Nov 24, 2025

16s Metagenomic Sequencing using Illumina iSeq100 Platform

DOI
https://dx.doi.org/10.17504/protocols.io.j8nlkyxexg5r/v1

Jasmine Velo[1], Ma. Carmel F. Javier[1], Nadelene Faith N. Cartago[1], Victor Marco Emmanuel N. Ferriols[1]

[1]Philippine Genome Center Visayas

Jasmine Velo
Philippine Genome Center Visayas

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.j8nlkyxexg5r/v1

Protocol Citation: Jasmine Velo, Ma. Carmel F. Javier, Nadelene Faith N. Cartago, Victor Marco Emmanuel N. Ferriols 2025. 16s Metagenomic Sequencing using Illumina iSeq100 Platform. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.j8nlkyxexg5r/v1

Protocol status: Working
We use this protocol and it's working

Created: November 24, 2025

Last Modified: November 24, 2025

Protocol Integer ID: 233349

Keywords: Illumina, 16s metagenomics, V4, iSeq100, scale 16s metagenomic study, metagenomic sequencing, cornerstone of microbiome research, metagenomic study, microbiome research, quality reads suitable for accurate taxonomic profiling, routine microbial community monitoring, microbial community, rrna gene, using illumina iseq100 platform, accurate taxonomic profiling, illumina iseq100 platform the characterization, effective illumina iseq100 system, sequencing parameter, broader adoption of the iseq100, iseq100, sequencing cost, indexed pcr approach

Abstract

The characterization of microbial communities through 16S rRNA gene sequencing is a cornerstone of microbiome research. While established protocols are available for larger Illumina platforms, there remains a lack of published workflows optimized for the compact and cost-effective Illumina iSeq100 system. Here, we present a standardized protocol for 16S metagenomic sequencing using the iSeq100, detailing sample preparation, library construction, and sequencing parameters. Amplicons targeting the V4 hypervariable regions of the 16S rRNA gene with a size of ~300 bp were generated using a dual-indexed PCR approach adapted for the iSeq100's 2 × 150 bp read chemistry. Library quality was evaluated through fluorometric quantification and fragment analysis prior to sequencing. Using this workflow, the iSeq100 produced high-quality reads suitable for accurate taxonomic profiling, with sufficient depth for community structure assessment across diverse environmental and biological samples. Results demonstrate that the iSeq100 is a reliable and accessible platform for small to medium-scale 16S metagenomic studies, providing consistent performance while reducing sequencing costs and turnaround time. This protocol fills a current gap in published methodologies, enabling broader adoption of the iSeq100 for microbiome research and routine microbial community monitoring.

Materials

STEP 1: AMPLICON PCR

- Genomic DNA (5 ng/µl) -15° to -25°C
- 16S V4 Reverse Primer (10 µM) -15° to -25°C
- 16S V4 Forward Primer (10 µM) -15° to -25°C
- NEB Q5 High Fidelity DNA Polymerase -15° to -25°C
- Microseal 'A' film
- 96-well 0.2 ml PCR plate

STEP 2: PCR CLEAN-UP 1

- 10 mM Tris pH 8.5 -15° to -25°C
- AMPure XP beads 2° to 8°C
- Freshly Prepared 80% Ethanol (EtOH)
- 96 well 0.2 ml PCR plate
- Microseal 'A' film
- [Optional] 96 well MIDI plate

Before start

- Prepare all PCR reagents on ice.
- Bring the AMPure XP beads to room temperature. 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 reservoir depending on the number of samples being processed.

STEP 1: AMPLICON PCR

1 Label a new PCR plate with PCR1, DATE, and APPLICATION (Ex: PCR1 04/26/2015 16S Metagenomics).

2 Prepare a mastermix using the Q5 NEB High Fidelity PCR Master mix as follows:

	A	B
	Item	Volume
	☐ PCR Buffer	5 µl
	☐ 16S Forward Primer 10 µM	1.25 µl
	☐ 16S Reverse Primer 10 µM	1.25 µl
	☐ dNTPs	0.5 µl
	☐ NEB Q5 High fidelity DNA Polymerase	0.25µl
	☐ Nuclease free water	15.75µl
	☐ Genomic DNA (5 ng/µl)	1 µl
	Total	**25 µl**

List of Primers used for PCR Amplification.

	Target Gene	Sequence	Expected Length	References

	16S rRNA (V4 primers with overhangs)	16SV4_Forward: TCGTCGGCAGCGTCAGATG TGTATAAGAGACAGGTGCCAGCMGCCGCGGTA A16SV4_Reverse: GTCTCGTGGG CTCGGAGATGTGTATAAGAGACAGGGACTACHV GGGTWTCTAAT	~450 bp	Caporaso et al. 2012; Cole et al. 2014; Kim et al. 2021

3 Load 24 µl PCR mastermix into each well of the PCR1 Plate.

4 Add 1 µl gDNA into the mastermix.

5 Seal the plate, place on a thermal cycler and run the PCR1 Program:

- 98°C for 30 seconds
- 30 cycles of:
  98°C for 10 seconds
  55°C for 30 seconds
  72°C for 30 seconds
- 72°C for 2 minutes
- Hold at 4°C

Set the reaction volume to 25 µL

STEP 2: AMPLICON PCR CLEAN-UP 1

6

	Item	Storage
	☐ 10 mM Tris pH 8.5	-15° to -25°C
	☐ AMPure XP beads	2° to 8°C
	☐ Freshly Prepared 80% Ethanol (EtOH)	
	☐ 96 well 0.2 ml PCR plate	
	☐ Microseal 'A' film	
	☐ [Optional] 96 well MIDI plate	

7 Add 20 µl of AMPure XP beads to each well of the PCR1 plate. Change tips in between samples.
- Gently pipette the entire volume up and down 10 times.
- Incubate at room temperature without shaking for 5 minutes.
- Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
- Use a pipette to remove and discard the supernatant. Change tips between samples.

8 With the PCR1 plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
- Using a pipette, add 100 µl of freshly prepared 80% ethanol to each sample well.
- Incubate the plate on the magnetic stand for 30 seconds.
- Carefully remove and discard the supernatant.

9 With the PCR1 plate on the magnetic stand, perform a second ethanol wash as follows:
- Using a pipette, add 100 µl of freshly prepared 80% ethanol to each sample well.
- Incubate the plate on the magnetic stand for 30 seconds.
- Carefully remove and discard the supernatant.
- Using a filtered tip, remove excess ethanol.

10 With the PCR1 plate still on the magnetic stand, allow the beads to air dry for 10 minutes.
- Remove the PCR 1 plate from the magnetic stand.

11 Add 25 µl of 10 mM Tris pH 8.5 to each well of the PCR1 plate.

12 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.

12.1 Incubate at room temperature for 2 minutes.

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

13 Label new PCR plate PCR CLEAN-UP 1, DATE and APPLICATION

14 Carefully transfer 20 µl of the supernatant from the PCR 1 plate to the PCR CLEAN-UP 1 plate. Change tips between samples to avoid cross contamination.

15 Perform gel electrophoresis to view the PCR Amplicons from the PCR CLEAN-UP 1 plate.

16 *SAFE STOPPING POINT.* Seal plate and store at -25°C for up to 7 days.

STEP 3: INDEX PCR

17 Label a new PCR plate INDEX PCR and DATE

18
- **Prepare a mastermix as follows:**

	A	B
	Item	Volume
	☐ PCR Buffer	5 µl
	☐ dntp	0.5 µl
	☐ Nuclease free water	4.25 µl
	☐ High fidelity DNA Polymerase	0.25 µl
	☐ IDT for Illumina UD Indexes	10 µl
	☐ PCR Clean-Up 1 product	5 µl
	Total	**25 µl**

19 Transfer **10 µl master mix** on each well of the new INDEX PCR plate.
- Gently pipette up and down 10 times to mix.

20 Add **10 µl index adapters** to each well.

21 . Add **5 µl PCR product** from the PCR CLEAN-UP 1 Plate to each well.
- Cover the plate with plastic sealing film.
- Centrifuge the plate at 1,000 × g at 20°C for 1 minute.

22 Place on a thermal cycler and run the INDEX PCR Program using the following conditions:

- **95°C for 3 minutes**
- **8 cycles of:**
  95°C for 30 seconds
  56°C for 30 seconds
  72°C for 30 seconds
- 72°C for 5 minutes
- **Hold at 4°C**

STEP 4: INDEX PCR CLEAN UP

23
- **CONSUMABLES AND REAGENTS**

	Item	Storage
	☐ 10 mM Tris pH 8.5	15° to 25°C
	☐ AMPure XP beads	2° to 8°C
	☐ Freshly Prepared 80% Ethanol (EtOH)	
	☐ 96 well 0.2 ml PCR plate	
	☐ Microseal 'A' film	

24 **THINGS TO DO BEFORE DOING INDEX PCR CLEAN UP:**
- Centrifuge the INDEX PCR plate at 280 × g at room temperature for 1 minute to collect condensation.
- 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 reservoir.

25 3. Add **56 µl of AMPure XP beads** to each well of the INDEX PCR plate.
- Gently pipette mix up and down 10 times.
- Incubate at room temperature for 5 minutes.
- Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

26 With the INDEX PCR plate on the magnetic stand, use a pipette to remove and discard the supernatant. Change tips between samples.

27 **With the INDEX PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:**
- Add 100 µl of freshly prepared 80% ethanol to each sample well.
- Incubate the plate on the magnetic stand for 30 seconds.
- Carefully remove and discard the supernatant.

28 **With the INDEX PCR plate on the magnetic stand, perform a second ethanol wash as follows:**
- Add 100 µl of freshly prepared 80% ethanol to each sample well.
- Incubate the plate on the magnetic stand for 30 seconds.
- Carefully remove and discard the supernatant.
- Use a pipette with fine pipette tips to remove excess ethanol.
- Allow the beads to air dry for 10 minutes.
- Remove the Index PCR plate from the magnetic stand.

29 Add **27.5 µl of 10 mM Tris pH 8.5** to each well of the INDEX PCR plate.
- Gently mix up and down 10 times until beads are fully resuspended, changing tips after each column.

30 Incubate at room temperature for 2 minutes.
- Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
- Label a new PCR plate PCR CLEAN-UP 2, and DATE

31 Carefully transfer **25 µl of the supernatant** from the INDEX PCR plate to the new PCR CLEAN-UP 2 plate.
- Change tips between samples to avoid cross contamination.

32 Perform gel electrophoresis to view the PCR Amplicons from the PCR CLEAN-UP 2 plate.

33 **SAFE STOPPING POINT. SEAL PLATE AND STORE AT -25°C for up to 7 days**

STEP 5: POOL AND QUANTIFY LIBRARIES

34 **Pool libraries as follows:**
- Transfer **5 µl of cleaned up PCR amplicons** from each well of the PCR CLEAN-UP 2 plate into a 1.5 ml microcentrifuge tube (**Label as Pooled Lib/PL**).

35 Analyze **1 µl library pool**, **NTC and PC** using a Qubit dsDNA HS Assay kit.
- If libraries are outside the standard range, dilute to 1:10 concentration, and analyze again.

36 Analyze **1 µl library pool**, **NTC and PC** using an Agilent Bioanalyzer DNA 1000 Assay Kit.

37 Calculate the molarity value using the following formula:

$$\text{Library concentration (nM)} = \frac{Library\ Concentration\ (\frac{ng}{ul})}{660\frac{g}{mol}\ x\ Average\ library\ size\ (bp)} x10^6$$

38 Dilute library to **4nM,** then to final library concentration of **100 pM**.

STEP 6: LOADING THE LIBRARY IN iSeq100

39 Tear open the foil packaging of the cartridge.

40 Tilt the cartridge 5 times.

41 Using a pipette tip, pierce the foil of the library reservoir.

42 Load **20 µl of diluted library** with final concentration of **100 pM** at the bottom of the library reservoir.

43 Insert the flow cell into the cartridge.

44 Load the cartridge with flow cells into the sequencer.

45 Wait for 19-24 hours until run is finished and analyze.

Protocol references

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, et al. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme J. 6 (8):1621–24.

Illumina. (2013, November 27). *16S Metagenomic Sequencing Library Preparation: Preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system* (Part # 15044223 Rev. B). Illumina, Inc. Retrieved from **https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf**

Kim, J.Y., Yi, Mh., Mahdi, A.A.S. et al. iSeq 100 for metagenomic pathogen screening in ticks. Parasites Vectors 14, 346 (2021). https://doi.org/10.1186/s13071-021-04852-w

New England Biolabs. Q5® High-Fidelity DNA Polymerase (M0491) Protocol. Available from: https://www.neb.com/en/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491