Handbook

Version: 1.0 *Ref: HB_RC-ONT-16SFL* *Revision date: 2026-04-24*

EasySeq™

Full-length 16S Library Prep Kit

For long-read sequencing platforms

NimaGen.

Innovators in DNA Sequencing Technologies

Contents

Product and Company Information

EasySeq™ Full-length 16S Library Prep Kit

REF RC-ONT-16SFL096

Research Use Only

NimaGen B.V.
Hogelandseweg 88
6545 AB Nijmegen
The Netherlands
Tel: +31 (0)24 820 02 41
Email: info@nimagen.com

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
	GS1 DataMatrix, containing information about the product

Reverse Complement PCR Kit Contents

NimaGen Part# RC-ONT-16SFL096 (store at -20 °C)	Contents
Full-length 16S Probe Panel (REF: PM-ONT-16SFL)	1x Tube (24 µL)
2x Master Mix HiFi Polymerase (REF: MM096)	1x Tube (1150 µL)
Probe Dilution Buffer enhanced (REF: PDB-Enh)	1x Tube (216 µL)

Storage

All RC-PCR components of the kit should be stored at a temperature of -20 °C. The expiry date of each individual tube is stated on the label.

The dehydrated barcode plates should be stored dry, at room temperature (15–25 °C) and are stable for 1 year under these conditions. The expiry date is stated on the box label and plate.

Intended Use

EasySeq™ Full-length 16S Library Prep Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention or treatment of a disease. Research Use Only (RUO).

General Precautions

Read the Material Safety Data Sheet (MSDS) and follow the handling instructions. Adhere to good laboratory practice when handling both the reagents supplied in this kit and other reagents required.

Use a Pre-PCR environment for setting up the RC-PCR. Sample pooling, purification and library quantification should be performed in a Post-PCR environment.

All reagents need to be thawed and centrifuged before use.
Make sure to mix reagents and reactions properly.

Make sure to place the remaining strip(s) of the index plate(s) back into the provided plastic bag and include the silica gel pouch. Try to leave as little air as possible inside the plastic bag, while sealing it.

Quality Control

In accordance with NimaGen's ISO-certified Quality Management System, each lot of the EasySeq™ Full-length 16S Library Prep Kit is tested against predetermined specifications to ensure consistent product quality.

Required Materials, Not Included

Description	Vendor
EasySeq™ Barcode Plate for long read NGS, dehydrated with 96 barcodes available. **Note:** The barcode sequences are available from the download section of the NimaGen website.	NimaGen
Adjustable Pipette Set (P10, P20, P100, P200, P1000)	Multiple Vendors
TapeStation, Bioanalyzer Instrument, incl. consumables.	Agilent
Ethanol Absolute, Molecular Biology Grade	Multiple Vendors
AmpliClean™ LF	NimaGen
General plasticware, DNAse free (1.5 mL tubes, pipette tips etc.)	Multiple Vendors
Mini Spinner for 1.5 mL tubes and 8-well PCR strips or PCR plates	Multiple Vendors
Magnetic stand for 1.5 mL tubes and/or 96-wells plates	Multiple Vendors / NimaGen
Molecular grade H_2O	Multiple Vendors
Qubit Fluorometer incl. dsDNA High Sensitivity consumables	Thermo Fisher Scientific
Thermal cycler with heated lid, (0.2 mL standard PCR tubes), compatible with semi-skirted ABI style PCR plates and option for ramp rate programming. **Note:** Kit is validated for Applied Biosystems™ QuantStudio™, MiniAmp™ and SimpliAmp™ Thermal Cyclers, and Bio-Rad C1000 Touch™ Thermal Cyclers.	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors
Optional: ZymoBIOMICS Microbial Community DNA Standard (D6305/D6306)	Zymo research

Product Description

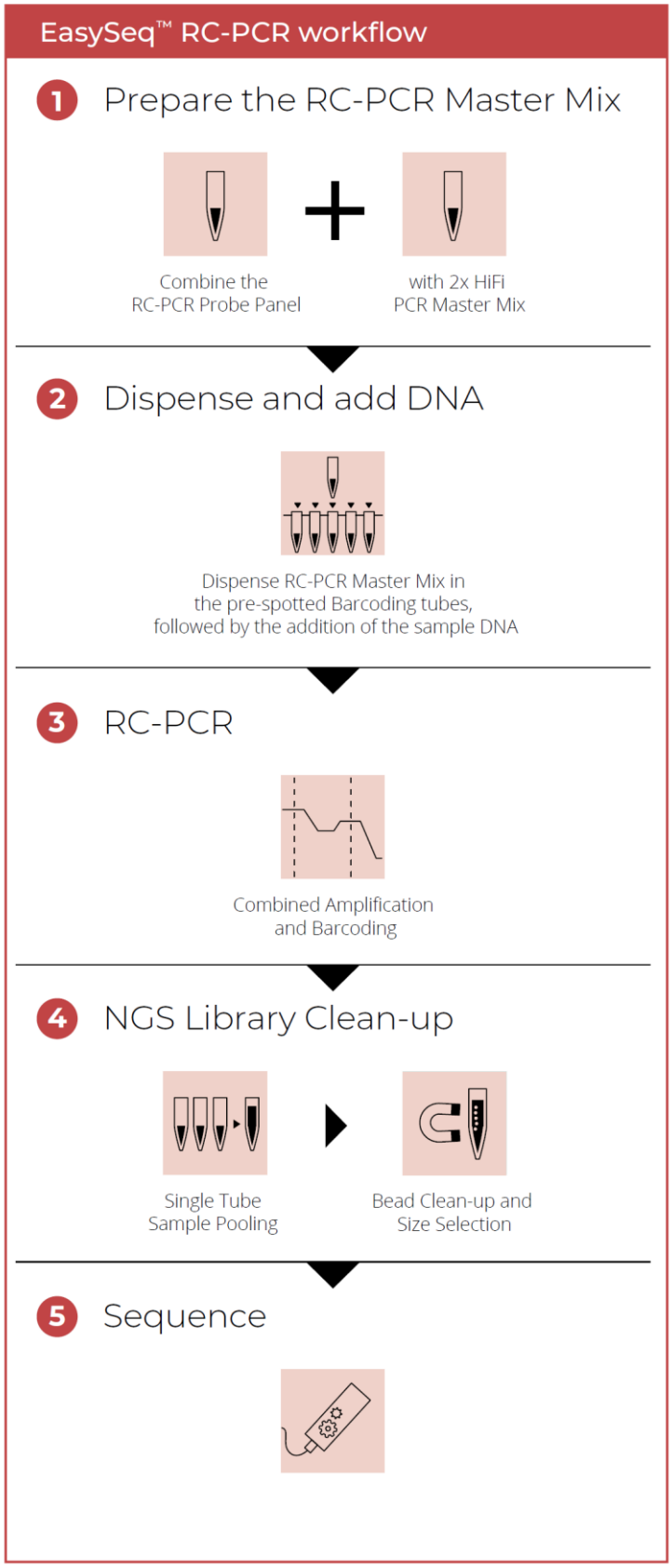
The kit is based on the unique and patented Reverse Complement PCR (RC-PCR) technology to create Next-Generation Sequencing (NGS) libraries for sequencing. The RC-PCR technology provides a safe, robust and simple workflow, combining amplification with barcoding in a single reaction, decreasing the risk of PCR contamination and sample swapping.

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, for example 27F and 1492R, 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 identify bacterial species and analyze the bacterial composition of a variety of samples. The EasySeq™ Full-length 16S Library Prep Kit contains all reagents to generate libraries ready for the Oxford Nanopore Technologies (ONT) or PacBio SMRTbell methods for amplicon sequencing. Our method supports an NGS driven analysis of whole 16S rRNA gene that allows both identification and deconvolution 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 safe and straightforward workflow. The kit is not intended for diagnostic use, but for research use only.

A single fragment is generated comprising the whole 16S rRNA gene, and is optimized to amplify a very broad range of bacterial taxa.

EasySeq™ Workflow



Important Notes

Sample Collection and Storage

Optimal results are obtained with fresh samples or those that have been immediately frozen and stored at temperatures between -80 °C and -15 °C. Repeated freezing and thawing should be avoided, as this can diminish template integrity and availability. Additionally, using low-quality starting material may result in shorter DNA fragments and lower overall yield.

Input DNA Guidelines

DNA samples should ideally be free of PCR inhibitors and only contain microbial DNA. Additionally, all DNA samples should be normalized to 1 ng/µL for optimal results. Use of a fluorescent quantification method like Qubit® prior normalization is recommended.

Non-ideal Sample Types

Low biomass or high host DNA containing samples are difficult to normalize prior PCR. The maximum DNA input is 100 ng total DNA, including host DNA. However, diluting these samples 10 or 100 times can improve microbial DNA amplification.

Processing of Samples

The standard protocol of this kit has been optimized for sample types containing high levels of microbial DNA but the kit can also be used for non-ideal samples. Please refer to the table below to determine the appropriate protocol. The samples in an experiment should be grouped and handled per sample category for optimal results. An extended protocol is provided for users who require full normalization control but this significantly increases hands-on-time.

	Ideal samples	Non-ideal samples	
Sample category	High microbial DNA	Low microbial DNA / high host DNA	Low microbial DNA & high host DNA / PCR inhibitors in DNA sample
Example sample type	Fecal, soil, culture	Water samples, intestinal tissue	Skin swabs, biopsies
Normalization method	Prior PCR	After PCR	After PCR
Recommended protocol	Standard	Non-ideal	Non-ideal with additional cleanup

The following protocols are included in this handbook:

- Standard for ideal samples that can be normalized prior PCR
- Non-ideal for samples that can't be normalized prior PCR
- Non-ideal for challenging samples that can't be normalized prior PCR
- Extended for the most control of normalization

Standard Protocol

RC-PCR Thermal Cycling

This thermal cycling program is specifically designed for the EasySeq™ Full-Length 16S Library Prep workflow and has been tested on Bio-Rad C1000, Applied Biosystems SimpliAmp, MiniAmp, Quantstudio thermal cyclers at NimaGen. Using a different thermal cycler might require other settings.

This protocol takes approximately **2.5 hours** to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program without samples, to check the predicted duration of 2.5 hours.

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	4 °C/sec*	1 x
80 °C	1 second	4 °C/sec	
55 °C	10 minutes	0.1 °C/sec (or 2% of Max)	
72 °C	1 minute	4 °C/sec	
95 °C	10 seconds	4 °C/sec	2 x
80 °C	1 second	4 °C/sec	
55 °C	**30 minutes**	0.1 °C/sec (or 2% of Max)	
72 °C	30 seconds	4 °C/sec	
95 °C	10 seconds	4 °C/sec	25 x
55 °C	30 seconds	4 °C/sec	
72 °C	30 seconds	4 °C/sec	
16 °C	∞	4 °C/sec	1 x

Heated lid at 105 °C.

***Note:** Use a max ramp rate of 4 °C/sec. If this rate is not an option for your thermal cycler, choose the highest ramp rate possible but not exceeding 4 °C/sec.

Sample Normalization

DNA samples should be free of PCR inhibitors and only contain microbial DNA. Use a fluorescent quantification method (Qubit®) to quantify each sample prior normalization. Dilute all DNA samples to equal concentration, 1 ng/µL is recommended.

Reverse Complement PCR Reaction Set-up

In a single, closed tube reaction, the target specific RC-probes are working as a template to extend the universal barcode primers to synthesize functional, tailed and barcoded PCR primers. This will be followed by two long hybridization/extension steps of 30 minutes and subsequently a further DNA amplification of the target regions, meanwhile synthesizing more primers.

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

   Note: The HiFi Master Mix contains iso-stabilizers and may not freeze completely, even when stored at -15 °C to -25 °C. It may contain precipitates when thawed at +2 °C to +8 °C.

   Note: Always ensure all components are fully thawed and thoroughly mixed before use.

2. Take the Barcode PCR plate and break off the number of strips needed.

   Note: Register the barcodes used (strip-column number and well position for each sample).

   Note: Before breaking off 8-well strips, cut the seal at the breaking line with a sharp knife.

3. Prepare in a fresh 1.5 mL tube the RC-PCR mix by combining and mixing:
   - 0.2 µL RC-PCR Probe Panel per reaction (Black cap)
   - 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
   - 10 µL HiFi Master Mix per reaction (Purple cap)

Example: 24 samples + 10% extra volume*
- 5.28 µL RC-PCR Probe Panel
- 47.52 µL Probe Dilution Buffer
- 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. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100 pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. Place the samples in the thermal cycler(s) and start the RC-PCR program.

After the RC-PCR, samples have been amplified and tagged with sample-specific barcodes. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Purification using AmpliClean LF

The purification involves one-sided size selection using magnetic beads, minimizing the number of reads lost to residual primers and dimers.

Note: Before pooling, optionally check the unpurified PCR products on agarose.

1. Bring the magnetic bead solution (AmpliClean™ LF) to **room temperature** for at least one hour**.**

   Note: It is important to allow the magnetic bead solution equilibrate to room temperature before the purification. A colder bead solution will lead to inaccurate size selection.

2. Pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube (increase volume to 15 µL when processing less than 24 samples).

3. Mix well and transfer 100 µL of this pool to a new 1.5 mL tube.

4. Beads purification using AmpliClean LF:

Purification

a. Vortex the beads thoroughly to resuspend.
b. Add 180 µL bead solution to the 100 µL pool (from step 3) and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
c. Incubate for 10 minutes.

On magnet:
d. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.
e. Remove and discard the liquid carefully, without disturbing the beads.
f. Add 300 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. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
j. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry.** Immediately continue with the Elution.

5. Elution:

a. On magnet: Add 32 µL Low TE buffer or Tris-HCl pH 8.0 buffer and close the tube.
b. Off magnet: Resuspend the beads by flicking, or by short vortexing.
c. Off magnet: Incubate for 2 minutes.
d. On magnet: Wait for 1-3 minutes, or until the solution is fully cleared.
e. On magnet: Carefully bring 30 µL of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

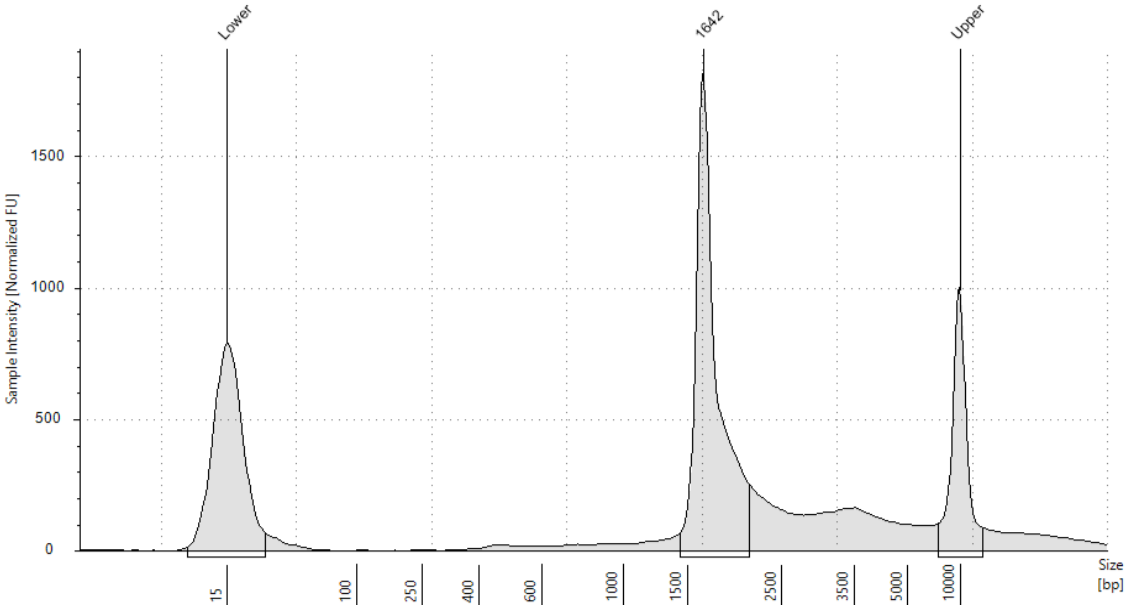
The libraries are now ready for a quantitative and qualitative check, followed by NGS.

Safe stopping point: The libraries can be stored at 4 °C for up to a week after elution. For longer storage, keep the samples at -20 °C. It is best to sequence the library within a month. Perform a new quantification after storing the library for longer than a day at either temperature.

Library Quantitative and Qualitative Check

Quantify the pooled and cleaned library with a Qubit® HS measurement. Verify the quality of the library using a TapeStation, Bioanalyzer or equivalent according to manufacturer's protocol.

Example of a clean library on TapeStation:



Downstream Processing using ONT Ligation Sequencing kit (SQK-LSK114)

Transfer 200 ng of the pooled library into a new tube and adjust the volume to 49 µL with nuclease-free water. Proceed with Section 3 (End-Prep) of the platform-specific (e.g. MinION or PromethION) Ligation sequencing amplicons V14 protocol. An adjusted protocol of version ACDE_9163_v114_revN has been used for validation (see appendix A).

Downstream Processing using PacBio SMRTbell Prep Kit 3.0

Depending on the PacBio sequencer (see table 1 of the SMRTbell 3.0 protocol), transfer 50-200 ng of the pooled library in a new 0.2 mL PCR tube strip and adjust the volume to 46 µL with low TE buffer. Proceed with Section 2 (Repair & A-tailing) of the SMRTbell 3.0 protocol.

Data Analysis

After sequencing on an ONT or PacBio platform and demultiplexing, the data could be processed by *in-house* or open-source pipelines like EMU. Commercial options for Full-length 16S analysis are compatible with our library prep kit, examples are 1928, GenomeDetective, and BugSeq.

Non-ideal Samples Protocol

RC-PCR Thermal Cycling

This thermal cycling program is specifically designed for the EasySeq™ Full-Length 16S Library Prep workflow and has been tested on Bio-Rad C1000, Applied Biosystems SimpliAmp, MiniAmp, Quantstudio thermal cyclers at NimaGen. Using a different thermal cycler might require other settings.

This protocol takes approximately **2.5-3 hours** to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program without samples, to check the predicted duration of 2.5-3 hours.

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	4 °C/sec*	1 x
80 °C	1 second	4 °C/sec	
55 °C	10 minutes	0.1 °C/sec (or 2% of Max)	
72 °C	1 minute	4 °C/sec	
95 °C	10 seconds	4 °C/sec	2 x
80 °C	1 second	4 °C/sec	
55 °C	**30 minutes**	0.1 °C/sec (or 2% of Max)	
72 °C	30 seconds	4 °C/sec	
95 °C	10 seconds	4 °C/sec	35 x
55 °C	30 seconds	4 °C/sec	
72 °C	30 seconds	4 °C/sec	
16 °C	∞	4 °C/sec	1 x

Heated lid at 105 °C.

***Note:** Use a max ramp rate of 4 °C/sec. If this rate is not an option for your thermal cycler, choose the highest ramp rate possible but not exceeding 4 °C/sec.

Reverse Complement PCR Reaction Set-up

In a single, closed tube reaction, the target specific RC-probes are working as a template to extend the universal barcode primers to synthesize functional, tailed and barcoded PCR primers. This will be followed by two long hybridization/extension steps of 30 minutes and subsequently a further DNA amplification of the target regions, meanwhile synthesizing more primers.

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

   Note: The HiFi Master Mix contains iso-stabilizers and may not freeze completely, even when stored at -15 °C to -25 °C. It may contain precipitates when thawed at +2 °C to +8 °C.

   Note: Always ensure all components are fully thawed and thoroughly mixed before use.

2. Take the Barcode PCR plate and break off the number of strips needed.

   Note: Register the barcodes used (strip-column number and well position for each sample).

   Note: Before breaking off 8-well strips, cut the seal at the breaking line with a sharp knife.

3. Prepare in a fresh 1.5 mL tube the RC-PCR mix by combining and mixing:
   - 0.2 µL RC-PCR Probe Panel per reaction (Black cap)
   - 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
   - 10 µL HiFi Master Mix per reaction (Purple cap)

Example: 24 samples + 10% extra volume*
- 5.28 µL RC-PCR Probe Panel
- 47.52 µL Probe Dilution Buffer
- 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. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. Place the samples in the thermal cycler(s) and start the RC-PCR program.

After the RC-PCR, samples have been amplified and tagged with sample-specific barcodes. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Quantification and Normalization

To optimize the read depth distribution across samples with variable or unknown input, we recommend performing a DNA quantification step after RC-PCR and prior to pooling. Quantification using the Qubit™ High Sensitivity (HS) assay, followed by equimolar pooling, improves barcode read distribution within the EasySeq™ Full-Length 16S Library Prep workflow.

Normalization procedure

Determine the concentration of RC-PCR product using a Qubit (HS) measurement:

a. Bring the Qubit reagents to room temperature
b. Label the Qubit tubes on the lid according to the number of samples
c. Measure according to manufacturer's instructions
d. Perform equimolar pooling ("Full-length 16S pooling calculator.xlsx" can be used [downloadable from the NimaGen website]).
e. Proceed with the purification using AmpliClean LF

In case equal volume pooling is preferred, pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube (increase volume to 15 µL when processing less than 24 samples). Transfer 100 µL to a new tube and proceed with the purification using AmpliClean LF.

Purification using AmpliClean LF

The purification involves one-sided size selection using magnetic beads, minimising the number of reads lost to residual primers and dimers.

Note: Before pooling, optionally check the unpurified PCR products on agarose.

Bring the magnetic bead solution (AmpliClean™ LF) to **room temperature** for at least one hour**.**

Note: It is important to allow the magnetic bead solution equilibrate to room temperature before the purification. A colder bead solution will lead to inaccurate size selection.

Purification

a. Vortex the beads thoroughly to resuspend.
b. Add 180 µL bead solution to the 100 µL pool and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
c. Incubate for 10 minutes.

On magnet:
d. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.
e. Remove and discard the liquid carefully, without disturbing the beads.
f. Add 300 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. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
j. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry.** Immediately continue with the Elution.

Elution

a. On magnet: Add 32 µL Low TE buffer or Tris-HCl pH 8.0 buffer and close the tube.
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The libraries are now ready for a quantitative and qualitative check, followed by NGS.

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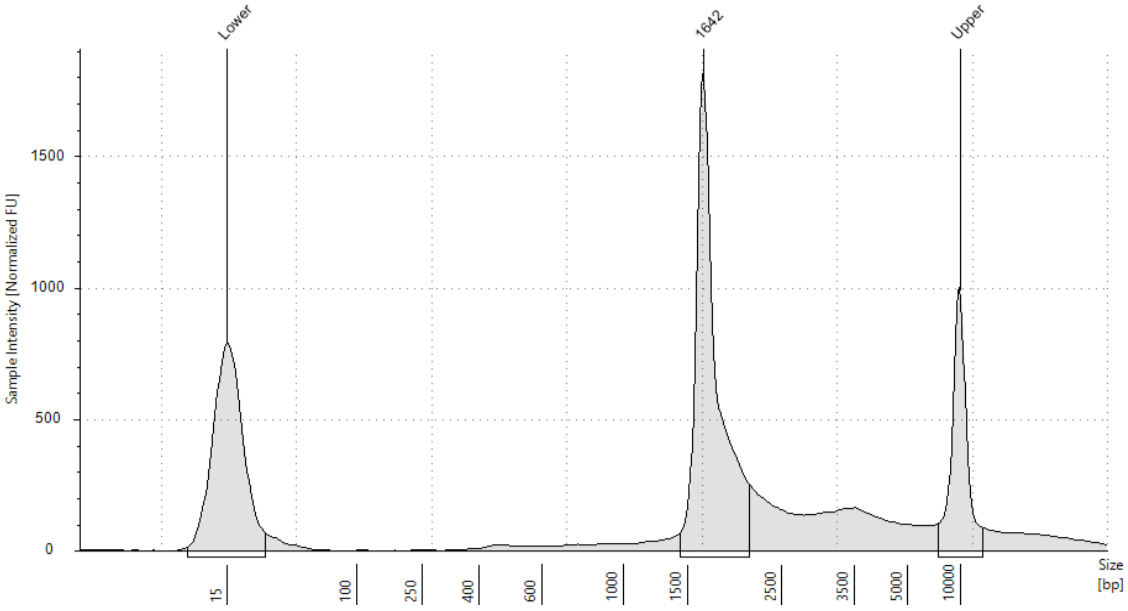

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After sequencing on a ONT or PacBio platform and demultiplexing, the data could be processed by *in-house* or open-source pipelines like EMU. Commercial options for Full-length 16S analysis are compatible with our library prep kit, examples are 1928, GenomeDetective, and BugSeq.

Non-ideal Samples Protocol with Additional Cleanup

RC-PCR Thermal Cycling

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72 °C	30 seconds	4 °C/sec	
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55 °C	30 seconds	4 °C/sec	
72 °C	30 seconds	4 °C/sec	
16 °C	∞	4 °C/sec	1 x

Heated lid at 105 °C.

***Note:** Use a max ramp rate of 4 °C/sec. If this rate is not an option for your thermal cycler, choose the highest ramp rate possible but not exceeding 4 °C/sec.

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   Note: Always ensure all components are fully thawed and thoroughly mixed before use.

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   Note: Register the barcodes used (strip-column number and well position for each sample).

   Note: Before breaking off 8-well strips, cut the seal at the breaking line with a sharp knife.

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- 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. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. Place the samples in the thermal cycler(s) and start the RC-PCR program.

After the RC-PCR, samples have been amplified and tagged with sample-specific barcodes. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Quantification and Normalization

To optimize the read depth distribution across samples with variable or unknown input, we recommend performing a DNA quantification step after RC-PCR and prior to pooling. Quantification using the Qubit™ High Sensitivity (HS) assay, followed by equimolar pooling, improves barcode read distribution within the EasySeq™ Full-Length 16S Library Prep workflow.

Normalization procedure

Determine the concentration of RC-PCR product using a Qubit (HS) measurement:

a. Bring the Qubit reagents to room temperature
b. Label the Qubit tubes on the lid according to the number of samples
c. Measure according to manufacturer's instructions
d. Perform equimolar pooling ("Full-length 16S pooling calculator.xlsx" can be used [downloadable from the NimaGen website]).
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In case equal volume pooling is preferred, pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube (increase volume to 15 µL when processing less than 24 samples). Transfer 100 µL to a new tube and proceed with the purification using AmpliClean LF.

Purification using AmpliClean LF

The purification involves one-sided size selection using magnetic beads, minimizing the number of reads lost to residual primers and dimers.

Note: Before pooling, optionally check the unpurified PCR products on agarose.

Bring the magnetic bead solution (AmpliClean™ LF) to **room temperature** for at least one hour**.**

Note: It is important to first let the magnetic bead solution equilibrate to room temperature before continuing with the purification. A colder bead solution will lead to inaccurate size selection.

Purification #1

a. Vortex the beads thoroughly to resuspend.
b. Add 180 µL bead solution to the 100 µL pool and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
c. Incubate for 10 minutes.

On magnet:
d. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.

e. Remove and discard the liquid carefully, without disturbing the beads.
f. Add 300 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. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
j. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry.**
k. Add 105 µL Low TE buffer.

Off magnet:
l. Resuspend the beads by pipetting up and down, by flicking or by short vortexing.
m. Incubate for 2 minutes.

On magnet:
n. Wait for 1-3 minutes, or until the solution is fully cleared.
o. Carefully bring 100 µL of the clear solution into a new 1.5 mL tube, ensuring not to transfer any of the beads.

Purification #2

Off magnet:
p. Vortex the beads thoroughly to resuspend.
q. Add 180 µL bead solution to the 100 µL pool (from step **o.**) and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
r. Incubate for 10 minutes.

On magnet:
s. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.

t. Remove and discard the liquid carefully, without disturbing the beads.
u. Add 300 µL (freshly prepared) 75% ethanol, without disturbing the beads.
v. Wait for 1 minute.

w. Repeat steps **t.**, **u.** and **v.** for a second ethanol wash step.
x. Carefully remove all liquid without leaving traces of ethanol. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
y. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry**. Immediately continue with the Elution.

Elution

a. On magnet: Add 32 µL Low TE buffer or Tris-HCl pH 8.0 buffer and close the tube.
b. Off magnet: Resuspend the beads by flicking, or by short vortexing.
c. Off magnet: Incubate for 2 minutes.
d. On magnet: Wait for 1-3 minutes, or until the solution is fully cleared.
e. On magnet: Carefully bring 30 µL of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

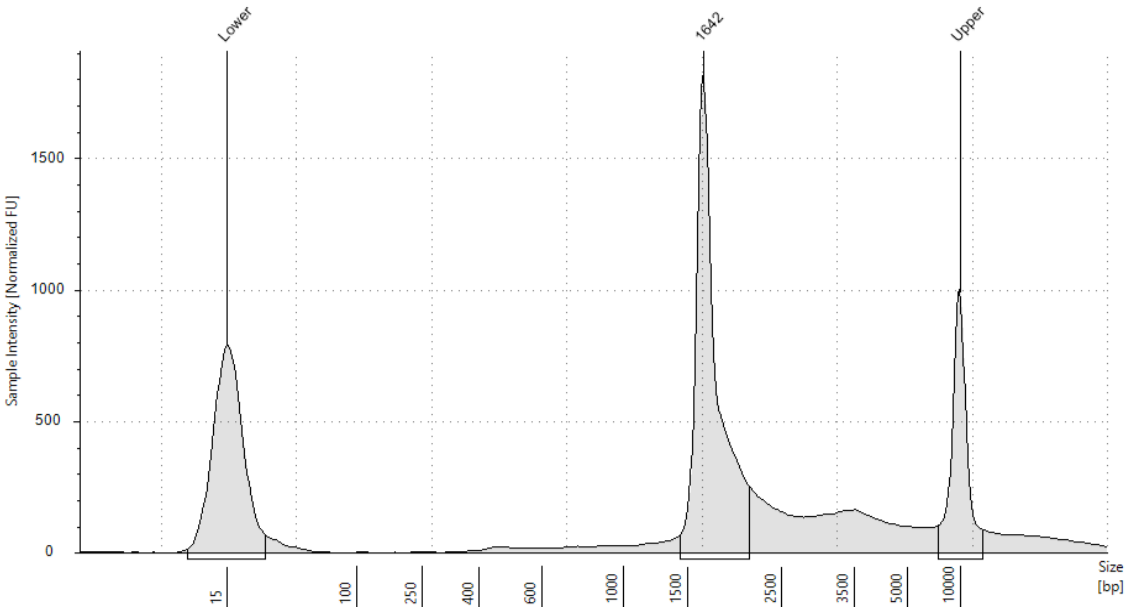
The libraries are now ready for a quantitative and qualitative check, followed by NGS.

Safe stopping point: The libraries can be stored at 4 °C for up to a week after elution. For longer storage, keep the samples at -20 °C. It is best to sequence the library within a month. Perform a new quantification after storing the library for longer than a day at either temperature.

Library Quantitative and Qualitative Check

Quantify the pooled and cleaned library with a Qubit® HS measurement. Verify the quality of the library using a TapeStation, Bioanalyzer or equivalent according to manufacturer's protocol.

Example of a clean library on TapeStation:



Downstream Processing using ONT Ligation Sequencing Kit (SQK-LSK114)

Transfer 200 ng of the pooled library in a new tube and adjust the volume to 49 µL with nuclease-free water. Proceed with Section 3 (End-Prep) of the platform-specific (e.g. MinION or PromethION) Ligation sequencing amplicons V14 protocol. An adjusted protocol of version ACDE_9163_v114_revN has been used for validation (see appendix A).

Downstream Processing using PacBio SMRTbell Prep Kit 3.0

Depending on the PacBio sequencer (see table 1 of the SMRTbell 3.0 protocol), transfer 50-200 ng of the pooled library in a new 0.2 mL PCR tube strip and adjust the volume to 46 µL with low TE buffer. Proceed with Section 2 (Repair & A-tailing) of the SMRTbell 3.0 protocol.

Data Analysis

After sequencing on a ONT or PacBio platform and demultiplexing, the data could be processed by *in-house* or open-source pipelines like EMU. Commercial options for Full-length 16S analysis are compatible with our library prep kit, examples are 1928, GenomeDetective, and BugSeq.

Extended Protocol

RC-PCR Thermal Cycling

This thermal cycling program is specifically designed for the EasySeq™ Full-Length 16S Library Prep workflow and has been tested on Bio-Rad C1000, Applied Biosystems SimpliAmp, MiniAmp, Quantstudio thermal cyclers at NimaGen. Using a different thermal cycler might require other settings.

This protocol takes approximately **2.5-3 hours** to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program without samples, to check the predicted duration of 2.5-3 hours.

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	4 °C/sec*	1 x
80 °C	1 second	4 °C/sec	
55 °C	10 minutes	0.1 °C/sec (or 2% of Max)	
72 °C	1 minute	4 °C/sec	
95 °C	10 seconds	4 °C/sec	2 x
80 °C	1 second	4 °C/sec	
55 °C	**30 minutes**	0.1 °C/sec (or 2% of Max)	
72 °C	30 seconds	4 °C/sec	
95 °C	10 seconds	4 °C/sec	35 x
55 °C	30 seconds	4 °C/sec	
72 °C	30 seconds	4 °C/sec	
16 °C	∞	4 °C/sec	1 x

Heated lid at 105 °C.

***Note:** Use a max ramp rate of 4 °C/sec. If this rate is not an option for your thermal cycler, choose the highest ramp rate possible but not exceeding 4 °C/sec.

Reverse Complement PCR Reaction Set-up

In a single, closed tube reaction, the target specific RC-probes are working as a template to extend the universal barcode primers to synthesize functional, tailed and barcoded PCR primers. This will be followed by two long hybridization/extension steps of 30 minutes and subsequently a further DNA amplification of the target regions, meanwhile synthesizing more primers.

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

   Note: The HiFi Master Mix contains iso-stabilizers and may not freeze completely, even when stored at -15 °C to -25 °C. It may contain precipitates when thawed at +2 °C to +8 °C.

   Note: Always ensure all components are fully thawed and thoroughly mixed before use.

2. Take the Barcode PCR plate and break off the number of strips needed.

   Note: Register the barcodes used (strip-column number and well position for each sample).

   Note: Before breaking off 8-well strips, cut the seal at the breaking line with a sharp knife.

3. Prepare in a fresh 1.5 mL tube the RC-PCR mix by combining and mixing:
   - 0.2 µL RC-PCR Probe Panel per reaction (Black cap)
   - 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
   - 10 µL HiFi Master Mix per reaction (Purple cap)

Example: 24 samples + 10% extra volume*
- 5.28 µL RC-PCR Probe Panel
- 47.52 µL Probe Dilution Buffer
- 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. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. Place the samples in the thermal cycler(s) and start the RC-PCR program.

After the RC-PCR, samples have been amplified and tagged with sample-specific barcodes. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Purification using AmpliClean LF

The purification involves one-sided size selection using magnetic beads, minimizing the number of reads lost to residual primers and dimers. The procedure will be performed per individual sample instead of a pooled purification.

Bring the magnetic bead solution (AmpliClean™ LF) to **room temperature** for at least one hour**.**

Note: It is important to allow the magnetic bead solution equilibrate to room temperature before the purification. A colder bead solution will lead to inaccurate size selection.

Purification #1

a. Vortex the beads thoroughly to resuspend.
b. Add 36 µL bead solution to each sample and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
c. Incubate for 10 minutes.

On magnet:
d. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.

e. Remove and discard the liquid carefully, without disturbing the beads.
f. Add 80 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. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
j. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry.**
k. Add 22 µL Low TE buffer.

Off magnet:
l. Resuspend the beads by pipetting up and down, by flicking or by short vortexing.
m. Incubate for 2 minutes.

On magnet:
n. Wait for 1-3 minutes, or until the solution is fully cleared.
o. Carefully bring 20 µL of the clear solution into a new PCR plate, ensuring not to transfer any of the beads.

Purification #2

Off magnet:
p. Vortex the beads thoroughly to resuspend.
q. Add 36 µL bead solution to each sample and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
r. Incubate for 10 minutes.

On magnet:
s. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.

t. Remove and discard the liquid carefully, without disturbing the beads.
u. Add 80 µL (freshly prepared) 75% ethanol, without disturbing the beads.
v. Wait for 1 minute.

w. Repeat steps **t.**, **u.** and **v.** for a second ethanol wash step.
x. Carefully remove all liquid without leaving traces of ethanol. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
y. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry**. Immediately continue with the Elution.

Elution

a. On magnet: Add 12 µL Low TE buffer or Tris-HCl pH 8.0 buffer and close the tube.
b. Off magnet: Resuspend the beads by flicking, or by short vortexing.
c. Off magnet: Incubate for 2 minutes.
d. On magnet: Wait for 1-3 minutes, or until the solution is fully cleared.
e. On magnet: Carefully bring 10 µL of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

The libraries are now ready for a quantitative and qualitative check, followed by NGS.

Safe stopping point: The libraries can be stored at 4 °C for up to a week after elution. For longer storage, keep the samples at -20 °C. It is best to sequence the library within a month. Perform a new quantification after storing the library for longer than a day at either temperature.

Quantification and Pooling

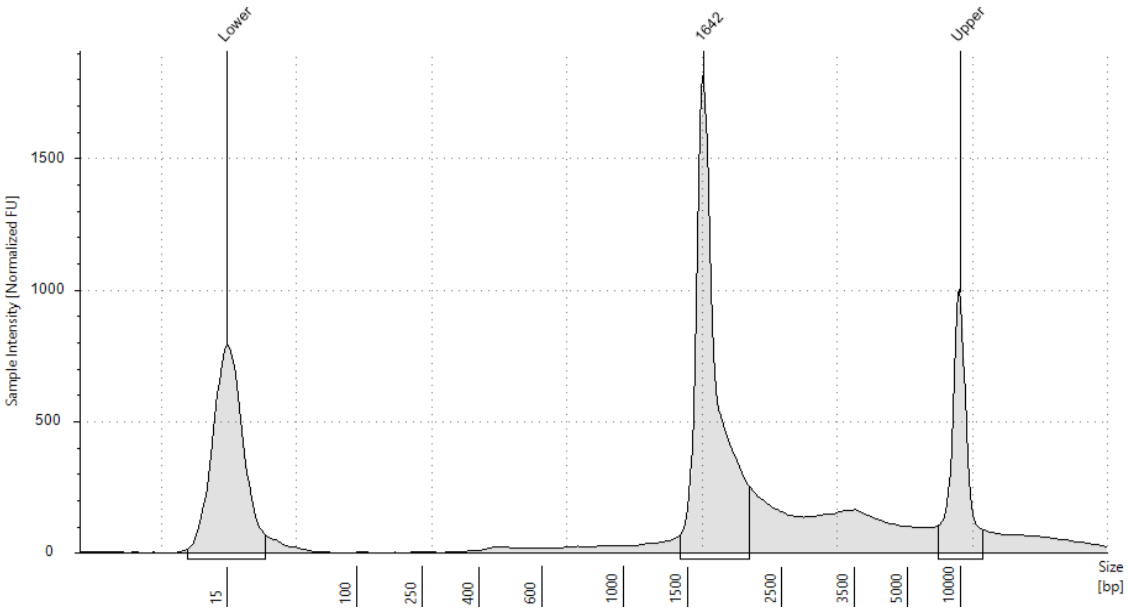
Determine the concentration of the purified samples using a Qubit (HS) measurement:

a. Bring the Qubit reagents to room temperature
b. Label the Qubit tubes on the lid according to the number of samples
c. Measure according to manufacturer's instructions
d. Perform equimolar pooling (“Full-length 16S pooling calculator.xlsx” can be used [downloadable from the NimaGen website]).

Library Quantitative and Qualitative Check

Quantify the pooled and cleaned library with a Qubit® HS measurement. Verify the quality of the library using a TapeStation, Bioanalyzer or equivalent according to manufacturer's protocol.

Example of a clean library on TapeStation:



Downstream Processing using ONT Ligation Sequencing Kit (SQK-LSK114)

Transfer 200 ng of the pooled library in a new tube and adjust the volume to 49 µL with nuclease-free water. Proceed with Section 3 (End-Prep) of the platform-specific (e.g. MinION or PromethION) Ligation sequencing amplicons V14 protocol. An adjusted protocol of version ACDE_9163_v114_revN has been used for validation (see appendix A).

Downstream Processing using PacBio SMRTbell Prep Kit 3.0

Depending on the PacBio sequencer (see table 1 of the SMRTbell 3.0 protocol), transfer 50-200 ng of the pooled library in a new 0.2 mL PCR tube strip and adjust the volume to 46 µL with low TE buffer. Proceed with Section 2 (Repair & A-tailing) of the SMRTbell 3.0 protocol.

Data Analysis

After sequencing on a ONT or PacBio platform and demultiplexing, the data could be processed by *in-house* or open-source pipelines like EMU. Commercial options for Full-length 16S analysis are compatible with our library prep kit, examples are 1928, GenomeDetective, and BugSeq.

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	Concentrate the sample or repeat DNA isolation with more input material. Alternatively, up to 40 PCR cycles could be used but will result in a higher percentage of background 16S reads.

Cleanup

Low yield	Increase pool volume from 100 µL to 200 µL and elute in the volume of lowTE or Tris-HCl pH 8.0.

Primer-dimers peak after cleanup

Insufficient input DNA	Because of very low input DNA or negative samples, primer-dimers may be formed.
Improper washing of beads	Ensure that the whole bead pellet is covered with fresh 75% EtOH. 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.

Low sequence output

Over or under loaded flow cell	Most likely this is caused by incorrect quantification. Make sure that the quantification method is calibrated. Also, insufficient removal of primer-dimers and adapters could result in low sequencing output. Furthermore, over or under loading of a flow cell could have detrimental effects on the pores.
Air bubbles introduced	Air bubbles will irreversibly damage the pores on a Nanopore flow cell resulting in low to no sequence output. Avoid introduction of air by checking liquid levels on the flow cell and pipettip.
Inefficient adapter ligation	Proper end-prep is necessary to ligate the sequencing adapter. Traces of ethanol after bead clean-up and incorrect handling of the NEB reagents will impact the efficiency.
Incompatible sequence kit used	Only the ligation kits with the Ligation Adapter are compatible with the EasySeq™ Full-length 16S Library Prep Kit. Native Barcoding or Rapid kits are not compatible and will not be attached to the 16S amplicons.
Expired flow cell	Expired flow cell could have low pore counts that impacts the sequence output. Also, the average Q-score could be lower.
Improper adapter wash	Resuspension of the beads after adapter ligation is needed to properly wash free adapter.

Customer Support

For technical questions, assistance, or to suggest enhancements, please contact us at techsupport@nimagen.com.

Revision History

Section	Summary of changes	Version	Date
All	New document.	1.0	2026-04-24

Appendix

Appendix A: Adjusted Ligation Sequencing Amplicons (version ACDE_9163_v114_revN)

1. End-prep:
    - **a.** Perform a flow cell check prior end-prep.
    - **b.** Prepare the NEBNext Ultra II End Repair / dA-tailing module according to manufacturer's instructions.
        - Thaw all reagents on ice.
        - Mix well by flicking or invert the tubes. **Do not vortex the Ultra II End Enzyme mix.**
        - Spin down the tubes before opening
        - The Ultra II End Prep Buffer might have some precipitate. Allow the buffer to come to room temperature and mix by pipetting or vortexing.
    - **c.** Transfer 100-400 fmol DNA (100-400 ng 1.6 kb) of cleaned RC-PCR products in a new PCR tube. Adjust to 50 µL with nuclease-free water if needed.
    - **d.** Add 7 µL Ultra II End-Prep reaction buffer.
    - **e.** Add 3 µL Ultra II End-prep Enzyme mix.
    - **f.** Mix well by pipetting with a sufficient volume (for example 40 µL).
    - **g.** Incubate at 20 °C for 5 minutes and 65 °C for 5 minutes in a thermocycler.

2. Bead purification 1:
    - **a.** Vortex the beads thoroughly to resuspend.
    - **b.** Add 60 µL beads solution to the end-prep tube 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 1-3 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 30 seconds.
    - **h.** Repeat steps **e.**, **f.** and **g.** for a second ethanol wash step.

      2x Ethanol wash
    - **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 1-3 minutes at Room Temperature.
      Do not over-dry as this will impact yield.

3. Elution 1:
    - **a.** On Magnet: Add 61 µL Molecular Grade water to the tube.
    - **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 minute or for the solution to be fully cleared.
    - **e.** Transfer 61 µL eluate to a new tube.
    - **f.** Quantify 1 µL of the eluted sample using a Qubit fluorometer.

4. Adapter ligation:
   - **a.** Prepare the Ligation Adapter, Ligation Buffer of the ONT ligation kit, and the Quick T4 ligase.
     - Thaw the Ligation adapter and Quick T4 ligase on ice. Thaw the Ligation Buffer at room temperature
     - Mix well by flicking or invert the tubes except the Ligation Buffer, mix the Ligation Buffer by pipetting. **Do not vortex any tube.**
     - Spin down the tubes before opening.
   - **b.** Add 25 µL Ligation buffer (LNB) to the 60 µL DNA of step 1.3.
   - **c.** Add 5 µL Ligation Adapter.
   - **d.** Add 10 µL Quick T4 ligase.
   - **e.** Mix well by gentle pipetting with a sufficient volume (for example 60 µL).
   - **f.** Incubate at room temperature 10 minutes.

5. Bead purification 2:
   - **a.** Vortex the beads thoroughly to resuspend.
   - **b.** Add 60 µL beads solution to the adapter ligation tube mix well by pipetting carefully up and down 5 times.
   - **c.** Incubate for 5 minutes, off magnet.
   - **d.** Prepare wash buffer mix by mixing 80 µL Short Fragment Buffer (SFB) with 240 µL Long Fragment Buffer (LFB).
   - **e.** Place the tube on magnet for 2 minutes or for the solution to be fully cleared.
   - **f.** Remove and discard all liquid carefully without disturbing the beads.
   - **g.** Add 160 µL SFB/LFB mix (1:3), flick or pipette to resuspend the beads.
     Note: Resuspension is needed to properly wash free adapter.
   - **h.** Place the tube on the magnet for 1-3 minutes.
   - **i.** Repeat steps **f.**, **g.** and **h.** for a second wash step.
   - **j.** Carefully remove all liquid without leaving traces of liquid. (Optionally a quick spin can be performed, then place the tube back on magnet and remove excess liquid)
   - **k.** Dry with open cap for 30 seconds at Room Temperature.
     Do not over-dry as this will impact yield.

6. Elution 2:
   - **a.** Off magnet: Add 15 µL Elution Buffer (EB) to the tube.
   - **b.** Re-suspend the beads by flicking or pipetting.
   - **c.** Incubate for 10 minutes, off magnet.
   - **d.** Put the tube on magnet and wait for 1 minute or for the solution to be fully cleared.
   - **e.** Transfer 15 µL eluate to a new tube.
   - **f.** Quantify 1 µL of the eluted sample using a Qubit fluorometer.

7. Prepare flow cell and library:
   - **a.** In a new tube add 1170 µL Flow cell flush with 30 µL Flow cell tether and mix by pipetting (prime mix).
   - **b.** Set a P1000 pipette to 200 µL and insert in the priming port. Drawback 20 µL from the flow cell or until you can see a small volume of storage buffer.

c. Load 800 µL prime mix via the priming port on the flow cell by turning the wheel slowly (priming should take >1 minute). **Note: ensure no air bubbles are introduced as it will damage the flow cell.**

d. Wait 5 minutes.

e. Open the SpotOn port and load 200 µL prime mix via the priming port on the flow cell by turning the wheel slowly.

f. Prepare the library by mixing 37.5 µL sequencing buffer, 25.5 µL library beads, 12 µL DNA library to load 50-100 fmol (55-110 ng for 16S amplicon + adapters), and adjust volume to 75 µL with Elution Buffer.

g. Add the library to the flow cell.

h. Start sequence run and use the following settings:
- Select LSK-114 (or future ligation kit) and PBC-096 as barcode expansion pack.
- Basecall with high or super-high accuracy model.
- If fast intermediate analysis is required, set frequency in the output section to 10 minutes.
- It is recommended to enable POD5 output files to be able to perform basecalling again if for example incorrect setting were used.

i. Check sequence run after 15 minutes. Select free pores, pores in strand (sequencing), and adapter. The percentage sequencing / total pores should be >70%. Wash performance can also be checked as a proper wash result in <3% adapter. Improper wash (no resuspension) results in >15% adapter.

Appendix B: Primer Sequences

Full-length 16S forward primers:
GAAGRGTTYGATYMTGGCTCAG
AGAGTTTGATCATGGCTTAG
AGAATTTGATCTTGGTTCAG

Full-length 16S reverse primer:
SGGYTACCTTGTTACGACTT

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NimaGen B.V.
Hogelandseweg 88
6545 AB Nijmegen
The Netherlands
www.nimagen.com