Instructions For Use

Version: 1.0 *Ref: IFU-16SMB096* *Revision date: 2023-10-04*

EasySeq™ 16S Microbiome Library Prep kit

V3-V4 hypervariable region – Illumina platform

NimaGen.

Innovators in DNA Sequencing Technologies

Product and Company Information

EasySeq™ 16S Microbiome Library Prep kit

REF RC-16SMB096

Research Use Only

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Symbols Used on Product Labels and in Instructions For Use

Symbol	Description
	Manufacturer
	Use-by date
	Lot number
	Reference number
	Temperature limit for storage
	Contains sufficient for <*n*> tests
	Matrix code containing the reference number, lot number and use-by date

Product Description

All species of archaea and bacteria have a 16S gene that codes for the small subunit of the ribosomes. This gene has highly variable regions (V1 till V9) used for taxonomical classifications. The gene also has conserved regions that can be used as targets for primers to amplify the highly variable regions. To classify the bacteria and unravel the composition of a sample, the sequences of the variable regions need to be known.

Next-generation sequencing (NGS) of the bacterial 16S ribosomal RNA gene is the gold standard to analyse the bacterial composition of a variety of samples. The EasySeq™ 16S Microbiome library prep kit contains all reagents to generate ready to sequence libraries for Illumina MiSeq™ or NextSeq™. The method supports an NGS driven analysis of the microbiome in DNA extracted from different kinds of specimen, like fecal, environmental, and sewage samples. The kit is developed for highly diverse microbial communities to enable researchers to deconvolute the composition in an unmatched easy and straightforward workflow.

The kit is based on the unique and patented Reverse Complement PCR technology, providing a safe, robust and simple workflow, combining amplification with indexing and adapter addition in a single reaction, decreasing the risk of PCR contamination and sample swapping.

A single fragment is generated comprising the V3-V4 region of the 16S rRNA gene, and is optimized to amplify a very broad range of bacterial taxa. Additionally, the kit is designed to preventing colour space issues by using phase shifting probes. Therefore, there is no need for a high PhiX spike-in thus improving sample throughput.

Kit Contents and Storage

1. NimaGen Part RC-16SMB096 (store at -20 °C)	Content
RC-PCR Probe Panel (REF: PM-16SMB)	Tube 24 µL
2x Master Mix HiFi Polymerase (REF: MM096)	Tube 1.15 mL
Probe Dilution Buffer Enhanced (REF: PDB-Enh)	Tube 216 µL

2. NimaGen Part# IDX96D-U0*x**	Content
IDX Dual Primer Plates, dehydrated. Choose one of the 8 available Index Plates for Illumina®. *Available SKU: IDX96-U01, IDX96-U02, IDX96-U03, IDX96-U04, IDX96-U05, IDX96-U06, IDX96-U07, IDX96-U08. Semi-skirted, "ABI style" PCR Plates, containing 96 different dehydrated, coloured, Unique Dual Index primer pairs, ready to use.	Sealed, breakable 96-well plate 12 strips of 8 caps

Note: When ordering multiple RC-16SMB096 kits, any combination of UDI's from the 8 available index plates can be used in a single Illumina run. This enables to combine up to 768 samples in one run.

Needed, but not included

Description	Vendor
Adjustable Pipette Set (P2, P10, P20, P100, P200, P1000)	Multiple Vendors
TapeStation, Bioanalyzer System, or equivalent, incl. consumables, or optional: agarose gel system	Agilent® or other
Ethanol absolute, mol. biol. grade	Multiple Vendors
AmpliClean™ or AMPureXP™ Bead Solution	NimaGen / Beckman Coulter
General plasticware, DNAse free (1.5 mL tubes, pipette tips w/filter)	Multiple Vendors
Ice or tabletop cooling block	Multiple Vendors
Mini Spinner for 1.5 mL tubes and 8-well PCR strips	Multiple Vendors
Magnetic Stand for 1.5 mL Eppendorf tubes	Multiple Vendors
PCR Grade Water	Multiple Vendors
Qubit™ Fluorometer including High Sensitivity consumables	Thermo Fisher
Thermocycler with heated lid (0.2 mL standard PCR tubes) compatible with semi-skirted ABI style PCR plates and option for ramp rate programming of 0.1 °C/sec (or 2% of max). The method is verified with SimpliAmp and MiniAmp thermocycler of Applied Biosystems.	Multiple Vendors

NaOH solution (2 N)	Multiple Vendors
Tris/HCl (200 mM), pH 7	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors
Illumina® MiSeq® or NextSeq®	Illumina®
Illumina® Sequencing Reagent kit (250 / 300 cycles)	Illumina®
Optional: ZymoBIOMICS Microbial Community DNA Standard (D6305/D6306)	Zymo research

General Precautions

Read the Material Safety Data Sheet (MSDS) and follow the handling instructions. Adhere to good laboratory practice and wear protective eyewear, gloves and lab coat when handling both the reagents supplied in this kit and other reagents required. Wash body parts with ample amount of water immediately if they come in contact with the reagents. Seek medical help if needed.

Use a Pre-PCR environment for setting up the RC-PCR. Pooling, cleaning and library preparation should be performed in a Post-PCR environment.

Protocol

Thermocycling program

Temp	Duration	Ramping rate (from previous step)	Cycles
98 °C	2 minutes	N/A	1 x
80 °C	1 second	Max	1 x
58 °C	10 minutes	**0.1 °C/sec (or 2% of max)**	
72 °C	1 minute	Max	
95 °C	10 seconds	Max	2 x
80 °C	1 second	Max	
58 °C	90 **minutes**	**0.1 °C/sec (or 2% of max)**	
72 °C	30 seconds	Max	
95 °C	10 seconds	Max	25 x*
80 °C	1 second	Max	
58 °C	2 minutes	**0.5 °C/sec (or 10% of max)**	
72 °C	30 seconds	Max	

Heated lid at 105 °C.

Double Check: Depending on the instrument, this protocol takes 6 hours to complete.

** Change number of cycles depending on input DNA. Use lowest number of cycles as possible. If input DNA is ≥1 ng use ≤20 cycles.*

The 16S V3-V4 RC-probe will be transformed into the functional, tailed and indexed PCR primers, followed by DNA amplification of the target region.

1. Thaw on ice:
   - RC-PCR Probe Panel (Black cap)
   - Probe Dilution Buffer Enhanced (Blue cap)
   - 2x HiFi Master Mix (Purple cap)

   Note: The Master Mix contains isostabilizers and may not freeze completely, even when stored at -15 °C to -25 °C. The Master Mix may contain precipitates when thawed at +2 °C to +8 °C. Always ensure that the Master Mix is fully thawed and thoroughly mixed before use.

2. Take the IDX PCR plate and cut off the number of strips needed.

   Note: Register the indexes used (IDX set/strip-column number and well position for each sample).

3. Prepare in a new 1.5 mL tube the Probe-Polymerase premix, by combining and mixing:
   - 0.2 µL RC-PCR Probe Panel per reaction (Black cap)
   - 1.8 µL Probe Dilution Buffer Enhanced per reaction (Blue cap)
   - 0 - 6 µL Molecular Grade Water, depending on volume of input DNA
   - 10 µL Master Mix per reaction (Purple cap)

Example: 24 samples + 10% extra volume*

- **Probe-Polymerase premix:**
  - **5.28 µL RC-PCR Probe Panel**
  - **47.52 µL Probe Dilution Buffer Enhanced**
  - **0 – 158.4 µL Molecular Grade Water**
  - **264 µL HiFi Master Mix**

***It is recommended to allow for a 10% excess when preparing the RC-PCR mix to correct for any pipetting loss. The kit contains extra reagent to facilitate this.**

4. Add to each tube of the plate: 12 - 18 µL of RC-PCR mix of the previous step.

5. Add 2 – 8 µL DNA to each well (at least 5 pg of DNA) and adjust volume to 20 µl with Molecular Grade Water if needed.

6. Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 5 pg total).

7. Close the tube strips carefully with caps provided and mix by short vortexing and a quick spin. Verify that the reaction mix has a homogeneous light pink colour.

8. Start the RC-PCR program in the thermal cycler(s).

The samples have now been amplified and tagged with sample specific indexes and sequencing adapters. From this point, PCR products can be pooled together in a single tube and purified by a bead purification to remove primers and salt.

Note: Before pooling, optionally check 3 µL of the unpurified PCR products on agarose (2%) or HSD1000 Tapestation according to manufacturer's specification.

1. Bring the beads solution to Room Temperature.

2. Combine 5 µL RC-PCR products of all the reaction wells in a 1.5 mL Eppendorf tube. (increase volume when combining less than 8 samples). If one has samples with varying but known input, three separate pools can be made. The subsequent steps should be performed per pool.

   - pool 1: ±1000 – 250 pg
   - pool 2: ±250 – 64 pg
   - pool 3: <64 pg

   Note: Appendix A can be used instead of step 2 if more control in balancing the samples is preferred but this will increase hands on time significantly.

3. Mix well and transfer 40 µL to a new 1.5 mL Eppendorf tube.

4. Add 60 µL Low TE buffer or molecular grade water to the tube and mix (total volume is now 100 µL).

5. Beads purification:
   - **a.** Vortex the beads thoroughly to resuspend.
   - **b.** Add 100 µL beads solution to the tube from step 3.5 and mix well immediately by pipetting up and down 5 times.
   - **c.** Incubate for 5 minutes, off magnet.
   - **d.** Place the tube on magnet for 3 minutes or for the solution to be fully cleared.
   - **e.** Remove and discard all liquid carefully without disturbing the beads.
   - **f.** Add 200 µL (freshly prepared) 75% ethanol, without disturbing the beads.
   - **g.** Wait for 1 minute.

   2x Ethanol wash

   - **h.** Repeat steps **e.**, **f.** and **g.** for a second ethanol wash step.
   - **i.** Carefully remove all liquid without leaving traces of ethanol. (Optionally a quick spin can be performed, then place the tube back on magnet and remove excess ethanol)
   - **j.** Dry with open cap for 2-3 minutes at Room Temperature. **Do not over-dry as this will impact yield.**

6. Elution
   - **a.** On Magnet: Add 45 µL Low TE buffer to the tubes.
   - **b.** Off Magnet: Re-suspend the beads by flicking or short vortexing.
   - **c.** Incubate for 2 minutes, off magnet.

d. Put the tube on magnet and wait for 1 - 3 minutes or for the solution to be fully cleared.

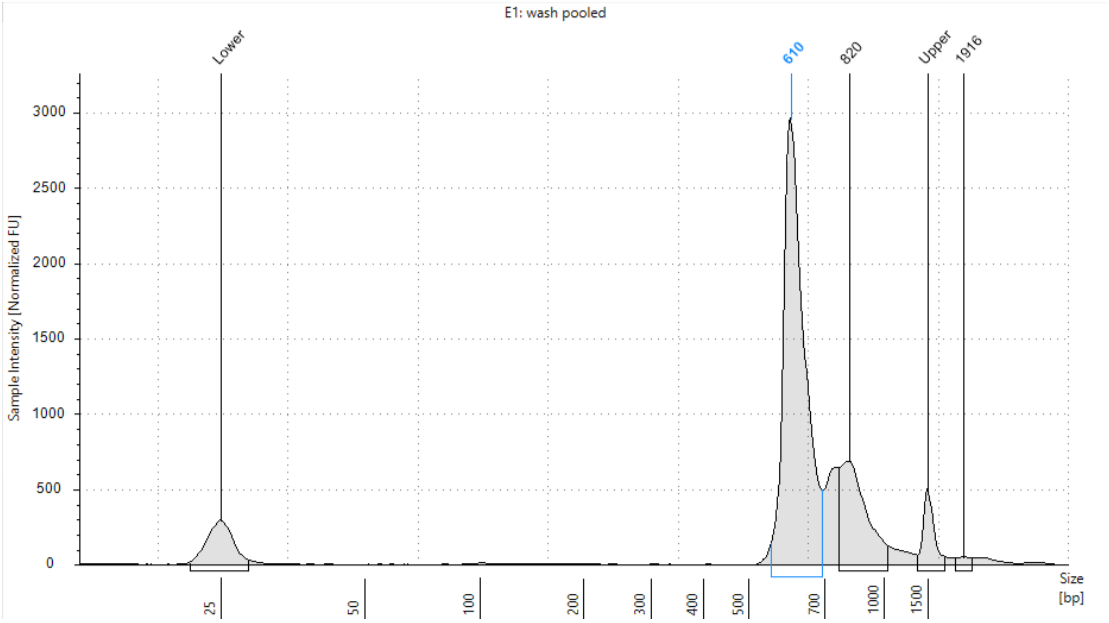
e. Carefully bring 40 µL of the clear solution to a new 1.5 mL Eppendorf tube, making sure not to transfer any of the beads.

The libraries are now ready for quantification and qualitative check

7. Determine the final concentration of the libraries by a double Qubit (HS) measurement according to manufacturer's manual and calculate the average. If three separate pools were made, pool equimolar in a new tube.

8. Verify the libraries on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the panels. Example: For TapeStation High Sensitivity kit, dilute to ~2 ng/µL.

Example of a clean library on TapeStation:



9. Sequence library on the Illumina MiSeq or NextSeq using a supported reagent kit (table 1).

a. Calculate loading with >100,000 reads per sample as it should be sufficient to deconvolute complex samples (table 1).

b. Though phase shifting primers are used to provide enough base diversity, a spike-in of 5% PhiX is recommended for QC purposes. Higher percentages up to 25% (MiSeq) or 40% (NextSeq) can be used to act as a safeguard against colour-space issues.

c. The user should start with a lower final library concentration for their initial sequence run. This avoids overclustering and potentially failure of the run. For V2 MiSeq a concentration of 4 pM, V3 MiSeq 6 pM, and NextSeq 650 pM** is suggested as a starting point and adjust in subsequent runs if needed.

Table 1 MiSeq sequence and sample multiplexing guidelines

Sequencer	Reagent kit	Run setup	Number of samples*	Paired-end reads	Library concentration
MiSeq	V3 600 cycles	301-10-10-301	250	25 million	10 pM
MiSeq	V2 500 cycles	251-10-10-251	150	15 million	6 pM
MiSeq	V2 Nano 500 cycles	251-10-10-251	10	1 million	6 pM
NextSeq 1000/2000	P1 600 cycles	301-10-10-301	768	100 million	1000 pM**
NextSeq 1000/2000	P2 600 cycles	301-10-10-301	768	300 million	1000 pM**

* Theoretical maximum with 100,000 paired-end reads per sample or maximum number of indices available (n=768) if less than 100,000 reads are needed.
** Based on Illumina recommendations and assuming onboard denature and dilution.

Note: For technical assistance contact our technical support at techsupport@nimagen.com.

Data analysis

Sequencing is performed on the Illumina MiSeq or NextSeq that can demultiplex the samples. After demultiplexing, the data could be processed by *in-house* or open-source pipelines like dada2 and QIIME2. Primer sequences (Appendix C) should be removed prior analysis using for example cutadapt.

Troubleshooting

This guide could be useful to solve potential issues that might occur. If problems persist, please contact our technical support team at techsupport@nimagen.com. If possible, provide details of the experiment, QC, and add data of the positive control.

Low PCR yield

DNA contains PCR inhibitors	Use an appropriate DNA isolation kit or method to remove PCR inhibitors. Check the DNA quality with a spectrophotometer like the Nanodrop.
Insufficient input DNA	At least 5pg of DNA should be used as input with 25 PCR cycles. Concentrate the sample or repeat DNA isolation with more input material. Alternatively, 30-35 PCR cycles could be used but will result in a higher percentage of chimeric reads.

Sequence errors

Over or under clustering	Most likely this is caused by incorrect quantification. It is therefore advised to quantify your library twice. Make sure that the quantification method is calibrated. Also, insufficient removal of primer-dimers could result in overclustering.

Primer-dimers peak after cleanup

Insufficient input DNA	At least 5 pg of DNA should be used as input. Because of too low input DNA, primer-dimers may be formed.
Improper washing of beads	Ensure that the whole bead pellet is covered with fresh 75% EtOH. If the problem persists a second cleanup could remove the remaining primer-dimers. Furthermore, resuspending the beads during washing could aid in primer-dimer removal.
Excess ethanol not removed	After the second wash, spin down the beads, return the tube on the magnet, and remove any residual liquid.

High percentage of chimeric reads

Too many PCR cycles	PCR artifacts like chimeric reads cannot be avoided but can be minimized by using the lowest number of cycles possible depending on the DNA input. Though 25 cycles is a good starting point, lower number of cycles should be used when high percentage of chimeric reads are observed.
High DNA input	Chimeric reads are also linked to amount of input DNA. Dilute the sample or decrease PCR cycles.

Appendix A: Individual sample purification and balancing

Beads purification:

a. Vortex the beads thoroughly to resuspend.
b. Add 20 µL beads solution to each well and mix immediately by pipetting up and down 5 times.
c. Incubate for 5 minutes, off magnet.
d. Place the PCR plate on the magnet for 3 minutes or until the solution is clear.

2x Ethanol wash

e. Remove and discard all liquid carefully without disturbing the beads.
f. Add 50 µL (freshly prepared) 75% ethanol, without disturbing the beads.
g. Wait for 1 minute.
h. Repeat steps **e.**, **f.** and **g.** for a second ethanol wash step.
i. Carefully remove all liquid without leaving traces of ethanol. (Optionally a quick spin can be performed, then place the tube back on magnet and remove excess ethanol)
j. Dry with open cap for 2 - 3 minutes at Room Temperature. **Do not over-dry as this will impact the yield.**

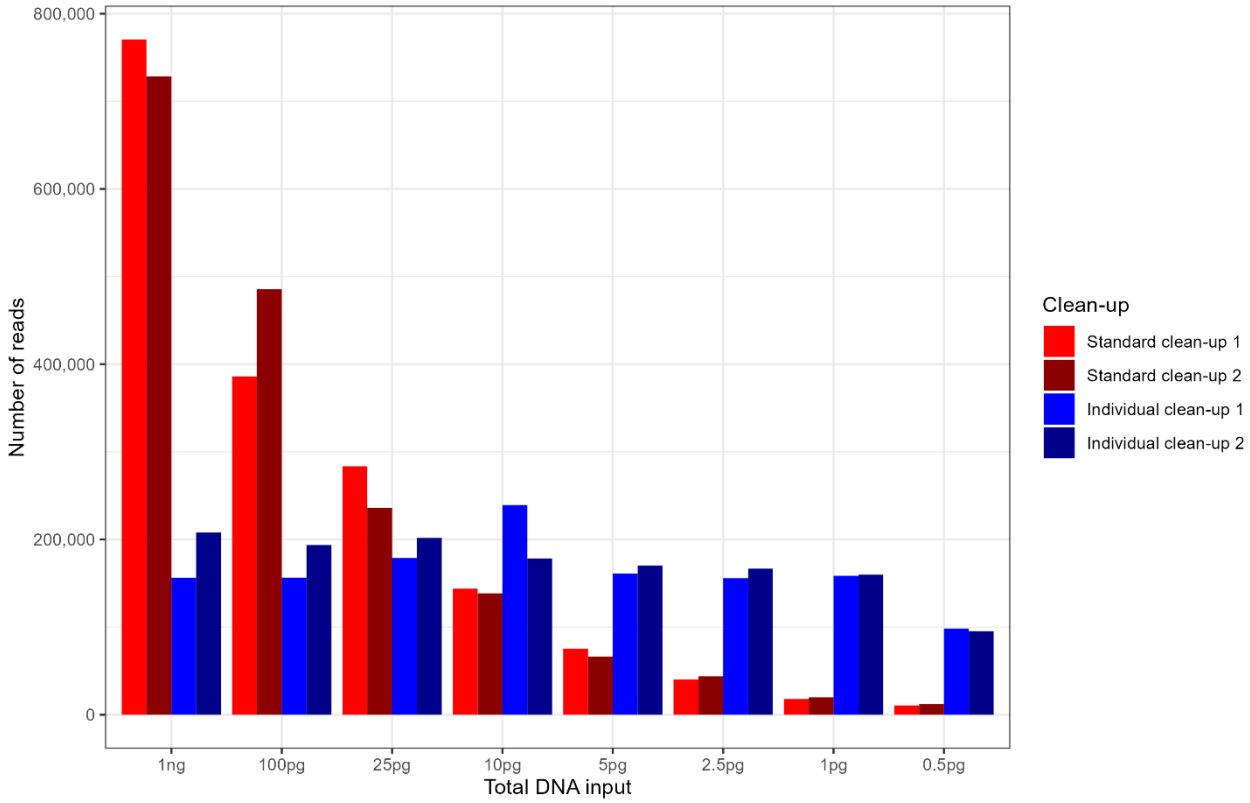
Elution

a. On Magnet: Add 25 µL Low TE buffer to all wells.
b. Off Magnet: Re-suspend the beads by flicking or short vortexing.
c. Incubate for 2 minutes, off magnet.
d. Put the tube on magnet and wait for 1-3 minutes or until the solution is clear.
e. Carefully bring 20 µL of the clear solution to a 96-well plate, making sure not to transfer any of the beads.

Pooling

a. Quantify each sample by a Qubit measurement, according to manufacturer's instructions.
b. Pool each sample equimolar into a new tube (variable µl per sample as DNA concentration might vary)
c. Continue at step 3.3

Example of standard purification versus individual clean-up and balancing:



Appendix B: Primer sequences

16S V3-V4 forward:

GACGCTCTTCCGATCTCTACCTACGGGNGGCWGCAG
GACGCTCTTCCGATCTGACCTACGGGNGGCWGCAG
GACGCTCTTCCGATCTTCCTACGGGNGGCWGCAG
GACGCTCTTCCGATCTCCTACGGGNGGCWGCAG

16S V3-V4 reverse:

CGATCGTCGAAATTCGCCATGACTACHVGGGTATCTAATCC
CGATCGTCGAAATTCGCACGACTACHVGGGTATCTAATCC
CGATCGTCGAAATTCGCTGACTACHVGGGTATCTAATCC
CGATCGTCGAAATTCGCGACTACHVGGGTATCTAATCC

Revision History

Section	Summary of changes	Version	Date
All	Not applicable. New document.	1.0	2023-10-04

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