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Working

# 16S Amplicon PCR for the V3-V4 region for the MicroCOPD samples

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dx.doi.org/10.17504/protocols.io.2sygefw

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ABSTRACT

This is the protocol for DNA extraction, PCR, and MiSeq sample preparation for the microbiome samples from sputum, and the bronchoscopies in the Bergen COPD Cohort study, Bergen COPD Exacerbation Study, the Bergen COPD Microbiome Study and the Bergen ILD Microbiome Study.

Slightly modified Illumina protocol for our low-biomass airways samples in the Bergen COPD & ILD studies.

The protocol that was the template for the PCR and sample preparation is:

**16S Metagenomic Sequencing Library Preparation**
Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System
Part # 15044223 Rev.B.

PDF

16s-metagenomic-library-prep-guide-15044223-b.pdf

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| HotStart ReadyMix (KAPA HiFi PCR kit) | KK2601 | Kapa Biosystems |
| Tris HCl Buffer 1M Solution, Sterile pH 8.5 | SD8141.SIZE.450ml | Bio Basic Inc. |
| PCR grade water | | |
| MicroAmp™ Clear Adhesive Film | View | Applied Biosystems |
| 16S Amplicon forward primer 10uM | View | Illumina, Inc. |
| 16S Amplion reverse primer 10uM | View | Illumina, Inc. |

## *DNA extraction*

1 Reagents:

| Reagent | Supplier | Catalog Number |
|---|---|---|
| Sputasol stock | Oxoid | SR0233 |
| Molecular grade H2O | Merck | H2OMB1001 |
| Lysozyme (10 mg/mL) | Sigma-Aldrich | L3790 - 10 x 1 mL |
| Mutanolysin (25,000 U/mL) | Sigma-Aldrich | M9901-50KU |
| Lysostaphin (4000 U/mL) | Sigma-Aldrich | L4402-5MG |
| FastDNA SPIN kit | MP Biomedicals | 6540-600 |

2 Take the samples out from the -80 °C freezer and leave to thaw at room temperature.

| Sample | Abbreviation | Volume for extraction (µl) |
|---|---|---|
| Negative control PBS | CTRL | 450 µl |
| Oral wash | OW | 1800 µl |
| Protected bronchoalveolar lavage | PBAL (PBAL1 & PBAL2) | 1800 µl |
| Protected sterile brush | PSB (rPSB & lPSB) | 450 µl |
| Small volume lavage | SVL | 1800 µl |

3 Vortex the samples for 10 seconds and spin down briefly.

4 Transfer 450 µL of the CTRL sample to a sterile 1.5 mL eppendorf tube.

5 Split the 1800 µL OW sample into three aliquots of 600 µL each and transfer to sterile 1.5 mL eppendorf tubes.

6 Split the 1800 µL PBAL sample into three aliquots of 600 µL each and transfer to sterile 1.5 mL eppendorf tubes.

7 Prepare a working solution of Sputasol according to the number of samples being processed:

| # of samples: | 7-8 | 9-12 | 13-16 | 17-20 | 21-24 |
|---|---|---|---|---|---|
| Sputasol stock (ml) | 0.750 | 1.125 | 1.500 | 1.875 | 2.250 |
| Molecular grade H2O (ml) | 9.250 | 13.875 | 18.500 | 23.125 | 27.750 |
| *Total (ml)* | *10* | *15* | *20* | *25* | *30* |

8 Add 450 µL of the Sputasol working solution to the CTRL sample prepared in step 4. Mix by vortexing for 5 seconds.

9 Add 600 µL of the Sputasol working solution to each of the three aliquots of OW sample prepared in step 5. Mix by vortexing for 5 seconds.

10 Add 600 µL of the Sputasol working solution to each of the three aliquots of PBAL sample prepared in step 6. Mix by vortexing for 5 seconds.

11 Incubate the samples on a thermoshaker at 37°C with shaking at 1000 rpm for 15 minutes.

12 To pellet the bacterial cells, centrifuge the samples at 15700 x g at room temperature for 8 minutes.

13 Discard the supernatant from each sample, taking care not to disturb the pellet.

14 Resuspend the pellet in the CTRL sample with 250 µL PBS.

15 Carefully pipette the entire volume up and down 10 times to mix.

16 Resuspend and combine the three pellets from the OW sample into one eppendorf tube in a total of 250 µL PBS.

17 Carefully pipette the entire volume up and down 10 times to mix.

18 Resuspend and combine the three pellets from the PBAL sample into one eppendorf tube in a total of 250 µL PBS.

19 Carefully pipette the entire volume up and down 10 times to mix.

20 To ensure proper mixing, vortex each sample for 15 seconds and spin down briefly.

21 Prepare an enzyme cocktail solution according to the number of samples being processed:

| Components | Volume/Sample (µl) |
|---|---|
| Lysozyme (10 mg/ml) | 25 |
| Mutanolysin (25 000 U/ml) | 3 |
| Lysostaphin ( 4 000 U/ml in NaAC) | 1.5 |
| TE5 buffer (10 mM Tris HCl, 5mM EDTA, pH 8.0) | 20.5 |
| Total | 50 |

22 Add 50 µl of the enzyme-cocktail solution to each sample. Mix by pipetting the entire volume up and down 10 times. Vortex samples for 1 second.

23 Incubate the samples on a thermomixer at 37°C with shaking at 350 rpm for 1 hour.

24 Centrifuge the samples at 15700 x g at room temperature for 15 minutes to pellet any bacterial cells not lysed.

25 For each sample, transfer the supernatant containing bacterial DNA (about 300 µL) to a separate 2 mL eppendorf tube and store at 4°C. Proceed with pellet.

26 Resuspend the pellet in 800 µL CLS-TC buffer (FastDNA SPIN kit) by pipetting up and down 10 times.

27 For each sample, transfer the suspension from step 26 to a Lysing Matrix A tube (FastDNA SPIN kit).

28 Homogenize the samples in the FastPrep-24 instrument at a speed setting of 6.0 m/s for 40 seconds.

29 Centrifuge the samples at 14000 x g at room temperature for 10 minutes.

30 Carefully pipette out 650 µL of the supernatant and transfer to the corresponding 2 mL eppendorf tube set aside in step 25. The total volume is now about 950 µL per sample (650 µL + 300 µL).

31 Add 950 µL Binding Matrix (FastDNA SPIN kit) to each sample and invert to mix.

32 Place the samples on a rotator and incubate for 5 minutes at room temperature at low speed.

33 Transfer 700 µl of each suspension to a SPIN filter (FastDNA SPIN kit).

34 Centrifuge the SPIN filter at 14000 x g for 1 minute.

35 Discard flow-through.

36 Repeat steps 33-35 until the whole sample has been spun through the SPIN filter.

37 Add 500 µl prepared SEWS-M (FastDNA SPIN kit) and resuspend each pellet by pipetting up and down several times.

38 Centrifuge at 14000 x g for 1 minute. Discard flow-through.

39 Without adding any liquid, centrifuge the SPIN filters a second time at 14000 x g for 2 minutes.

40 Replace the catch tube with a new catch tube.

41 Add 100 µL of DES (FastDNA SPIN kit) and gently resuspend the Binding Matrix by pipetting up and down several times.

42 Incubate at 55°C for 5 minutes on a heating block.

43 Centrifuge at 14000 x g for 1 minute to elute the DNA.

44 Check the purity of the DNA sample using Nanodrop (260/280).

45 Measure the DNA concentration using the Qubit fluorometer.

### *Amplicon PCR*

46 Create an overview excel sheet and a plate- and pipetting-layout for the PCR run.

47 Select and thaw DNA samples for PCR.

48 Spin DNA samples at 13000 x g for 1 minute at room temperature.

49 Pipette 5 µl of each DNA sample into a well on a PCR plate, following the plate- and pipetting-layout.

50 Prepare a master mix containing the following components, in 2 Eppendorf tubes in a prePCR-hood/room. Vortex briefly to mix, and spin briefly to bring down the content.

| Component | Vol (µl) per tube | Total vol (µl) |
|---|---|---|
| 16S Amplicon F primer (10uM) | 27.5 | 55 |
| 16S Amplicon R primer (10uM) | 27.5 | 55 |
| 2X KAPA HiFi HotStart ReadyMix | 687.5 | 1375 |
| PCR grade water | 357.5 | 715 |
| *Total* | *1100* | *2200* |

51 Combine the master mix from the two Eppendorf-tubes in a sterile trough. Using a multichannel pipette, transfer 20 µl to each well of the PCR plate. Pipette up and down 10 times to mix.

52 Seal the PCR plate with MicroAmp film.

53 Centrifuge the PCR plate at 1000 x g for 1 minute at 20 °C.

54 Run PCR in a thermal cycler using the following program:

| | | |
|---|---|---|
| 95°C | 3 min | |
| 95°C | 30 sec | 45 cycles, row 2->4 |
| 55°C | 30 sec | |
| 72°C | 30 sec | |

| | | |
|---|---|---|
| 72°C | 5 min | |
| 4°C | indef | |

55 Continue with Agarose Gel Electrophoresis for checking of 16S PCR amplicons or store PCR plate at -20°C until use.

## *Agarose electrophoresis*

56 Thaw and spin down 16S amplicons in Amplicon PCR plate at 1000 x g at 20°C for 1 minute.

57 Cast two big 1.5% agarose gels containing GelRed (per gel use: 3g agarose + 200 ml 1xTAE + 10 µl GelRed). Use four 15-lane combs per gel.

58 On a new 96-well plate, add 1 µl 5x DNA loading buffer (use a multichannel pipette and pipette from buffer aliquots in 8 PCR tubes).

59 Using a multichannel pipette, transfer 5 µl of 16S amplicons from the Amplicon PCR plate to the 96-well plate containing 5x DNA loading buffer. Pipette up and down gently to mix.

60 Centrifuge plate briefly (10 seconds at 1000 x g) to bring down the content.

61 Load EZ 100bp ladder (3 µl) on the first lane of each lane-row.

62 Load samples using a multichannel pipette (remember to note down the sample order in loading).

63 Run gel in 1xTAE buffer at 80V for 15 minutes.

64 Increase to 100V and run until the bromophenol blue marker has moved ca. 2/3 of the gel (i.e. 1 hour).

If 1% is used, shorten running time!

## *PCR Clean-Up to purify the 16S V3 & V4 amplicon away from free primers and primer dimer species*

65 Consumables:

| Item | Quantity (per 96 samples) | Storage |
|---|---|---|
| 10 mM Tris pH 8.5 (EB buffer) | 6 ml | -15°C to -25°C |
| AMPure XP Beads | 2.5 ml | 2°C - 8°C |
| Freshly Prepared 80% Ethanol (EtOH) | 50 ml | |
| 96-well 0.2 ml PCR plate | 2 plates | |

| | | |
|---|---|---|
| Microseal 'B' film | | |
| Sterile trough | | |
| 10 µl pipette tips with filter | 3 boxes | |
| 200 µl pipette tips with filter | 7 boxes | |

Preparation: Bring the AMPure XP beads to room temperature.

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

67 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 (*ca 2.5 ml for 96 samples*).

68 Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate, and gently pipette entire volume up and down 10 times. Change tips between columns.

69 Incubate at room temperature without shaking for 5 minutes.

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

71 With the Amplicon PCR plate on the magnetic stand, use a 100 µl-multichannel pipette to remove first 30 µl and then a 10 µl-multichannel pipette to remove 10 µl and discard the supernatant. Change tips between samples.

72 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 195 µl the supernatant.

73 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 195 µl the supernatant. Use a 10 µl-multichannel pipette to remove the rest of supernatant and excess ethanol.

(Check for excess ethanol in each well by lifting up the plate from magnetic stand and looking from the bottom of each well. Try to remove all supernatant from wells.)

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

75 Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.

76 Leave stand for 1-2 minutes, and then gently pipette mix up and down 10 times, changing tips after each column. *Make sure that beads are fully resuspended.*

77 Incubate at room temperature for 2 minutes.

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

79 Using a multichannel pipette, carefully transfer 45 µl of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.
*Avoid taking AMPure beads along when pipetting the supernatant.*

80 Centrifuge the plate at 1000 x g at 20°C for 1 minute (to bring down the content and remove bubbles).

81 Transfer 5 µl of the cleaned-up PCR amplicons to a new 96-well PCR plate for Index PCR in next steps.

82 Seal the plate with the rest with Microseal “B” adhesive seal and store at -20°C.

Safe stopping point.
If you do not immediately proceed to Index PCR, seal the plate with 5 µl amplicons with Microseal "B" adhesive seal and store it at 4°C for short-term (1 day) or at -15°C to -25°C until use (up to a week)

## Index PCR - attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit.

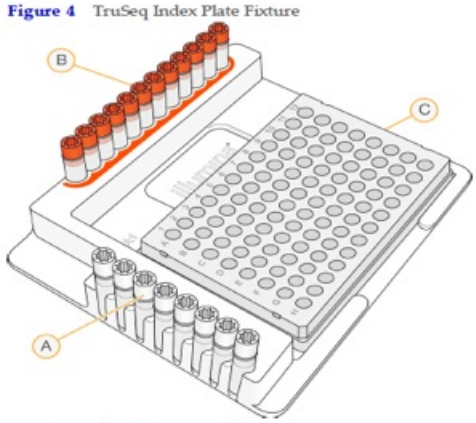
83 Consumables:

| Item | Quantity (for 96 samples) | Storage |
|---|---|---|
| 2x KAPA HiFi HotStart ReadyMix | 2 x 1325 µl | -15°C to -25°C |
| Nextera XT Index 1 Primers (N7XX) from the Nextera XT Index kit | 96 x 5 µl | -15°C to -25°C |
| Nextera XT Index 2 Primers (S5XX) from the Nextera XT Index kit | 96 x 5 µl | -15°C to -25°C |
| PCR grade water | 2 x 530 µl | -15°C to -25°C |
| TruSeq Index Plate Fixture (FC-130-1005) | 1 | |
| MicroAmp Adhesive Clear Film | 1 | |
| Sterile trough | 3 | |
| 10 µl pipette tips with filter | 2 boxes + | |
| 200 µl pipette tips with filter | 1 box + | |

84 Centrifuge the PCR plate with 5 µl of 16S PCR amplicon after clean-up, at 1000 x g at 20°C for 1 minute.

85 Place the plate on a TruSeq Index Plate Fixture (as shown in Fig. 4 below).

**Figure 4** TruSeq Index Plate Fixture




**A** Index 2 primers (white caps)
**B** Index 1 primers (orange caps)
**C** 96-well plate

86 Spin tubes containing Index primers briefly (1 second in a minicentrifuge) to bring down the content (use 2 ml-tubes without caps as "holding adapters").

87 Arrange the Index 2 primer tubes (white caps, clear solution) vertically, aligned with rows A through H in the TruSeq Index Plate Fixture (Fig. 4). Using a multichannel pipette, pipette 5 µl of Index 2 primers to the wells on the PCR plate.

88 Arrange the Index 1 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12. Using a multichannel pipette, pipette 5 µl of Index 1 primers to the wells on the PCR plate.

For pipetting convenience, begin first with the first 6 primer tubes aligned with columns 1-6 only. Then remove these, and continue with the next 6 primers tubes aligned with columns 7-12

89 Prepare a master mix in two 2 ml-tubes, containing:

| Component | Volume per tube (µl) |
|---|---|
| 2 x KAPA HiFi HotStart Ready Mix | 1325 µl |
| PCR Grade water | 530 µl |
| *Total* | *1855 µl* |

Vortex to mix and spin down.

90 Transfer the master mix to a sterile trough, and pipette 35 µl to each well on the PCR plate using a multichannel pipette. Gently pipette up and down 10 times to mix.

91 Cover the plate with MicroAmp Adhesive Clear film.

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

93 Perform PCR on a thermal cycler using the following program:

| | | |
|---|---|---|
| 95°C | 3 min | |
| 95°C | 30 sec | 8 cycles, row 2 -> 4 |
| 55°C | 30 sec | |
| 72°C | 30 sec | |
| 72°C | 5 min | |
| 4°C | indef | |

94 Store Index PCR plate at -20°C until the next steps.

## *PCR Clean-Up for Index PCR Samples*

95 Consumables:

| Item | Quantity (per 96 samples) | Storage |
|---|---|---|
| 10 mM Tris pH 8.5 (EB buffer) | 3 ml | -15°C to -25°C |
| AMPure XP beads | 6 ml | 2°C to 8°C |
| Freshly Prepared 80% Ethanol (EtOH) | 50 ml | |
| 96-well 0.2 ml PCR plate | 3 plates | |
| Microseal 'B' film | | |
| Sterile trough | 4 | |
| 10 µl pipette tips with filter | 3 boxes | |
| 200 µl pipette tips with filter | 7 boxes | |

Preparation: Bring the AMPure XP beads to room temperature.

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

97 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 (ca 6 ml for 96 samples).

98 Using a multichannel pipette, add 56 µl of AMPure XP beads to each well of the Index PCR plate and gently pipette entire volume up and down 10 times. Change tips between columns.

99 Incubate at room temperature without shaking for 5 minutes.

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

101 With the Amplicon PCR plate on the magnetic stand, use a 100 µl-multichannel pipette to remove first 90 µl and then a 10 µl-multichannel pipette to remove 10 µl and discard the supernatant. Change tips between samples.

102 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 195 µl the supernatant.

103 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 195 µl the supernatant.

d. Use a 10 µl-multichannel pipette to remove the rest of supernatant and excess ethanol.
*(Check for excess ethanol in each well by lifting up the plate from magnetic stand and looking from the bottom of each well. Try to remove all supernatant from wells.)*

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

105 Remove the Index PCR plate from the magnetic stand. Using a multichannel pipette, add 27.5 µl of 10 mM Tris pH 8.5 to each well of the Index PCR plate.

106 Leave stand for 1-2 min, and then gently pipette mix up and down 10 times, changing tips after each column. *Make sure that beads are fully resuspended.*

107 Incubate at room temperature for 2 minutes.

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

109 Using a multichannel pipette, carefully transfer 10 µl of the supernatant from the Index PCR plate to a new 96-well PCR plate. Label this plate with “first plate”. Change tips between samples to avoid cross-contamination.

110 Repeat step 109 (to obtain 2 PCR plates with 10 µl each Index PCR per plate). Label the plate with “second plate”. Change tips between samples to avoid cross-contamination.

Avoid taking XP beads along when transferring the samples

111 Seal (with Microseal B film) and store the plate with minimal XP beads (usually “first plate”) at -20°C for library normalization (dilution) and pooling for use in sequencing with MiSeq.

112 Use the second plate for assessment of DNA concentration and analysis with BioAnalyzer (if not proceeding to these steps right after, seal and store the plate at -20°C until use).

## *Library Quantification, Normalization & Pooling*

113 Calculate DNA concentration (C) in each Index PCR sample using the corresponding average concentration value (PicoGreenAve) ng/µl obtained from PicoGreen.

***We use Qubit!***

C (ng/µl) = PicoGreen Ave x 100

114 Convert concentrations in ng/µl to nM, using the following formula:
[(concentration in ng/µl)/(660 mol × 630)] $\times 10^6$= concentration in nM

115 For normalization, dilute concentrated final library (i.e. index PCR) using 10 mM Tris pH 8.5 (EB buffer) to 4nM.
i. Transfer a desired volume (e.g. 5 µl) of cleaned index PCR samples from the Index PCR plate with minimal contaminated XP beads after

clean-up to a new PCR plate.
ii. Add an appropriate amount of EB buffer to each well to make a dilution to 4nM. Pipette up and down gently 5 times to mix.
iii. Label the plate with diluted index PCR samples with “IndexPCR” PLATE #, and “4nM”.

116 Aliquot 5 µl of each diluted index PCR sample and mix aliquots in an Eppendorf tube for pooling libraries.

117 Make 5 5µl-aliquots of the library pool, and store at -20°C until use in sample preparation for MiSeq sequencing.

## *Sample Preparation for MiSeq Sequencing - preparation*

118 Create a txt/excel file containing the following info separated by tab/in column:

| *Sample_ID* | *Sample_Name* | *Sample_Plate* | *Sample_Well* | *I7_Index_ID* | *index* | *I5_Index_ID* | *index2* | *Sample_Project* | *Description* |
|---|---|---|---|---|---|---|---|---|---|

* Tip: use a previous sample sheet as a template and copy over info for the following fields:

| *I7_Index_ID* | *index* | *I5_Index_ID* | *index2* |
|---|---|---|---|

(index order is meant to be the same for all runs)

* If there is any notice about samples (DNA extraction, PCR product, etc), use the *Description* field

* Leave empty fields: *Sample_Plate, Sample_Well,* and *Sample_Project*

119 **Thawing the MiSeq Reagent Cartridge**

An alternative to the thawing steps is to thaw reagents overnight in 2°C to 8°C storage. Reagents are stable up to one week when stored at this temperature.

19.1 Remove the reagent cartridge from -15°C to -25°C storage.

19.2 Place the reagent cartridge in a water bath containing enough room temperature deionized water to submerge the base of the reagent cartridge. Do not allow the water to exceed the maximum water line printed on the reagent cartridge.

19.3 Allow the reagent cartridge to thaw in the room temperature water bath for approx 60-90 minutes or until it is thawed completely.

19.4 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.

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

19.6 Visually inspect the reagents in positions 1,2, and 4 to make sure that they are fully mixed and free of precipitates.

19.7 Gently tap the cartridge on the bench to reduce air bubbles in the reagents.

The MiSeq 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.

19.8 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.

### *Library Denaturing and MiSeq Sample Loading*

120 Consumables

| Item | Quantity | Storage |
|---|---|---|
| 10 mM Tris pH 8.5 (EB buffer, Qiagen) | 6 µl | -15°C to -25°C |
| HT1 (Hybridization buffer) | 1540 µl | -15°C to -25°C (attached to the MiSeq reagent cartridge) |
| 0.2 N NaOH (less than a week old) | 10 µl | -15°C to -25°C |
| PhiX Control Kit v3 (FC-110-3001) | 4 µl | -15°C to -25°C |
| MiSeq reagent cartridge | 1 cartridge | -15°C to -25°C |
| 1.7 ml microcentrifuge tubes (screw cap recommended) | 3 tubes | |
| 2.5 L ice bucket | | |
| | | |

121 Set a heat block suitable for 1.7 ml microcentrifuge tubes to 96°C.

122 Prepare 0.2 N NaOH by diluting 2 µl 10M NaOH in 98 µl PCR-grade H2O (total volume = 100 µl).

123 In an ice bucket, prepare an ice-water bath by combining 3 parts ice and 1 part water.

124 Thaw a tube with 5 µl pooled library on ice.

125 Thaw HT1 (Hybridization Buffer) at room temperature. Keep HT1 on ice (to chill) after thawing.

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

127 Set aside the remaining dilution of 0.2 N NaOH to prepare a PhiX control within the next 12 hours.

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

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

130 Add the following volume of pre-chilled HT1 to the tube containing denatured DNA:
• Denatured DNA (10 µl)
• Pre-chilled HT1 (990 µl)
*Adding the HT1 results in a 20 pM denatured library in 1 mM NaOH.*

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

132 Dilute the denatured DNA to 10 pM by combining:
• 20 pM denatured library (300 µl)
• Pre-chilled HT1 (300 µl)

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

134 Place the denatured and diluted DNA on ice.

135 Combine the following volumes to dilute the PhiX library to 4 nM:
• 10 nM PhiX library (2 µl)
• 10 mM Tris pH 8.5 (3 µl)

136 Combine the following volumes of 4 nM PhiX and 0.2 N NaOH in a microcentrifuge tube:
• 4 nM PhiX library (5 µl)
• 0.2 N NaOH (5 µl)

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

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

139 Add the following volumes of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library:
• Denatured PhiX library (10 µl)
• Pre-chilled HT1 (990 µl)

140 Dilute the denatured 20 pM PhiX library to 10 pM as follows:
• 20 pM denatured PhiX library (300 µl)
• Pre-chilled HT1 (300 µl)

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

142 Place the denatured and diluted PhiX on ice.

143 Combine the following volumes of denatured PhiX control library and the denatured amplicon library in a microcentrifuge tube:
• Denatured and diluted PhiX control (90 µl) – i.e 15% of a total vol of 600µl
• Denatured and diluted amplicon library (510 µl)

*15% PhiX control spike-in is used*

144 Set the combined sample library and PhiX control aside on ice until you are ready to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge.

145 Using a heat block, incubate the combined library and PhiX control tube at 96°C for 2 minutes.

Perform the heat denaturation step immediately before loading the library into the MiSeq reagent cartridge to ensure efficient template loading on the MiSeq flow cell

146 After the incubation, invert the tube 1–2 times to mix and immediately place in the icewater bath.

147 Keep the tube in the ice-water bath for 5 minutes.