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16S Sequencing on Illumina MiSeq Platform

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We use this protocol and it's working

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Abstract

The 16S protocol detailed here is designed to amplify bacteria using V4-V5 hyper-variable regions of the 16S rRNA gene on the Illumina MiSeq DNA sequencer.

This protocol uses 518F and 926R primers previously published: <https://doi.org/10.1371/journal.pone.0094249>

Materials

Amplicon PCR:

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)		

Protocol and table from :

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

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	

Protocol and table from :

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

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	

Protocol and table from :

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf



Before Starting

- 1 Clean the working area and all equipment: wipe down with 10% bleach and let dry. Wipe down with 70% ethanol and let dry. Then, wipe down using RNase AWAY and let dry.
- 2 Prepare ice bucket to thaw all samples and mastermix.

Amplicon PCR

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

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

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

- 4 Seal plate and perform PCR in a thermal cycler using the following program:
94°C for 4 minutes
30 cycles of:
— 94°C for 30 seconds
— 57°C for 45 seconds
— 72°C for 60 seconds
72°C for 2 minutes
Hold at 4°C

Purify PCR product with Agencourt Ampure XP beads

- 5 Before beginning work-up, make fresh 80% ethanol, and bring AMPure XP beads to room temperature.
- 6 Centrifuge the Amplicon PCR plate at 1,000×g at 20°C for 1 minute to collect condensation, then carefully remove seal.
- 7 Transfer the entire Amplicon PCR product from the PCR plate to the MIDI plate.
- 8 Vortex the AMPure XP beads for 30 seconds to mix. Add an appropriate volume of beads to a trough.
- 9 Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate.
- 10 Seal plate and shake at 1800 rpm for 2 minutes, then incubate at room temperature without shaking for 5 minutes.
- 11 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
- 12 With the Amplicon PCR plate on the magnetic stand, remove and discard the supernatant.
- 13 With the Amplicon PCR plate on the magnetic stand, wash the beads by
 - a. Adding 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.
- 14 Repeat step 13 for a total of two washes, then remove excess ethanol with a pipette.
- 15 With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.
- 16 Remove the Amplicon PCR plate from the magnetic stand, and add 52.5 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.



- 17 Gently pipette mix up and down 10 times, or seal plate and shake at 1800 rpm for 2 minutes to mix. Make sure that beads are fully resuspended before incubating for 2 minutes at room temperature.
- 18 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
- 19 Carefully transfer 50 μ l of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate.

NOTE: Plate can be sealed and stored at -15° to -25°C for up to a week prior to indexing.

Index PCR

- 20 Transfer 5 μ l from each well to a new 96-well plate.

NOTE: The remaining 45 μ l is not used in the protocol and can be stored for other uses.
- 21 Arrange the Index 1 and 2 primers in a rack (i.e. the TruSeq Index Plate Fixture):
 - a. Arrange Index 2 primer tubes (white caps) vertically, aligned with rows A through H.
 - b. Arrange Index 1 primer tubes (orange caps) horizontally, aligned with columns 1 through 12.

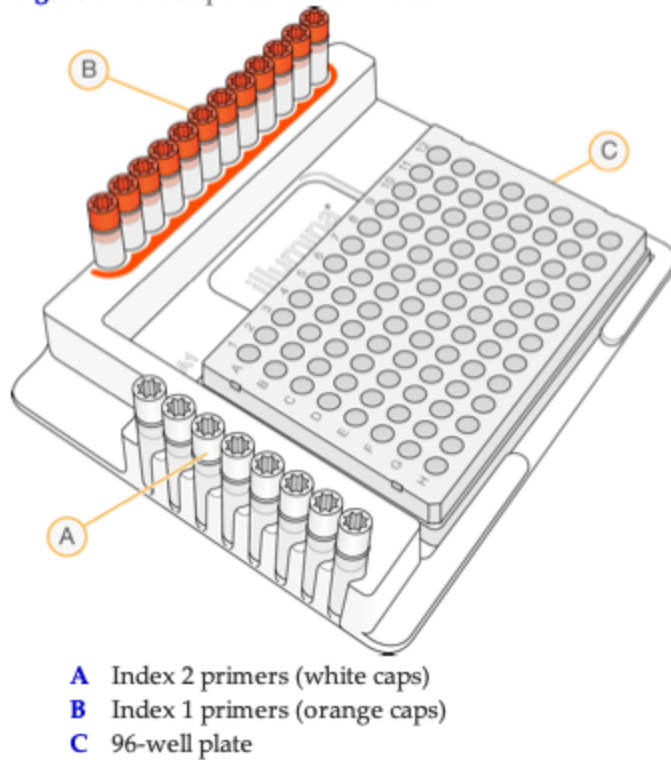
Figure 4 TruSeq Index Plate Fixture

Figure from Illumina Protocol:

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

- 22 Place the 96-well PCR plate with the 5 μ l of resuspended PCR product DNA in the TruSeq Index Plate Fixture.
- 23 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

Figure from Illumina Protocol:

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

- 24 Gently pipette up and down 10 times to mix, and seal plate with Microseal 'A'.
- 25 Centrifuge the plate at 1,000×g at 20°C for 1 minute.
- 26 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 seconds72°C for 5 minutes
Hold at 4°C

Purify PCR product with Agencourt Ampure XP beads

- 27 Before beginning work-up, make fresh 80% ethanol and bring AMPure XP beads to room temperature.
- 28 Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.
- 29 Transfer the entire Index PCR product from the PCR plate to the MIDI plate.

- 30 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.
- 31 Using a multichannel pipette, add 56 μ l of AMPure XP beads to each well of the Index PCR plate.
- 32 Seal plate and shake at 1800 rpm for 2 minutes, then incubate at room temperature without shaking for 5 minutes.
- 33 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
- 34 With the Index PCR plate on the magnetic stand, remove and discard the supernatant.
- 35 With the Index PCR plate on the magnetic stand, wash the beads by:
 - a. Adding 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.
- 36 Repeat step 35 to conduct a second wash, then use pipette with fine pipette tips to remove excess ethanol.
- 37 With the Index PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.
- 38 Remove the Index PCR plate from the magnetic stand, and add 27.5 μ l of 10 mM Tris pH 8.5 to each well of the Index PCR plate.
- 39 Seal plate and shake at 1800 rpm for 2 minutes. Then incubate at room temperature for 2 minutes.
- 40 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
- 41 Carefully transfer 25 μ l of the supernatant from the Index PCR plate to a new 96-well PCR plate.

NOTE: Plate can be sealed and stored at -15° to -25°C for up to a week prior to sequencing.

Normalize Libraries Using SequalPrep Normalization Plate Kit and Pool

- 42 Transfer 20 μ l of each Index PCR product to a SequalPrep Normalization Plate.
- 43 Add 20 μ l of SequalPrep Normalization Binding Buffer. Seal plate, vortex to mix and briefly centrifuge.
- 44 Incubate plate for 1 hour at room temperature.
- 45 Remove the liquid from the wells. Be sure to not scrape the well sides during aspiration.

Note: Amplicon/Binding Buffer mixture can be discarded or stored at -20°C for addition normalization at a later time.
- 46 Add 50 μ l of SequalPrep Normalization Wash Buffer to each well. Mix by pipetting up and down twice to improve removal of contaminants. Completely aspirate buffer from each well and discard.
- 47 Add 20 μ l of SequalPrep Normalization Elution Buffer to each well. Seal plate, vortex to mix, and centrifuge.
- 48 Incubate at room temperature for 5 minutes.
- 49 Transfer normalized DNA to a new 96-well plate. Each library should now be normalized to approximately 1 ng/ μ l
- 50 Aliquot 5 μ l of normalized DNA from each library and mix aliquots to create a pooled library with unique indices.

Quantify Pooled Library Using Qubit 2 Fluorometer

- 51 Set up the required number of QubitTM tubes for standards and samples. The QubitTM dsDNA HS Assay requires 2 standards.
NOTE: Use only thin-wall, clear, 0.5-mL PCR tubes (Cat. No. Q32856) for the QubitTM 4 Fluorometer and 8 $\times$ 200- μ L tube strips (Cat No. Q33252) for the QubitTM Flex Fluorometer.
- 52 Allow all reagents to warm up to room temperature.

- 53 Label the top of each tube lid, ensuring sides are clean to ensure accurate reading of the fluorometer.
- 54 Prepare working solution by diluting the Qubit™ dsDNA HS Reagent 1:200 in Qubit™ dsDNA HS Buffer. Use a sterile plastic tube each time you prepare the Qubit™ working solution.
- 55 Prepare each tube using the following reaction volumes for standards and samples:
Standard: 190uL working solution, 10uL standard
Sample: 198uL working solution, 2uL sample
- 56 Vigorously vortex for 3–5 seconds, avoiding the creation of bubbles. Incubate all tubes at room temperature for 2 minutes.
- 57 On Qubit instrument, select **dsDNA High Sensitivity** as the assay type, and read standards:
a. Touch **Read standards**
b. Insert the tube containing Standard #1 into the sample chamber, close the lid, then touch **Read standard**. When the reading is complete (~3 seconds), remove Standard #1.
c. Insert the tube containing Standard #2 into the sample chamber, close the lid, then touch **Read standard**. When the reading is complete, remove Standard #2.
- 58 Run Samples:
a. Touch **Run samples**.
b. On the assay screen, select the **Sample volume** and set to 2uL.
c. Insert a sample tube into the sample chamber, close the lid, then touch **Read tube**.
d. When the reading is complete (~3 seconds), remove the sample tube, and repeat until all samples have been read.

Size Verify Pooled Library using Agilent 2100 Bioanalyzer

- 59 Bring High Sensitivity DNA reagents to room temperature, allowing them to equilibrate for 30 min before preparing reactions.
- 60 After 30 minutes of equilibration at room temperature, prepare the Gel-Dye Mix:
a. Vortex DNA dye concentrate and add 25 µl of the dye to a DNA gel matrix vial.
b. Vortex solution well and spin down. Transfer to spin filter.
c. Centrifuge at 2240 g ± 20 % for 15 min. Protect the solution from light.
- 61 Load the gel dye mix into the chip priming station:
a. Put a new High Sensitivity DNA chip on the chip priming station.
b. Pipette 9.0 µl of gel-dye mix in the well third from top on the far right.

- c. Confirm plunger is positioned at 1mL, then close the chip priming station.
- d. Press the plunger until it is held by the clip, and wait for exactly 60 sec before releasing the clip.
- e. Wait for 5 sec, then slowly pull back plunger to 1ml position.
- f. Open the chip priming station and pipette 9.0 μ l of gel-dye mix in the top two wells and bottomwell on the right.



https://www.agilent.com/cs/library/usermanuals/public/2100_Bioanalyzer_HSDNA_QSG.pdf

Top image: step b

Bottom image: step f

- 62 Load the markers by pipetting 5uL of marker in all 11 sample wells and ladder wells. Ensure all wells are filled.
- 63 Load the ladder and the samples:
 - a. Pipette 1 μ l of DNA ladder in the bottom right well
 - b. In each of the 11 sample wells, pipette 1 μ l of sample. If you have unused wells, add 1 μ l of de-ionized water.
 - c. Put the chip horizontally in the adapter and vortex for 1 min at 2400 rpm.
 - d. Load chip into the Agilent 2100 bioanalyzer, and run within 5 min.

Library Denaturing and MiSeq Sample Loading

- 64 Prepare by
 - a. setting heat block to 96°C
 - b. remove a MiSeq reagent cartridge from -20°C storage and thaw at room temperature
 - c. preparing an ice bath
- 65 Calculate DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer and concentration measured by the Qubit

and dilute to 4nM if needed

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

For example:

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

- 66 Combine 5 uL of 4 nM pooled library of pooled final DNA library and 5 uL freshly diluted 0.2 N NaOH in a microcentrifuge tube. Set aside the remaining dilution of 0.2 N NaOH to prepare a PhiX control within the next 12 hours.
- 67 Vortex briefly to mix the solution, then centrifuge the sample solution at $280 \times g$ at 20°C for 1 minute.
- 68 Incubate for 5 minutes at room temperature to denature the DNA into single strands.
- 69 Add 990 uL pre-chilled HT1. This results in a 20 pM denatured library in 1 nM NaOH. Store on ice until ready to proceed to final dilution.
- 70 Dilute the denatured DNA to 3pM by using 90uL 20 pM denatured library and 510 uL pre-chilled HT1. Invert several times to mix and then pulse centrifuge the DNA solution, and store on ice.
- 71 Dilute the PhiX library to 4 nM by combining 2 uL of 10 nM PhiX library and 3 uL of 10 mM Tris pH 8.5. Then combine 5 uL of 4 nM PhiX library with 5 uL 0.2 N NaOH. Vortex briefly to mix, and incubate for 5 minutes at room temperature to denature the PhiX library.
- 72 Create a 20 pM PhiX library by adding 990 uL pre-chilled HT1 to the denatured PhiX, then dilute to 3 pM to match the concentration of the amplicon library. Invert several times to mix and then pulse centrifuge the DNA solution, and store on ice.
- 73 Combine 150 uL denatured and diluted PhiX control with 450 uL denatured and diluted amplicon library in a microcentrifuge tube, and store on ice until ready to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge.
- 74 Using a heat block, incubate the combined library and PhiX control tube at 96°C for 2 minutes.
- 75 Remove from heat block, invert 1-2 times to mix, and immediately place in the ice water bath. Keep on ice for 5 minutes.



76 Load into the MiSeq using a 500 cycle kit

Protocol references

DNA extraction protocol:

518F and 926R primers: <https://doi.org/10.1371/journal.pone.0094249>

Illumina 16S Sequencing Protocol:

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

Qubit™ dsDNA HS Assay Kit: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

Agilent DNA 1000 Kit Quick Start Guide: https://www.agilent.com/library/usermanuals/Public/G2938-90015_DNA1000Assay_QSG.pdf