Illumina 16S Metagenomics Sequencing Library Preparation Protocol for Canine/Feline Feces and Plaque samples

RELATED PROCEDURES & REFERENCES:
Refer to Illumina 16S Metagenomics Sequencing Library Preparation protocol. Illumina Protocol # 15044223 Rev. A

EQUIPMENT & MATERIALS:
Nuclease Free Water, Ambion AM9932
P1000 Pipetman pipette, Rainin P1000
P200 Pipetman pipette, Rainin P200
P100 Pipetman pipette, Rainin P100
P10 Pipetman pipette, Rainin P10
P2 Pipetman pipette, Rainin P2
100-1000uL filtered pipette tips, sterile, USA Scientific 1126-7810
50-200uL filtered pipette tips, sterile, USA Scientific 1120-8810
100µL filtered pipette tips, sterile, USA Scientific 1120-1840
0.1µL-10µL filtered pipette tips, sterile, USA Scientific 1121-3810
20mL automatic pipet, Rainin LTS 2-20mL
20mL pipet tip, sterile, Rainin RC-L20MLS
10mL automatic pipet, Rainin LTS 1-10mL
10mL pipet tip, sterile, Rainin RC-L10MLS
Hard Shell Low Profile Thin Walled Skirted 96-well plate, BioRad HSP-9601
Microseal 'B' Adhesive Seal, BioRad MSB-1001
Microcentrifuge tube rack(s)
Eppendorf Microcentrifuge, model #5417C
BioRad C1000 Touch Thermal Cycler, BioRad 184-0197
Vortex mixer, VWR 58816-121
Agencourt AMPure XP, Beckman Coulter A63881
Ethanol 200 proof (molecular grade), Sigma-Aldrich E7023
Nextera XT Index Kit, Illumina FC-131-1002
PhiX Control Kit v3, Illumina FC-110-3001
Ultra Clean PCR Water, MoBio 17000-11
Magnetic stand-96, Life Technologies AM10027
TruSeq Index Plate Fixture, Illumina FC-130-1005

PCR Clean-up

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	52.5 µl per sample	-15° to -25°C
AMPure XP beads	20 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Preparation

1. Bring the AMPure XP beads to room temperature at least 30 minutes prior.

Procedure

1. Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.
2. Using a multichannel pipette set to 25 µl, transfer 25 uL of Amplicon PCR product from the PCR plate to the MIDI plate. Change tips between samples.

NOTE
Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.

3. 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 sample reservoir depending on the number of
samples processing.
4. Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate. Change tips between columns.
5. Gently pipette entire volume up and down 10 times if using a 96-well PCR plate or seal
plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
6. Incubate at room temperature without shaking for 5 minutes.
7. Place the MIDI plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
8. With the MIDI plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
9. With the MIDI 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. Do not disturb the beads!
b. Incubate the plate on the magnetic stand for 30 seconds.

c. Carefully remove and discard the supernatant.

10. With the MIDI 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. Do not disturb the beads!
    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.
11. With the MIDI plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.
12. Remove the MIDI 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 MIDI plate.
13. 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.
14. Incubate at room temperature for 2 minutes.
15. Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
16. Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the MIDI plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination. Label new PCR Plate with test and PCR Clean #1.

SAFE STOPPING POINT
If you do not immediately proceed to *Index PCR*, seal plate with Microseal “B” adhesive seal and store it at -15° to -25°C for up to a week.

Index PCR

Consumables

Item	Quantity	Storage
2x KAPA HiFi HotStart ReadyMix	25 µl per sample	-15° to -25°C
Nextera XT Index 1 Primers (N7XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 µl per sample	-15° to -25°C
Nextera XT Index 2 Primers (S5XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 µl per sample	-15° to -25°C
PCR Grade Water	10 µl per sample	
TruSeq Index Plate Fixture (FC-130-1005)	1	
96-well 0.2 ml PCR plate	1 plate	
Microseal 'A' film	1	

Procedure

1. Using a multichannel pipette, transfer 5 µl from each well of PCR Clean #1 plate to a new 96-well plate. The remaining 45 µl is not used in the protocol and can be stored at -20 °C for other uses.
2. 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.
3. Place the 96-well PCR plate with the 5 µl of resuspended PCR product DNA in the TruSeq Index Plate Fixture.
4. Set up the following reaction of DNA, Index 1 and 2 primers, 2x KAPA HiFi HotStart ReadyMix, and PCR Grade water:

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

5. Gently pipette up and down 10 times to mix.
6. Cover the plate with Microseal 'A'. Centrifuge the plate at 1,000 × g at 20°C for 1 minute.
7. Perform PCR on a thermal cycler using the following program:
   • 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 minutes
   • Hold at 4°C

PCR Clean-up 2

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	27.5 µl per sample	-15° to -25°C
AMPure XP beads	56 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Preparation

1. Bring the AMPure XP beads to room temperature at least 30 minutes prior.

Procedure

1. Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.
2. Using a multichannel pipette set to 50 µl, transfer the entire Index PCR product from the PCR plate to the MIDI plate. Change tips between samples.

NOTE
Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.

3. 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 reagent reservoir.
4. Using a multichannel pipette, add 56 µl of AMPure XP beads to each well of the Index PCR plate.
5. Gently pipette mix up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
6. Incubate at room temperature without shaking for 5 minutes.
7. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
8. With the Index PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
9. 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. Do not disturb the beads!!
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.

10. Repeat the entirety of Step 9 for a total of 2 washes with 80% ethanol.
11. With the Index PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.
12. 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.
13. If using a 96-well PCR plate, gently pipette mix up and down 10 times until beads are fully resuspended, changing tips after each column. If using a MIDI plate, seal plate and shake at 1800 rpm for 2 minutes.
14. Incubate at room temperature for 2 minutes.
15. Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
16. 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.

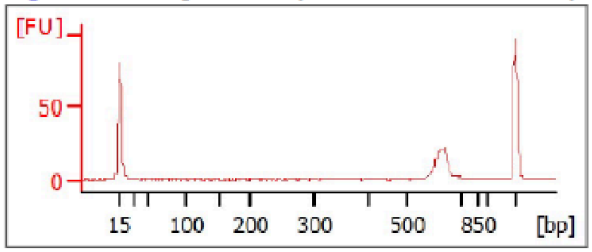
SAFE STOPPING POINT

If you do not plan to proceed to *Library Quantification, Normalization, and Pooling* seal the plate with Microseal "B" adhesive seal. Store the plate at -15° to -25°C for up to a week.

[Optional] Validate Library

Run 1 µl of a 1:50 dilution 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.

Figure 5 Example Bioanalyzer Trace of Final Library



*Quantify library using the *Thermo Life Technologies Quant-iT dsDNA HS* quantification Kit following manufacturers instructions. Alternatively, use Qubit for quantification.

Library Quantification, Normalization, and Pooling

Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes.

Calculate DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace:

$$\frac{(\text{concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size})} \times 10^6 = \text{concentration in nM}$$

For example:

$$\frac{15\ \text{ng/µl}}{(660\ \text{g/mol} \times 500)} \times 10^6 = 45\ \text{nM}$$

Dilute concentrated final library using Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 to 4 nM. Aliquot 5 µl of diluted DNA from each library and mix aliquots for pooling libraries with unique indices. Depending on coverage needs, up to 96 libraries can be pooled for one MiSeq run.

For metagenomics samples, >100,000 reads per sample is sufficient to fully survey the bacterial composition. This number of reads allows for sample pooling to the maximum level of 96 libraries, given the MiSeq output of > 20 million reads.

16S V3V4 Library size ~ 539bp

Library Denaturing and MiSeq Sample Loading

Consumables

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

Preparation

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

2. Remove a MiSeq reagent cartridge from -15°C to -25°C storage and thaw at room
temperature.
3. In an ice bucket, prepare an ice-water bath by combining 3 parts ice and 1 part water.

Denature DNA

1. 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)
2. Set aside the remaining dilution of 0.2 N NaOH to prepare a PhiX control within the
next 12 hours.
3. Vortex briefly to mix the sample solution, and then centrifuge the sample solution at 280
× g at 20°C for 1 minute.
4. Incubate for 5 minutes at room temperature to denature the DNA into single strands.
5. 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 1 mL of 20 pM denatured library in 1 mM NaOH.
6. Place the denatured DNA on ice until you are ready to proceed to final dilution.

Dilute Denatured DNA

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

   NOTE
   Illumina recommends targeting 800–1000 K/mm² raw cluster densities using MiSeq v3 reagents. Start the run using a 6 pM loading concentration. To optimize for different libraries, refer to the full chart at the end.

Final Concentration	6 pM
20 pM denatured library	180 µl
Pre-chilled HT1	420 µl

2. Invert several times to mix and then pulse centrifuge the DNA solution.
3. Place the denatured and diluted DNA on ice.

Denature and Dilution of PhiX Control

Use the following instructions to denature and dilute the 10 nM PhiX library to the same loading concentration as the Amplicon library. The final library mixture must contain at 10% PhiX.

1. 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)

2. 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)

3. Vortex briefly to mix the 2 nM PhiX library solution.
4. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.
5. 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)

6. Dilute the denatured 20 pM PhiX library to the same loading concentration as the Amplicon library as follows:

Final Concentration	6 pM
20 pM denatured library	180 µl
Pre-chilled HT1	420 µl

7. Invert several times to mix and then pulse centrifuge the DNA solution.
8. Place the denatured and diluted PhiX on ice.

Combine Amplicon Library and PhiX Control

NOTE

The Illumina recommended PhiX control spike-in of ≥ 5% for low diversity libraries is possible with RTA v1.17.28 or later, which is bundled with MCS v2.2. For optimal performance, update to v3 software (MCS 2.3). If you are using an older version of the MiSeq software or sequencing these libraries on the GA or HiSeq, Illumina recommends using ≥ 25% PhiX control spike-in.

1. The PhiX control library will be added at 10%. Combine the following volumes of denatured PhiX control library and your denatured

amplicon library in a microcentrifuge tube:
• Denatured and diluted PhiX control (60 µl)
• Denatured and diluted amplicon library (540 µl)

2. 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.

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

minutes.

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

bath.

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

NOTE
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.

*For Fecal 16S start with 6pM loading concentration and optimize if needed.

Full Illumina chart for loading concentrations and volumes.

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 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

Continue loading the MiSeq reagent cartridge and set up the instrument following manufacturers instructions.
See *Illumina MiSeq User System Guide* for instructions.