reagents for extraction.**
   a. Label PowerBead Tube with sample number. Add 60 µl of Solution C1 to the. PowerBead Tube.
   b. Label a 2 mL collection tube with the sample number, and add 250 µl Solution C2 to the tube.
   c. Label a 2 mL collection tube with the sample number, and add 200 µl Solution C3 to the tube.
   d. Label a 2 mL collection tube with the sample number. Shake Solution C4, and add 1200 µl Solution C4 to the tube.
   e. Insert a MB Spin Column into a 1.5 mL Collection Tube, and label the spin column lid with the sample number.
   f. Label a 1.5 mL tube with the sample number and attach a LIMS barcode. This tube will be used for final elution.

2. **Add 0.25 g of soil sample to the labeled PowerBead Tube containing 60 µl of Solution C1. If the original sample is a swab already in the PowerBead tube, add 60 uL of Solution C1 to the PowerBead tube containing the swab. Gently vortex to mix. Change gloves to prevent carry-over of any sample to other tubes.**

3. **Secure PowerBead Tubes horizontally using a Tissue lyser.**

4. **Shake tubes in Tissue lyser at maximum speed for 10 min, rotating tubes after 5 minutes.**

5. **Centrifuge tubes at 10,000 x g for 30 s.**

6. **Avoiding the debris, transfer the supernatant to the 2 mL tube containing Solution C2. Vortex for 5 s. Incubate at 2–8°C for 5 min.**

   Note: Expect between 400–500 µl of supernatant. Supernatant may still contain some soil particles.

7. **Centrifuge the tubes for 1 min at 10,000 x g.**

8. **Avoiding the pellet, transfer up to 600 µl of supernatant to the 2 mL tube containing Solution C3. Vortex briefly. Incubate at 2–8°C for 5 min.**

9. **Centrifuge the tubes for 1 min at 10,000 x g.**

10. **Avoiding the pellet, transfer up to 750 µl of supernatant to the 2 mL tube containing Solution C4. Vortex for 5 s or pipet mix the solution carefully.**

11. **Load 675 µl onto the MB Spin Column and centrifuge at 10,000 x g for 1 min. Discard flow through.**

12. **Repeat step 11 twice, until all of the sample has been processed.**

13. **Add 500 µl of Solution C5 to the column. Centrifuge for 30 s at 10,000 x g.**

14. **Discard the flow through. Centrifuge again for 1 min at 10,000 x g.**

15. **Carefully place the MB Spin Column into the pre-labeled 1.5 mL tube for elution. Avoid splashing any Solution C5 onto the column.**

16. **Add 100 µl of Solution C6 to the center of the white filter membrane.**

17. **Centrifuge at room temperature for 30 s at 10,000 x g. Discard the MB Spin Column.**

18. **The DNA is now ready for downstream applications.**

Normalize the DNA for Library Prep:

19. **QC the DNA using Quantit Broad Range kit and agarose gel and determine the amount of DNA to add to the plate to equal 12.5 ng of DNA.**

    Controls

    a. Positive Process Control: 12.5 ng of DNA is dried down and resuspended in 2.5 µl of water
    b. Negative Process Control: (from the DNA extraction, no sample was added, just the reagents from the kit were added). 5 µl of the elution is dried down and resuspended in 2.5 µl of water
    c. Zymo Control: ZymoBiomics, catalog D6305 DNA standard 200 ng in 20 µl (10 ng/µl), use 1.25 µl stock and add 1.25 µl water to make it a 2.5 µl total
    d. NTC- 2.5 µl of water is added to the well on the plate.

20. **Create normalization plate:**

    a. Transfer 12.5 ng of DNA for each sample into the appropriate well of the normalization plate.
    b. Speed vac dry the plate for 30-45 min, until the liquid evaporates.
    c. Add 2.5 µl of water to each well and seal the plate with a foil seal.
    d. Vortex plate.
    e. Centrifuge plate to collect sample.
    f. Store plate at room temp for 4 h-overnight.
    g. Vortex plate.
    h. Centrifuge plate to collect sample.
    i. Proceed to amplification PCR.

16S Metagenomic Sequencing Library Preparation

Please follow EIGC.011_Appendix_D, 16s-metagenomic-library-prep-guide-15044223-b.

Required Reagents and Consumables:

Nextera XT Index Primers (FC-131-1001 or FC-131-1002)
Amplicon PCR Reverse Primer (1 µM)
Amplicon PCR Forward Primer (1 µM)
2x KAPA HiFi HotStart ReadyMix
AMPure XP beads
Freshly Prepared 80% Ethanol (EtOH)
10 mM Tris pH 8.5
HT1 (Hybridization Buffer)
0.2 N NaOH (less than a week old)
PhiX Control Kit v3 (FC-110-3001)
MiSeq V3 600 cycle reagent cartridge

Amplicon PCR: These steps are to be performed in PRE.

21. **Set up the following reaction of DNA, 2x KAPA HiFi HotStart ReadyMix, and primers:**

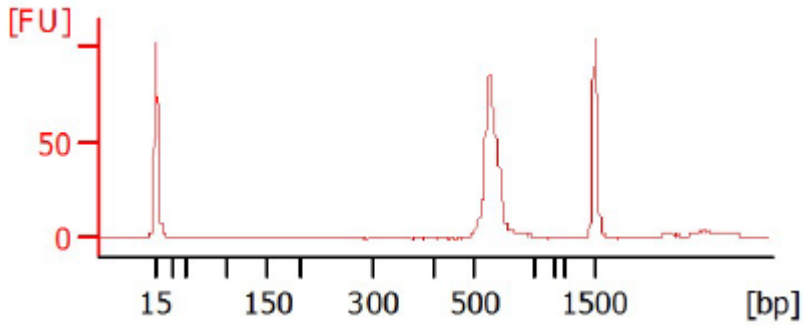
Reagent	**Volume (µl) per reaction**
DNA	2.5 µl at 5 ng/µl for 12.5 ng total
Amplicon Forward 1uM (be sure to dilute from 100 µM stock)	5
Amplicon Reverse 1uM (be sure to dilute from 100 µM stock)	5
2x KAPA HiFi HotStart ReadyMix	12.5
Total	**25**

Make 15% extra master mix for pipetting error.

22. **Seal plate, briefly vortex plate, and centrifuge the plate at 1,000 × g briefly. Perform PCR in a thermal cycler using the following program (BioRad Tetrad 2 machine: Run-> Illumina folder ->16S_PCR; 1 h, 18 min):**

    •95°C for 3 minutes
    • 25 cycles of:
    — 95°C for 30 seconds
    — 55°C for 30 seconds
    — 72°C for 30 seconds
    • 72°C for 5 minutes
    • Hold at 4°C

23. **Run 1 µl of the PCR product on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a bioanalyzer trace after the Amplicon PCR step is ~550 bp. Run all controls, and a selection of samples. Confirm that the samples and positive controls did amplify but the negative controls did not.**



PCR Clean-Up: These steps are to be performed in POST.

This step uses AMPure XP beads to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species.

Preparation

- Equilibrate the AMPure XP beads to room temperature.

Procedure

24. **Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.**

25. **Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough depending on the number of samples processing.**

26. **Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate. Change tips between columns.**

27. **Gently pipette entire volume up and down 10 times.**

28. **Incubate at room temperature without shaking for 5 minutes.**

29. **Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.**

30. **With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.**

31. **With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:**
    a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    b. Incubate the plate on the magnetic stand for 30 seconds.
    c. Carefully remove and discard the supernatant.

32. **With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:**
    a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    b. Incubate the plate on the magnetic stand for 30 seconds.
    c. Carefully remove and discard the supernatant.
    d. Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

33. **With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.**

34. **Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of water to each well of the Amplicon PCR plate.**

35. **Gently pipette mix up and down 10 times, changing tips after each column (or seal plate and shake at 1800 rpm for 2 minutes). Make sure that beads are fully resuspended.**

36. **Incubate at room temperature for 2 minutes**

37. **Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.**

38. **Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.**

Index PCR

Steps 1-7 are to be performed in PRE. Steps 8 and beyond are to be performed in POST.

For microbiome samples, >100,000 reads per sample is sufficient to fully survey the bacterial composition. This number of reads can allow for sample pooling for 96-192 libraries, given the MiSeq output of > 20 million reads. Using index sets A and B will allow for unique indexing for up to 192 samples.

This step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit.

Thaw Indexes at RT.

Reagent	**Volume µl per reaction**
DNA	5
Nextera XT Index Primer 1 (N7xx)	5
Nextera XT Index Primer 2 (S5xx)	5
2x KAPA HiFi HotStart ReadyMix	25
PCR Grade water	10
Total	**50**

Procedure

39. **Make a master mix of 2x KAPA HiFi HotStart ReadyMix and water.**

Reagent	**Volume µl per reaction**
2x KAPA HiFi HotStart ReadyMix	25
PCR Grade water	10

40. **Transfer 35 µl of the master mix to each sample well of a 96-well PCR plate.**

41. **Arrange the Index 1 and 2 primers in a rack (i.e. the TruSeq Index Plate Fixture) using the following arrangements as needed:**
    a. Arrange Index 2 primer tubes (white caps, clear solution) vertically, aligned with rows A through H.
    b. Arrange Index 1 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.

    ********For more information on index selection, see Dual Indexing Principle, on page 23.**

42. Place the 96-well PCR plate with the 35 µl of master mix into the TruSeq Index Plate Fixture. Take a picture of the index primers in the fixture, and post this to the PPMS folder for the project.

43. Add 5 µl of the correct Nextera XT Index Primer 1 (N7xx) to the appropriate wells.

44. Add 5 µl of the correct Nextera XT Index Primer 2 (S5xx) to the appropriate wells.

45. Place fresh caps onto the index primers. DO NOT reuse the caps to the primers.

46. Take the 96-well plate containing the PCR reaction mix into POST.

47. Using a multichannel pipette, transfer 5 µl of cleaned DNA from the Amplicon PCR plate into the 96-well plate containing the PCR reaction mix. Freeze the remaining 45 µl of Amplicon for future use if needed.

48. Cover the plate with a foil seal.

49. Vortex plate.

50. Centrifuge the plate at 1,000 × g at 20°C for 1 minute.

51. Perform PCR on a thermal cycler using the following program (Tetrad, reaction is about 30 min):

- 95°C for 3 minutes
- 8 cycles of:
— 95°C for 30 seconds
— 55°C for 30 seconds
— 72°C for 30 seconds
- 72°C for 5
- Hold at 4°C

PCR Clean-Up 2:

This step uses AMPure XP beads to clean up the final library before quantification

Procedure

52. **Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.**

53. **Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough.**

54. **Using a multichannel pipette, add 56 µl of AMPure XP beads to each well of the Index PCR plate.**

55. **Gently pipette mix up and down 10 times.**

56. **Incubate at room temperature without shaking for 5 minutes.**

57. **Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.**

58. **With the Index PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.**

59. **With the Index PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:**
    a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each\ sample well.
    b. Incubate the plate on the magnetic stand for 30 seconds.
    c. Carefully remove and discard the supernatant.

60. **With the Index PCR plate on the magnetic stand, perform a second ethanol wash as follows:**
    a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    b. Incubate the plate on the magnetic stand for 30 seconds.
    c. Carefully remove and discard the supernatant.
    d. Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

61. **With the Index PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.**

62. **Remove the Index PCR plate from the magnetic stand. Using a multichannel pipette, add 27.5 µl of water to each well of the Index PCR plate.**

63. **Gently pipette mix up and down 10 times until beads are fully resuspended, changing tips after each column.**

64. **Incubate at room temperature for 2 minutes.**

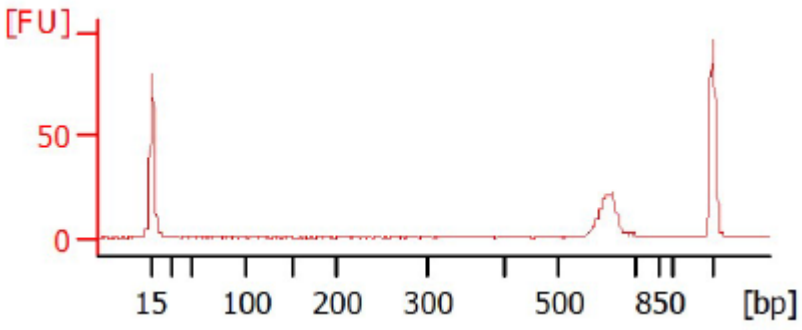
65. **Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.**

66. **Using a multichannel pipette, carefully transfer 25 µl of the supernatant from the Index PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.**

67. **Validate Library**

    a. Run 1 µl of the final library on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a Bioanalyzer trace of the final library is ~630 bp. Run all controls, and a selection of samples. Confirm that the samples and positive controls did amplify but the negative controls did not.

    b.

    c. 

SAFE STOPPING POINT

If you do not plan to proceed to Library Quantification, Normalization, and Pooling, on page 16, seal the plate with foil adhesive seal. Store the plate at -15° to -25°C for up to a week.

Library Quantification, Normalization, and Pooling

68. **Quantify libraries using Broad Range Quantit, and calculate the DNA concentration in ng/uL.**

69. **Dilute concentrated final library to ~10 nM using Normalization worksheet.**
    a. Normalize the libraries to the lowest concentration sample that had successful amplification.
    b. Refer to the Normalization worksheet for the amount of each library and water to add to the Library Normalization plate. Add appropriate amount of water to each well of the Library Normalization plate. Then, add indicated amount of each individual library. The final concentration of each individual library should be equal, and pipetting 1 µl from each well of the Library Normalization plate should result in equimolar pooling of the library
    c. Pipet 1 µl of diluted DNA from each library on the Library Normalization plate to pool libraries with unique indices.
        i. Using a multichannel pipet, pipet across rows to transfer 1 µl of diluted DNA from each well on the Library Normalization plate into an 8-tube PCR strip, and then
        ii. Combine the 8 PCR strip tubes into a 1.5 mL tube
    d. Add 5 µl of NTC, 5 µl of Negative Control(s) to the library pool

70. **Determine the final concentration of the library pool using KAPA qPCR.**

Library Denaturing and MiSeq Sample Loading

In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing. Each run must include 20% PhiX to serve as an internal control for these low diversity libraries. Illumina recommends using MiSeq v3 reagent kits for improved run metrics.

Preparation

71. **Set a heat block suitable for 1.5 ml microcentrifuge tubes to 96°C**

72. **Prepare the MiSeq sample sheet using Illumina Experiment Manager or using a previous order as a template.**

73. **Confirm that the MiSeq instrument has recently been washed. If not washed within a week, perform wash.**

74. **Check the disk space on the MiSeq instrument. The computer will need 40-50% free disk space. Moving files can take 4-5 hours, so you do not want to thaw reagents if you need to move files.**

75. **Confirm that the MiSeq has been power cycled within the past 2 weeks. Power cycle procedure: Shut down the software. Shut down the computer. Shut down the instrument, it will click, wait 1 min. Turn back on. Log back on immediately so the chiller will stay on. CNTR-ALT-DEL, "sbs123".**

76. **Confirm that the waste container has been emptied.**

77. **Make a fresh dilution of 0.2 N NaOH from 2 N stock.**

78. **Thaw HT1 solution at RT and then place on ice.**

79. **Thaw final library pool and PhiX on ice.**

80. **Remove a MiSeq reagent cartridge from -20°C storage and thaw in RT water bath.**

81. **Prepare an ice water bath.**

Denature DNA

82. **Combine the following volumes of pooled final DNA library and freshly diluted 0.2 N NaOH in a microcentrifuge tube:**
    a. 4 nM pooled library (5 µl)
    b. 0.2 N NaOH (5 µl)

83. **Vortex briefly to mix the sample solution, and then centrifuge the sample solution at 280 × g at 20°C for 1 minute.**

84. **Incubate for 5 minutes at room temperature to denature the DNA into single strands.**

85. **Add the following volume of pre-chilled HT1 to the tube containing denatured DNA. This dilution makes a final library concentration of 20 pM denatured library in 1 mM NaOH:**
    a. Denatured DNA (10 µl)
    b. Pre-chilled HT1 (990 µl)

86. **Place the denatured DNA on ice until you are ready to proceed to final dilution.**

Dilute Denatured DNA

87. Dilute the denatured DNA to the desired concentration using the following example:

NOTE: Illumina recommends targeting 800-1000 K/mm² raw cluster densities for 16S libraries using MiSeq v3 reagents (600 cycle kit for V3-V4 libraries). It is suggested to start your first run using a 6 pM loading concentration and adjust subsequent runs appropriately.

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
Denatured library	60 µl	120 µl	180 µl	240 µl	300 µl
Pre-chilled HT1	540 µl	480 µl	420 µl	360 µl	300 µl

88. Invert several times to mix and then pulse centrifuge the DNA solution.

89. Place the denatured and diluted DNA on ice.

Denaturation and Dilution of PhiX Control

Use the following instructions to denature and dilute the 10 nM PhiX library to 20 pM.

90. Combine the following volumes to dilute the PhiX library to 4 nM:

a. 10 nM PhiX library (2 µl)

b. 10 mM Tris pH 8.5 (3 µl)

91. Combine the following volumes of 4 nM PhiX and 0.2 N NaOH in a microcentrifuge tube:

a. 4 nM PhiX library (5 µl)

b. 0.2 N NaOH (5 µl)

92. Vortex briefly to mix the 2 nM PhiX library solution.

93. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.

94. Add the following volumes of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library:

a. Denatured PhiX library (10 µl)

b. Pre-chilled HT1 (990 µl)

95. Make aliquots of 100 µl, label with the date, and store at -20°C.

Combine Amplicon Library and PhiX Control

Use the following instructions to dilute the PhiX to the same loading concentration as the Amplicon library. The final library should contain 20% PhiX.

96. Remove 120 µl of denatured library pool and discard.

97. Add the following amount of PhiX and HT1 to the library pool based upon the final desired loading concentration:

Final Loading Concentration	Amount of PhiX to add	Amount of HT1 to add	Total Volume	Final PhiX percentage
4 nM	24 µl	96 µl	600 µl	20% (v)
6 nM	36 µl	84 µl	600 µl	20% (v)
7 nM	42 µl	78 µl	600 µl	20% (v)
8 nM	48 µl	72 µl	600 µl	20% (v)

98. Place the final library pool on ice until you are ready to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge.

99. Once the MiSeq reagents are ready to load the instrument, incubate the final library on a 96°C heat block for 2 minutes.

100. After the incubation, invert the tube 1–2 times to mix and immediately place in the ice water bath.

101. Keep the tube in the ice-water bath for 5 minutes. Centrifuge the tube briefly to collect liquid, and immediately place back on ice.

102. Use a fresh p1000 tip to pierce the foil of the well labeled "Load Sample" on the MiSeq reagent cartridge.

103. Using a fresh p1000 tip, load the denatured final library into the well of the MiSeq reagent cartridge labeled "Load Sample". Do not touch the tip to the remaining foil.

104. Insert reagent cartridge into machine.

105. Obtain the flow cell.

106. Prep the flow cell.

a. Rinse front and back with water.

b. Lightly dry with kimwipe.

c. Clean glass with 80% EtOH.

d. Dry with kimwipe, ensuring all liquid is removed from the plastic edges/borders.

107. Load the flow cell by opening the small box compartment. Push the white button to release the old flow cell. Throw away the old flow cell and insert the new flow cell. Gently push the hinge down to secure the flow cell.

108. Follow the instructions on the screen for a run. Requires an Illumina login.

109. Change the sample sheet to the one prepared in step 72.

110. Confirm that the run parameters are correct.

111. Start run.

112. You will need to select Start Run once the initiation steps are completed. Do not leave the instrument until you do this.

Table 1: MiSeq System performance parameters

Read length	Total time[a]	Output	Quality scores[b]	Single reads[c]	Paired-end reads[c]
MiSeq Reagent Kit v2					
2 × 25 bp	~5.5 hours	750-850 Mb	>90% bases higher than Q30	12-15 M	24-30 M
2 × 150 bp	~24 hours	4.5-5.1 Gb	>80% bases higher than Q30		
2 × 250 bp	~39 hours	7.5-8.5 Gb	>75% bases higher than Q30		
MiSeq Reagent Kit v3					
2 × 75 bp	~21 hours	3.3-3.8 Gb	>85% bases higher than Q30	22-25 M	44-50 M
2 × 300 bp	~56 hours	13.2-15 Gb	>70% bases higher than Q30		

QC of MiSeq after sequencing is finished:

113. **Review the run details in the Sequence Analysis Viewer.**

114. **See link for some helpful info on the parameters: https://support.illumina.com/content/dam/illumina-marketing/documents/products/other/MiSeq-overclustering-primer-770-2014-038.pdf**

115. **Review run Summary to confirm run is within spec.**

116. **Density: Optimal is 800-1000 K/mm^2 for 16S libraries with v3 reagents.**

117. **PhiX/% Aligned: (the percentage of the sample that aligned with PhiX). Should be close to the percent we added- 20%.**

118. **Clusters Passing Filter: Should be higher than 80%.**

119. **Review Indexing: You should see an entry for each of the samples that you entered on the sample sheet. The indexes should match what you added to each sample when you prepared the libraries.**

120. **The % Reads Identified (PF): Should be significantly higher for each experimental sample than for the NTC.**

121. **If one or two samples are very low compared to the other samples, check to make sure that the indexes are listed correctly, and check to see how the samples performed on Quantit for library pooling.**

122. **The NTC should be <0.05.**

123. **The Zymo should be similar to the sample level, and the Neg Process Control should be <0.05. The % Reads Identified (PF) should be similar between each experimental sample.**

124. **Transfer data: Use 7-Zip software to compress and post data to the PPMS ClientData or SequenceData project folder. Transfer a full data copy to the PPMS order folder.**

125. **Record the Density, PhiX, and Clusters Passing Filter information to the MiSeq prep sheet. Post a scan to PPMS order folder, and place the original sheet in the MiSeq notebook.**