



# Instructions For Use

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EasySeq™

## 16S rRNA

NGS Library Prep by Reverse Complement PCR

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<i>Version:</i>	3
<i>REF:</i>	IFU-16S
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## Product and Company Information

Product name:	16S rRNA
REF:	RC-16S096
Product use:	For Research Use Only
Company:	NimaGen B.V. Lagelandseweg 56 6545 CG Nijmegen The Netherlands
Telephone:	+31 (0)24 820 02 41
Email:	info@nimagen.com

## Product Use





Sequencing of the 16S ribosomal RNA gene is the gold standard for the analysis of bacterial samples: for Identification of pure cultures, mixed sample analysis and phylogeny.

EasySeq™ 16S rRNA NGS Library Prep provides the workflow for creating Illumina® compatible libraries for the sequencing analysis of the variable regions 1-6 and 9 of the bacterial 16S rRNA gene. It supports an NGS driven bacterial identification strategy for infectious disease, contamination investigation and root cause analysis testing.

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

## Kit Content

**Note: One complete kit consists of two part numbers, to be ordered separately:**

1. NimaGen Part RC-16S096 (store at -20 °C)	Content
RC-PCR Probe Panel A (REF: PM-16S-A)	Tube 24 µL 
RC-PCR Probe Panel B (REF: PM-16S-B)	Tube 24 µL 
2x Master Mix (Hot Start HiFi) (REF: MMHS096)	2 Tubes 1.15 mL 
Probe Dilution Buffer (REF: RC-PDB)	Tube 500 µL 

2. NimaGen Part# IDX96D-U0Dx*	Content
<b>IDX Dual Primer Plates, dehydrated.</b> <i>Choose one of the 8 available Index Plates for Illumina®.</i>  *Available SKU: IDX96-U01D, IDX96-U02D, IDX96-U03D, IDX96-U04D, IDX96-U05D, IDX96-U06D, IDX96-U07D, IDX96-U08D.  Semi-skirted, "ABI style" PCR Plates, containing 96 different dehydrated, coloured, Unique Dual Index primer pairs, ready to use.	2 x Sealed, breakable 96-well plates  2x 12 strips of 8 caps

**Note: When ordering multiple RC-16S096 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).	Multiple Vendors
Optional: LunaScript RT Supermix (5x)	NEB p/n E3010 or NimaGen p/n LSRT-096
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® NGS Sequencing instrument	Illumina®
Illumina® Sequencing Reagent kit (300 cycles)	Illumina®

## Procedure

### General precaution

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

### 1. Thermocycling program

Temp	Duration	Ramping rate (from previous step)	Cycles
98 °C	2 minutes	N/A	1 x
98 °C	10 seconds	Max	1 x
80 °C	1 second	Max	
58 °C	10 minutes	<b>0.1 °C/sec (or 2% of max)</b>	
72 °C	1 minute	Max	
95 °C	10 seconds	Max	2 x
80 °C	1 second	Max	
58 °C	<b>90 minutes</b>	<b>0.1 °C/sec (or 2% of max)</b>	
72 °C	30 seconds	Max	
95 °C	10 seconds	Max	34 x
80 °C	1 second	Max	
58 °C	2 minutes	<b>0.5 °C/sec (or 10% of max)</b>	
72 °C	30 seconds	Max	

Heated lid at 105 °C.

Double Check: Depending on the instrument, this protocol takes 6-7 hours to complete.

## 2. Reverse Complement PCR

The target specific RC-probes will be transformed into the functional, tailed and indexed PCR primers, followed by multiplex DNA amplification of the target regions.

### 2.1 Thaw on ice:

- RC-PCR Probe Panel A (Black cap)
- RC-PCR Probe Panel B (Red cap)
- Probe Dilution Buffer (Blue cap)
- 2x HiFi Master Mix (White 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.

### 2.2. Take the two identical IDX PCR plates and cut off the number of strips needed. Mark the plates with 'A' and 'B'.

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

**Note:** For each sample, two PCR reactions are needed (Panel A and Panel B). Always use the same well position for the same sample, in order to generate identical indexes for each sample in both panels .

### 2.3. Prepare in a fresh 1.5 mL Eppendorf tube the Probe-Polymerase premix Panel A, by combining and mixing:

- 0.2 µL RC-PCR Probe Panel A per reaction (Black cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Molecular Grade Water
- 10 µL Master Mix per reaction (White cap)

### 2.4. Prepare in a fresh 1.5 mL Eppendorf tube the Probe-Polymerase premix Panel B, by combining and mixing:

- 0.2 µL RC-PCR Probe Panel B per reaction (Red cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Molecular Grade Water
- 10 µL Master Mix per reaction (White cap)

#### Example: 24 samples + 10% extra volume\*

- Probe-Polymerase premix:
  - 5.28 µL RC-PCR Probe Panel
  - 47.52 µL Probe Dilution Buffer
  - 105.6 µL Molecular Grade Water
  - 264 µL Master Mix

\* It is recommended to allow for a 10% excess when preparing the Probe-Polymerase premix to correct for any pipetting loss. The kit contains extra reagent for this.

- 2.5. Add to each tube of plate A: 16 µL of Probe-Polymerase premix Panel A (from 2.3).
- 2.6. Add to each tube of plate B: 16 µL of Probe-Polymerase premix Panel B (from 2.4).
- 2.7. Add to each well 4 µL of DNA (100 pg total).
- 2.8. Close the tube strips carefully with caps and mix by flicking. Check for a homogeneous pink coloured reaction mix and then spin shortly.
- 2.9. Start the RC-PCR program in the thermal cycler(s) and place the samples in the cycler when the block is between 60 °C and 98 °C. Then close the lid.

### 3. Pool, Purify and Sequence

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%).

- 3.1. Bring the beads solution to Room Temperature.
- 3.2. Perform steps 3.3 to 3.7 for both Panel A and Panel B individually.
- 3.3. Combine 5 µL RC-PCR products from all the reaction wells from Panel A/B (except negative controls) in two separate 1.5 mL Eppendorf tubes.
- 3.4. Mix well and transfer 40 µL of the pools to two new 1.5 mL Eppendorf tubes.
- 3.5. Add 60 µL Low TE buffer or molecular grade water to the tubes and mix (total volume is now 100 µL).
- 3.6. Beads purification:
  - a. Vortex the beads thoroughly to resuspend.
  - b. Add 100 µL beads solution to both 100 µL pools (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.
  - 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.**

2x Ethanol wash

## 3.7. Elution

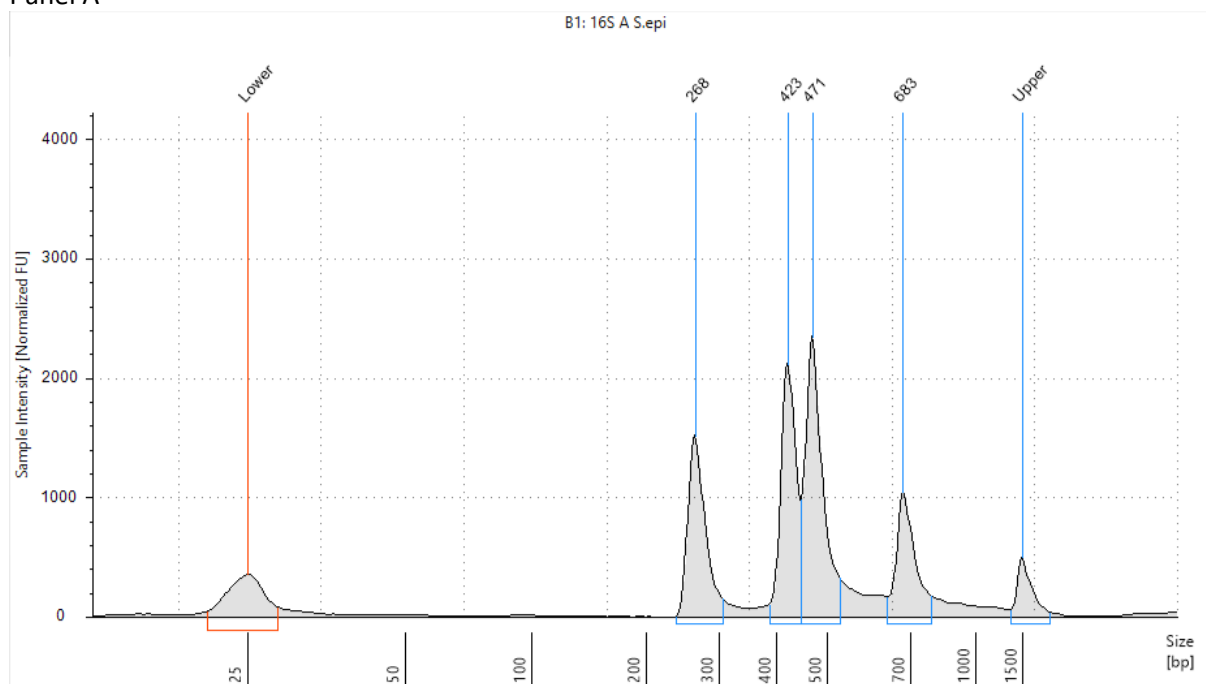
- On Magnet: Add 45  $\mu\text{L}$  Low TE buffer to the tubes.
- Off Magnet: Re-suspend the beads by flicking or short vortexing.
- Incubate for 2 minutes, off magnet.
- Put the tube on magnet and wait for 1-3 minutes or for the solution to be fully cleared.
- Carefully bring 40  $\mu\text{L}$  of the 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 qualification.

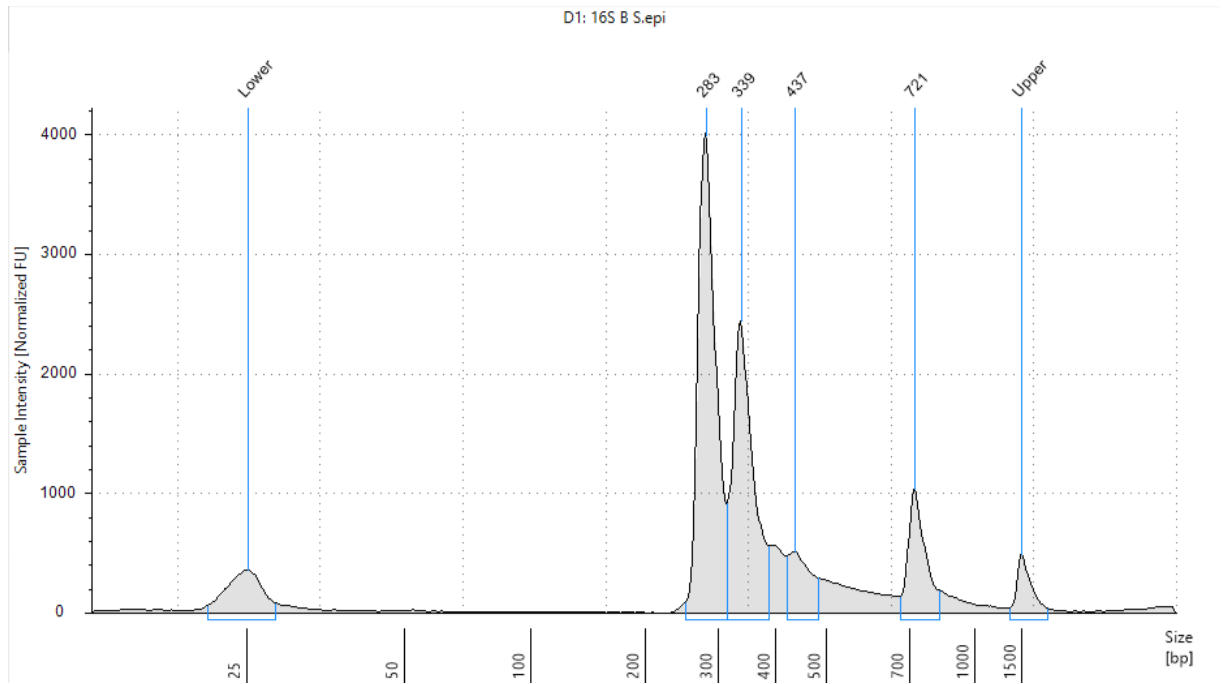
- Determine the final concentration of the libraries by a double Qubit (HS) measurement according to manufacturer's manual.
- Verify the libraries on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the panels. Example: For TapeStation High Sensitivity kit, dilute to  $\sim 2 \text{ ng}/\mu\text{L}$ .

*Examples of clean libraries on TapeStation:*

Panel A



Panel B



- Combine both purified pools equimolar and perform sequencing on an Illumina® NGS platform, according to the manufacturer's manual.
- Read at 151-8-8-151.
- Calculate loading with 2000 reads per target for mixed samples and 200 reads per target for cultures.

**Note:** For technical assistance contact our technical support at [techsupport@nimagen.com](mailto:techsupport@nimagen.com).



*Legal Notices:*

RC-PCR is patent protected (PCT/GB2016/050558, WO2016146968A1) and exclusively licensed to NimaGen B.V. Nijmegen

Qubit is a trademark of Thermo Fisher Scientific Inc.

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