

16S Metagenomic Sequencing Library Preparation

Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System

Introduction	2
16S Library Preparation Workflow	5
Amplicon PCR	6
PCR Clean-Up	8
Index PCR	10
PCR Clean-Up 2	13
[Optional] Validate Library	15
Library Quantification, Normalization, and Pooling	16
Library Denaturing and MiSeq Sample Loading	17
MiSeq Reporter Metagenomics Workflow	20
Supporting Information	21

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Introduction

Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations.

Which 16S rRNA region to sequence is an area of debate, and your region of interest might vary depending on things such as experimental objectives, design, and sample type. This protocol describes a method for preparing samples for sequencing the variable V3 and V4 regions of the 16S rRNA gene. This protocol can also be used for sequencing other regions with different region-specific primers. This protocol combined with a benchtop sequencing system, on-board primary analysis, and secondary analysis using MiSeq Reporter or BaseSpace, provides a comprehensive workflow for 16S rRNA amplicon sequencing.

Workflow Summary:

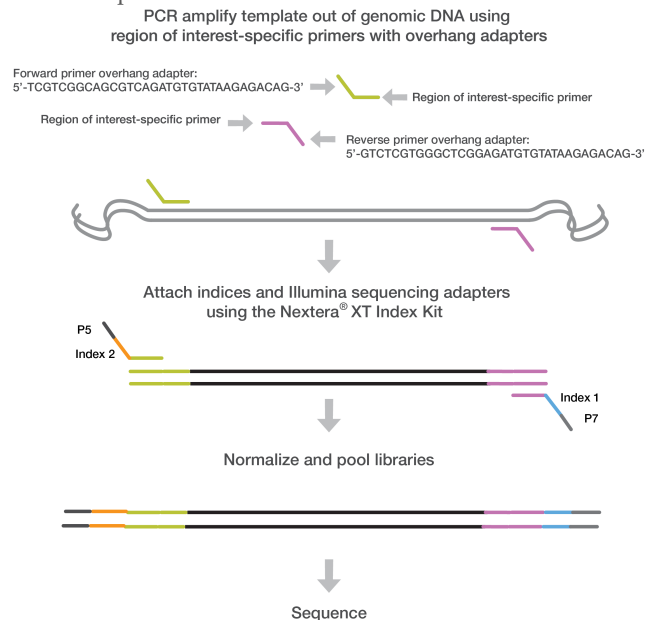
- 1 Order amplicon primers—The protocol includes the primer pair sequences for the V3 and V4 region that create a single amplicon of approximately ~460 bp. The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. Illumina does not sell these primers. They must be ordered from a third party. See [Amplicon Primers](#), on page 3 for more information on amplicon primers.
- 2 Prepare library—The protocol describes the steps to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual-index barcodes to the amplicon target. Using the full complement of Nextera XT indices, up to 96 libraries can be pooled together for sequencing.
- 3 Sequence on MiSeq—Using paired 300-bp reads, and MiSeq v3 reagents, the ends of each read are overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65-hour run. The MiSeq run output is approximately > 20 million reads and, assuming 96 indexed samples, can generate > 100,000 reads per sample, commonly recognized as sufficient for metagenomic surveys.
- 4 Analyze on MSR or BaseSpace—The Metagenomics workflow is a secondary analysis option built into the MiSeq Reporter (on-system software) or available on BaseSpace (cloud-based software). The Metagenomics Workflow performs a taxonomic classification using the Greengenes database showing genus or species level classification in a graphical format.

This protocol can be used to sequence alternative regions of the 16S rRNA gene and for other targeted amplicon sequences of interest. When using this protocol for amplicon sequencing other than 16S rRNA, use the Generate FASTQ Workflow (secondary analysis option). For more information, see [MiSeq Reporter Metagenomics Workflow](#), on page 20.



DISCLAIMER

The information in this Illumina Demonstrated Protocol is being provided as a courtesy; in some cases reagents are required to be purchased from non-authorized third-party suppliers. Illumina does not guarantee nor promises technical support for the performance of our products used with reagents purchased from a non-authorized third-party supplier.

Figure 1 16S V3 and V4 Amplicon Workflow

User-defined forward and reverse primers that are complementary upstream and downstream of the region of interest are designed with overhang adapters, and used to amplify templates from genomic DNA. A subsequent limited-cycle amplification step is performed to add multiplexing indices and Illumina sequencing adapters. Libraries are normalized and pooled, and sequenced on the MiSeq system using v3 reagents.

Amplicon Primers

- The gene-specific sequences used in this protocol target the 16S V3 and V4 region. They are selected from the Klindworth et al. publication (Klindworth A, Pruesse E, Schweer T, Peplles J, Quast C, et al. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41(1).) as the most promising bacterial primer pair. Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences. The full length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are:
 16S Amplicon PCR Forward Primer = 5'
 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
 16S Amplicon PCR Reverse Primer = 5'
 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
- This method can also be utilized to target other regions on the genome (either for 16S with other sets of primer pairs, or non-16S regions throughout the genome; ie any amplicon). The overhang adapter sequence must be added to the locus-specific primer for the region to be targeted (Figure 1). The Illumina overhang adapter sequences to be added to locus-specific sequences are:
 Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]
 Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]

- The following considerations are recommended for designing other locus-specific primers:
 - a Illumina recommends targeting regions that result in an amplicon that when sequenced with paired-end reads has at least ~50 bp of overlapping sequence in the middle. For example, if running 2x300 bp paired-end reads Illumina recommends having an insert size of 550 bp or smaller so that the bases sequenced at the end of each read overlap.
 - b The locus-specific portion of primer (not including overhang sequence) must have a melting temperature (T_m) of 60°–65°C. You can use online PCR primer sequence analysis tools (e.g. <http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>) to check the properties of primer designs. For the T_m calculation only, the gene-specific portion must be used in calculation. For hairpin and dimer calculations, the fully-assembled primer sequence (including the overhang) should be used.
 - c Illumina recommends using standard desalting purification when ordering oligo primer sets.



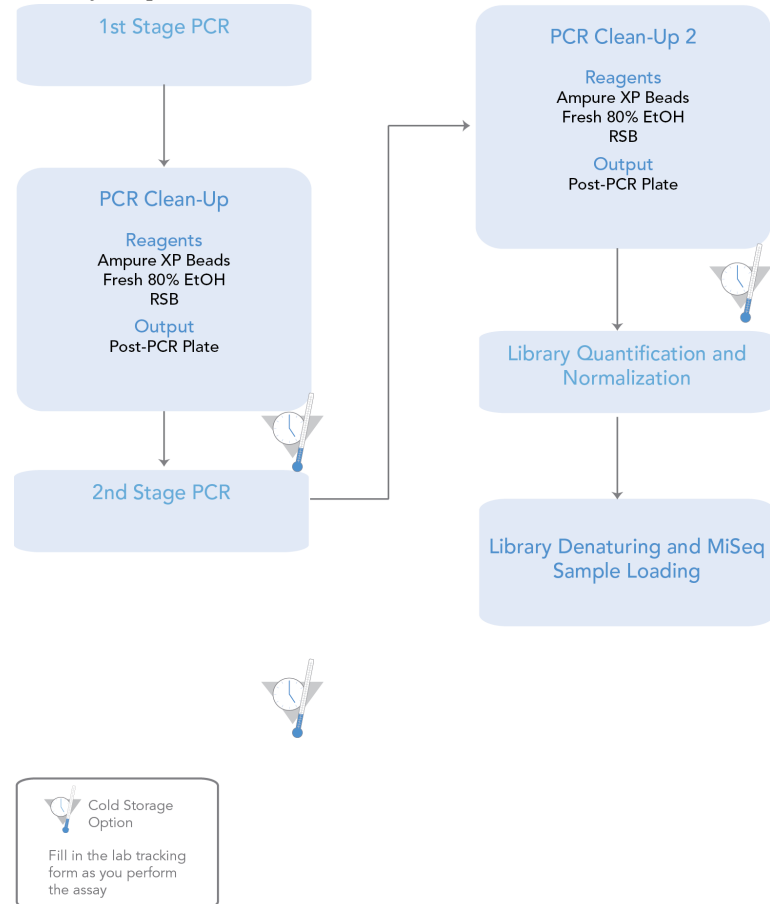
NOTE

For more information on reagents used in the protocol, see [Consumables and Equipment](#), on [page 21](#).

16S Library Preparation Workflow

The following diagram illustrates the workflow using the 16S Library Preparation Protocol. Safe stopping points are marked between steps.

Figure 2 16S Library Preparation Workflow



Amplicon PCR

This step uses PCR to amplify template out of a DNA sample using region of interest-specific primers with overhang adapters attached. For more information on primer sequences, see [Amplicon Primers](#), on page 3.

Consumables



NOTE

For more information on consumables and equipment for this protocol see [Consumables and Equipment](#), on page 21.

Item	Quantity	Storage
Microbial Genomic DNA (5 ng/μl in 10 mM Tris pH 8.5)	2.5 μl per sample	-15° to -25°C
Amplicon PCR Reverse Primer (1 μM)	5 μl per sample	-15° to -25°C
Amplicon PCR Forward Primer (1 μM)	5 μl per sample	-15° to -25°C
2x KAPA HiFi HotStart ReadyMix	12.5 μl per sample	-15° to -25°C
Microseal 'A' film		
96-well 0.2 ml PCR plate	1 plate	
[Optional] Bioanalyzer chip (Agilent DNA 1000 kit catalog # 5067-1504)		

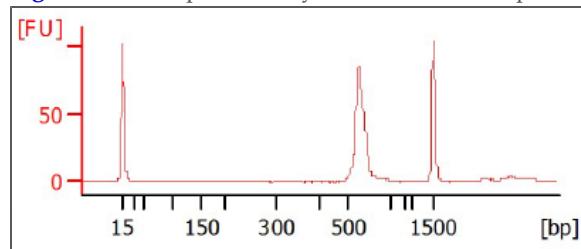
Procedure

- 1 Set up the following reaction of DNA, 2x KAPA HiFi HotStart ReadyMix, and primers:

	Volume
Microbial DNA (5 ng/μl)	2.5 μl
Amplicon PCR Forward Primer 1 μM	5 μl
Amplicon PCR Reverse Primer 1 μM	5 μl
2x KAPA HiFi HotStart ReadyMix	12.5 μl
Total	25 μl

- 2 Seal plate and perform PCR in a thermal cycler using the following program:
 - 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
- 3 **[Optional]** 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.

Figure 3 Example Bioanalyzer Trace after Amplicon PCR Step



PCR Clean-Up

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

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

- Bring the AMPure XP beads to room temperature.

Procedure

- 1 Centrifuge the Amplicon PCR plate at $1,000 \times g$ at 20°C for 1 minute to collect condensation, carefully remove seal.
- 2 **[Optional - for use with shaker for mixing]** Using a multichannel pipette set to 25 µl, transfer the entire 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 trough 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 plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
- 8 With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.

- 9 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 μ l 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.
- 10 With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:
 - a Using a multichannel pipette, add 200 μ l 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.
- 11 With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.
- 12 Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 μ l of 10 mM Tris pH 8.5 to each well of the Amplicon PCR 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 μ l of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

**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

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

Consumables

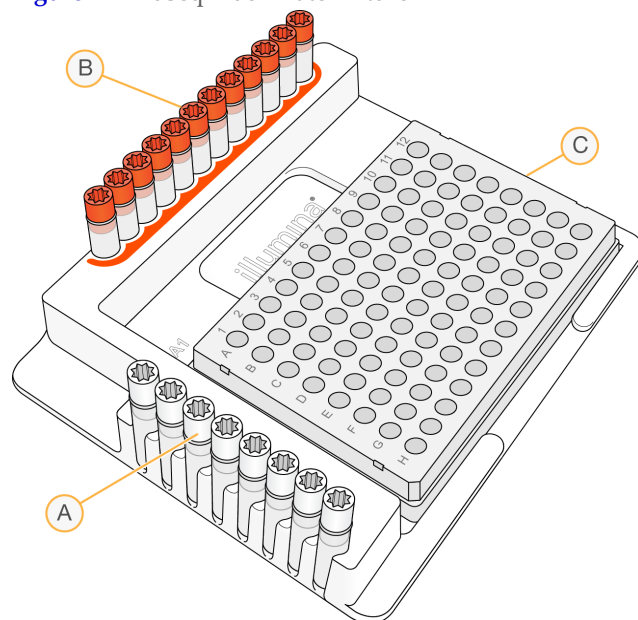
Item	Quantity	Storage
2x KAPA HiFi HotStart ReadyMix	25 µl per sample	-15° to -25°C
Nextera XT Index 1 Primers (N7XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 µl per sample	-15° to -25°C
Nextera XT Index 2 Primers (S5XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 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 to a new 96-well plate. The remaining 45 µl is not used in the protocol and can be stored 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.

For more information on index selection, see [Dual Indexing Principle](#), on page 23.

Figure 4 TruSeq Index Plate Fixture



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

- 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'.
- 7** Centrifuge the plate at 1,000 × g at 20°C for 1 minute.

- 8** 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

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

Consumables

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

Procedure

- 1 Centrifuge the Index PCR plate at $280 \times g$ at 20°C for 1 minute to collect condensation.
- 2 **[Optional - for use with shaker for mixing]** Using a multichannel pipette set to 50 μ l, 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 trough.
- 4 Using a multichannel pipette, add 56 μ l 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 μ l 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.

- 10 With the Index PCR plate on the magnetic stand, perform a second ethanol wash as follows:
 - a Using a multichannel pipette, add 200 μ l 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.
- 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 μ l 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 μ l of the supernatant from the Index PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.



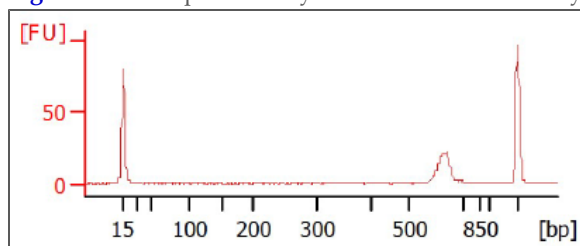
SAFE STOPPING POINT

If you do not plan to proceed to *Library Quantification, Normalization, and Pooling*, on page 16, 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 μ l 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



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:

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

For example:

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

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.

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 a minimum of 5% PhiX to serve as an internal control for these low-diversity libraries. Illumina recommends using MiSeq v3 reagent kits for improved run metrics.

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 μ l)
- Adding the HT1 results in a 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. It is suggested to start your first run using a 4 pM loading concentration and adjust subsequent runs appropriately.

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 μ l	120 μ l	180 μ l	240 μ l	300 μ l
Pre-chilled HT1	540 μ l	480 μ l	420 μ l	360 μ l	300 μ l

- 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 least 5% PhiX.

- 1 Combine the following volumes to dilute the PhiX library to 4 nM:
 - 10 nM PhiX library (2 μ l)
 - 10 mM Tris pH 8.5 (3 μ l)
- 2 Combine the following volumes of 4 nM PhiX and 0.2 N NaOH in a microcentrifuge tube:
 - 4 nM PhiX library (5 μ l)
 - 0.2 N NaOH (5 μ l)
- 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 μ l)
 - Pre-chilled HT1 (990 μ l)
- 6 Dilute the denatured 20 pM PhiX library to the same loading concentration as the Amplicon library as follows:

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

- 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 recommended PhiX control spike-in of $\geq 5\%$ 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 $\geq 25\%$ PhiX control spike-in.

- 1 Combine the following volumes of denatured PhiX control library and your denatured amplicon library in a microcentrifuge tube:
 - Denatured and diluted PhiX control (30 µl)
 - Denatured and diluted amplicon library (570 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 ice-water 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.

MiSeq Reporter Metagenomics Workflow

After samples are loaded, the MiSeq system provides on-instrument secondary analysis using the MiSeq Reporter software (MSR). MSR provides several options for analyzing MiSeq sequencing data. For this demonstrated 16S protocol, select the Metagenomics workflow.

By following this 16S Metagenomics protocol, the Metagenomics workflow classifies organisms from your V3 and V4 amplicon using a database of 16S rRNA data. The classification is based on the Greengenes database (<http://greengenes.lbl.gov/>). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. The analysis output includes:

- Clusters Graph – shows numbers of raw cluster, clusters passing filter, clusters that did not align, clusters not associated with an index, and duplicates.
- Sample Table – summarizes the sequencing results for each sample.
- Cluster Pie Chart – a graphical representation of the classification breakdown for each sample.

See the *MiSeq Reporter Metagenomics Workflow – Reference Guide* (Part # 15042317) for detailed instructions and guidance.

The method described in this 16S Metagenomics protocol can be used for any targeted amplicon sequencing, relevant to virus research, mutation detection, or other microbiology-related studies. If you use the protocol for other targeted amplicon sequencing studies, select the MiSeq Reporter Generate FASTQ Workflow for on-instrument generation of FASTQ files for downstream analysis. For specific guidance on the Generate FASTQ Workflow, see the *MiSeq Reporter Generate FASTQ Workflow – Reference Guide* (Part # 15042322).

Supporting Information

The protocols described in this guide assume that you are familiar with the contents of this section and have obtained all of the requisite equipment and consumables.

Acronyms

Table 1 Acronyms

Acronym	Definition
HT1	Hybridization Buffer
IEM	Illumina Experiment Manager
MSR	MiSeq Reporter
PCR	Polymerase Chain Reaction
rRNA	Ribosomal RNA
RSB	Resuspension Buffer

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation.

Table 2 User-Supplied Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier

Consumable	Supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
96-well 0.2 ml skirtless PCR plates or Twin.Tec 96-well PCR plates	Bio-Rad, part # MSP-9601
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Amplicon PCR Forward Primer (Standard desalting)	
Amplicon PCR Reverse Primer (Standard desalting)	
KAPA HiFi HotStart ReadyMix (2X)	KAPA Biosystems, part # KK2601
Microseal 'A' adhesive seals	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MiSeq Reagent Kit v3 (600 cycle)	Illumina, catalog # MS-102-3003
Nextera XT Index Kit	Illumina, catalog # FC-131-1001 or Illumina, catalog # FC-131-1002
PhiX Control Kit v3	Illumina, catalog # FC-110-3001
PCR grade water	General lab supplier
Fluorometric quantitation with dsDNA binding dye reagents	General lab supplier
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
[Optional] 96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859

Table 3 User-Supplied Equipment

Equipment	Supplier
2.5 L ice bucket	General lab supplier
96-well thermal cycler (with heated lid)	General lab supplier

Equipment	Supplier
Fluorometer for quantitation with dsDNA binding dyes	General lab supplier
Magnetic stand-96	Life Technologies, catalog # AM10027
Microplate centrifuge	General lab supplier
TruSeq Index Plate Fixture Kit (reusable)	Illumina, catalog # FC-130-1005
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Agilent DNA 1000 Kit	Agilent, part # 5067-1504
[Optional] High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) or VWR, catalog # 14216-214 (230V)

Dual Indexing Principle

The dual indexing strategy uses two 8 base indices, Index 1 (i7) adjacent to the P7 sequence, and Index 2 (i5) adjacent to the P5 sequence. Dual indexing is enabled by adding a unique Index 1 (i7) and Index 2 (i5) to each sample. The 96 sample Nextera XT Index Kit (FC-131-1002) use 12 different Index 1 (i7) adapters (N701–N712) and 8 different Index 2 (i5) adapters (S501–S508). The 24 sample Nextera XT Index Kit (FC-131-1001) uses 6 different Index 1 (i7) adapters (N701–N706) and 4 different Index 2 (i5) adapters (S501–S504). In the Index adapter name, the N or S refers to Nextera XT sample preparation, 7 or 5 refers to Index 1 (i7) or Index 2 (i5), respectively. The 01–12 refers to the Index number. A list of index sequences is provided for generating sample sheets to demultiplex the samples:

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCCGA	S501	TAGATCGC
N702	CGTACTAG	S502	CTCTCTAT
N703	AGGCAGAA	S503	TATCCTCT
N704	TCCTGAGC	S504	AGAGTAGA
N705	GGA CTCCT	S505	GTAAGGAG
N706	TAGGCATG	S506	ACTGCATA
N707	CTCTCTAC	S507	AAGGAGTA
N708	CAGAGAGG	S508	CTAAGCCT
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	G TAGAGGA		

Low Plexity Pooling Guidelines

Illumina uses a green laser or LED to sequence G/T and a red laser or LED to sequence A/C. At each cycle, at least one of two nucleotides for each color channel are read to ensure proper registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure. If you choose the dual-indexed sequencing workflow, always use at least two unique and

compatible barcodes for each index (index 1 and index 2). The following tables illustrate possible pooling strategies:

Table 4 Libraries Pooled: 6 or fewer; Sequencing Workflow: Single Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
1-plex (no pooling)	Any Index 1 adapter	Any Index 2 adapter
2-plex	<ul style="list-style-type: none"> • [option 1] N702 and N701 • [option 2] N702 and N704 	
3-plex	<ul style="list-style-type: none"> • [option 1] N701, N702, and N704 • [option 2] N703, N705, and N706 	
4- or 5-plex	<ul style="list-style-type: none"> • [option 1] N701, N702, N704, and any other Index 1 adapter • [option 2] N703, N705, N706, and any other Index 1 adapter 	
6-plex	N701, N702, N703, N704, N705, and N706	

Table 5 Sequencing Workflow: Single or Dual Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
7–12 plex, Dual Index	<ul style="list-style-type: none"> • [option 1] N701, N702, N704, and any other Index 1 adapter (as needed) • [option 2] N703, N705, N706, and any other Index 1 adapter (as needed) 	<ul style="list-style-type: none"> • [option 1] S501 and S502 • [option 2] S503 and S504 • [option 3] S505 and S506
7–12 plex, Single Index (96 sample Nextera Index adapter kit)	• N701–N706 and any other Index 1 adapter (as needed)	• Any Index 2 (i5) adapter
Greater than 12-plex	N701, N702, N703, N704, N705, N706, and any other Index 1 adapter	<ul style="list-style-type: none"> • [option 1] S501, S502, and any other Index 2 adapter (as needed) • [option 2] S503, S504, and any other Index 2 adapter (as needed) • [option 3] S505, S506, and any other Index 2 adapter (as needed)

These strategies represent only some of the acceptable combinations. Alternatively, check the real sequences of each index in the tables to make sure that each base position has a signal in both color channels for the index read:

Good				Bad			
Index 1		Index 2		Index 1		Index 2	
705	GGACTCCT	503	TATCCTCT	705	GGACTCCT	502	CTCTCTAT
706	TAGGCATG	503	TATCCTCT	706	TAGGCATG	502	CTCTCTAT
701	TAAGGCGA	504	AGAGTAGA	701	TAAGGCGA	503	TATCCTCT
702	CGTACTAG	504	AGAGTAGA	702	CGTACTAG	503	TATCCTCT
	√√√√√√√√		√√√√√√√√		√√√√√√√√		√√√√xxxx

√=signal in both color

x=signal missing in one color channel

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- **Physically Separate Pre-PCR and Post-PCR Areas**
 - Physically separate laboratory space where pre-PCR processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where PCR products are made and processed (post-PCR processes).
 - Never use the same sink to wash pre-PCR and post-PCR troughs.
 - Never share water purification systems for pre-PCR and post-PCR processes.
 - Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed.
- **Use Dedicated Equipment and Supplies**
 - Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes, and never share between processes.
 - Dedicate separate storage areas (freezers and refrigerators) to pre-PCR and post-PCR consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-PCR lab area. After unpacking the reagents, move the post-amplification reagents to the proper post-PCR storage area.

Pre-PCR and Post-PCR Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using

0.5% Sodium Hypochlorite (10% Bleach).



CAUTION

To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

Daily Cleaning of Pre-PCR Area

A daily cleaning of the pre-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-PCR area.

Identify pre-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-PCR processes. High-risk areas might include, but are not limited to, the following items:

- Benchtops
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Daily Cleaning of Post-PCR Area

Reducing the amount of PCR product in the post-PCR area helps reduce the risk of contamination in the pre-PCR area. Daily cleaning of the post-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps reduce the risk of contamination.

Identify post-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:

- Thermal cyclers
- Bench space used to process amplified DNA
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Weekly Cleaning of All Lab Areas

One time a week, perform a thorough cleaning of the pre-PCR and post-PCR areas using 0.5% Sodium Hypochlorite (10% Bleach).

- Clean all benchtops and laboratory surfaces.
- Clean all instruments that are not cleaned daily.
- Thoroughly mop lab floors.
- Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything falling to the floor must be treated as contaminated.

- Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.

- Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned. Use a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
- Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

Best Practices

When preparing libraries for sequencing, always adhere to good molecular biology practices. Read through the entire protocol before starting to make sure that all of the required materials are available and your equipment is programmed and ready to use.

Handling Liquids

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes ($\pm 0.5 \mu\text{l}$) can sometimes cause large differences in cluster numbers ($\sim 100,000$).
- Small volume pipetting can be a source of potential error in protocols requiring the generation of standard curves, such as qPCR, or small but precise volumes, such as the Agilent Bioanalyzer.
- If small volumes are unavoidable, use due diligence to make sure that pipettes are correctly calibrated.
- Make sure that pipettes are not used at the volume extremes of their performance specifications.
- Prepare the reagents for multiple samples simultaneously, to minimize pipetting errors, especially with small volume enzyme additions. As a result, pipette one time from the reagent tubes with a larger volume, rather than many times with small volumes. Aliquot to individual samples in a single pipetting movement to allow for standardization across multiple samples.

Handling Magnetic Beads



NOTE

Cleanup procedures have only been validated using the 96-well plates and the magnetic stand specified in the *Consumables and Equipment* list. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats, or other magnets.

- Before use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately before use, vortex the beads until they are well dispersed and the color of the liquid is homogeneous.
- When pipetting the beads, pipette slowly and dispense slowly due to the viscosity of the solution.
- Take care to minimize bead loss, which can affect final yields.
- Change the tips for each sample, unless specified otherwise.
- Let the mixed samples incubate at room temperature for the time indicated in the protocol for maximum recovery.

- When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 μ l of supernatant is left when the eluates are removed from the bead pellet.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.
- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, because the presence of ethanol affects the performance of the subsequent reactions. Illumina recommends at least minutes drying time, but a longer drying time can be required. Remaining ethanol can be removed with a 10 μ l pipette.
- Avoid over drying the beads, which can impact final yields.
- Do not scrape the beads from the edge of the well using the pipette tip.
- To maximize sample recovery during elution, incubate the sample/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Avoiding Cross-Contamination

Practice the following to avoid cross-contamination:

- Open only one adapter tube at a time.
- Change the tips for each sample, unless specified otherwise.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.

Potential DNA Contaminants

When handling and processing samples using this protocol, use best practices to avoid PCR contamination, as you would when preparing PCR amplicons.

Temperature Considerations

Temperature is an important consideration for making libraries:

- Keep libraries at temperatures $\leq 37^{\circ}\text{C}$, except where specifically noted.
- Place reagents on ice after thawing at room temperature.

Equipment

- Review the programming instructions for your thermal cycler user guide to make sure that it is programmed appropriately using the heated lid function.
- It is acceptable to use the thermal cycler tracked heating lid function.