Instructions For Use

Version: 1.0 *Ref: IFU-ONT-16SFL096* *Revision date: 2024-03-01*

EasySeq™

Full-length 16S Library Prep Kit

Oxford Nanopore Technologies platform

For NGS Library Prep by Reverse Complement PCR

NimaGen.

Innovators in DNA Sequencing Technologies

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 Instruction For Use

Symbol	Description
	Manufacturer
	Use-by date
LOT	Lot number
REF	Reference number
	Temperature limit for storage
Σ	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, 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 analyse 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) ligation method 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 faecal, 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.

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 whole 16S rRNA gene, and is optimized to amplify a very broad range of bacterial taxa.

Reverse Complement PCR Kit Contents

NimaGen Part# RC-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)

Required Materials, Not Included

Description	Vendor
EasySeq™ Barcode Plate for ONT, 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™ or AMPure XP Bead Solution	NimaGen / Beckman Coulter
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
Water, PCR Grade	Multiple Vendors
Qubit Fluorometer incl. High Sensitivity consumables	Thermo Fisher Scientific
Thermocycler 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 Cycler.	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors
Oxford Nanopore Technologies (ONT) Sequencing Instrument (Flongle/MinION/GridION/PromethION/P2 Solo)	ONT
Oxford Nanopore Technologies (ONT) Flow Cell	ONT
Oxford Nanopore Technologies (ONT) Ligation Sequencing Kit **Note:** The Full-length 16S Library Prep Kit is validated with the SQK-LSK114 Ligation Sequencing Kit. The kit is not compatible with the Native Barcoding Kit.	ONT
NEBNext Ultra II End repair/dA-tailing Module (E7546S or E7546L)	New England Biolabs
NEBNext Quick Ligation Module (E6056S or E6056L)	New England Biolabs
Optional: ZymoBIOMICS Microbial Community DNA Standard (D6305/D6306)	Zymo research
Optional: SYBR™ Green I, 10,000X in DSMO	Multiple Vendors

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.

Protocol

1. Thermocycling Program

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

Heated lid at 105 °C.

Note: 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 as a dummy run, to check the predicted duration of 2.5 hours.
*Change number of cycles depending on input DNA. Use lowest number of cycles as possible. If input microbial DNA input is ≥1 ng use ≤20 cycles.

2. Reverse Complement PCR

In a single, closed tube reaction, the target specific RC-probes are working as a template to extend the UDI 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.

2.1 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.

2.2. 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.

2.3. 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.

2.4. Remove the seal from the PCR plate or strip(s).

2.5. Dispense 12 µL of the RC-PCR mix (from step 2.3) to each well of the plate/strip(s).

2.6. Add to each well 8 µL of DNA solution.

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

2.7. Close the tube strips **carefully** with the caps provided, there should be an audible click (or use a(n) (optical) seal). Mix by short vortexing, followed by a quick spin. Verify that the colour of the reaction mix is homogenously pink.

2.8. 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.

3. Purification

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

3.1. Bring the magnetic bead solution (AmpliClean™ or AMpure XP) to room temperature.

3.2. Pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube.

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

3.3. Mix well and transfer 100 µL of this pool into a new 1.5 mL tube.

3.4. Beads purifications:

Purification #1

a. Vortex the beads thoroughly to resuspend.
b. Add 60 µL bead solution to the 100 µL pool (from step 3.2) and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
c. Incubate for 5 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 1-3 minutes at room temperature. **Do not over-dry.**
k. Add 102 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. Add 60 µL resuspended 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.
q. Incubate for 5 minutes.

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

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

v. Repeat steps **s.**, **t.** and **u.** for a second ethanol wash step.
w. 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).
x. Dry with open cap for 1-3 minutes at room temperature. **Do not over-dry**. Immediately continue with the Elution.

3.5. Elution:

a. On magnet: Add 32 µL Molecular Grade Water to the tube 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.

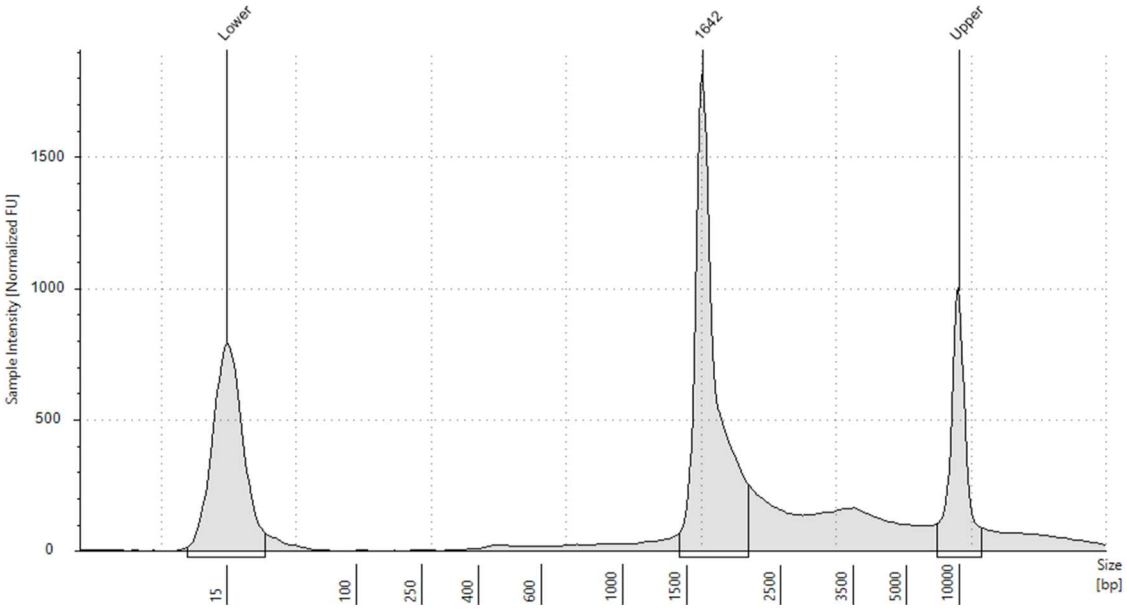
4. Sequencing

4.1. Determine the final concentration of the library or libraries by a double 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 to be used plus 2 standards.
- **c.** Dilute the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer for each sample/ standard. It is recommended to allow for >10% excess when preparing the working solution to correct for any pipetting loss.
- **d.** For the standards: mix 190 µL of the working solution with 10 µL of the standard.
- **e.** For the samples: mix 180-199 µL of the working solution with 1-20 µL sample (total 200 µL).
- **f.** Vortex the tubes thoroughly and incubate the tubes for 2 minutes.
- **g.** Measure the standards and the samples using the 'dsDNA High Sensitivity' settings making sure to select the correct sample volume used in step **e.**.

4.2. **Optional but recommended:** Perform a qualitative verification of the library on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the pool. E.g. for TapeStation High Sensitivity kit, dilute to ~2 ng/µL.

Example of a clean library on TapeStation:



Check the nanopore website for the latest protocols suiting your needs and sequence kits. Below an adjusted amplicon sequencing protocol for the Flongle R10.4.1 flow cell using LSK-114 ligation sequence kit is described. Appendix A describes the adjusted protocol for the R10.4.1. MinION flow cell.

4.3. 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.

4.3.b.1. Thaw all reagents on ice.

4.3.b.2. Mix well by flicking or invert the tubes. **Do not vortex the Ultra II End Enzyme mix.**
4.3.b.3. Spin down the tubes before opening
4.3.b.4. 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 50-200 fmol DNA (50-200 ng 1.6 kb) of cleaned RC-PCR products in a new PCR tube. Adjust to 25 µl with nuclease-free water if needed.
d. Add 3.5 µl Ultra II End-Prep reaction buffer.
e. Add 1.5 µl Ultra II End-prep Enzyme mix.
f. Mix well by pipetting with a sufficient volume (for example 10 µl).
g. Incubate at 20 °C for 5 minutes and 65 °C for 5 minutes in a thermocycler.

4.4 Bead purification 1:

a. Vortex the beads thoroughly to resuspend.
b. Add 30 µL beads solution to the tube from step 4.3 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.

2x Ethanol wash

e. Remove and discard all liquid carefully without disturbing the beads.
f. Add 100 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.

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.**

4.5. Elution 1:

a. On Magnet: Add 31 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 31 µL eluate to a new tube.
f. Quantify 1 µL of the eluted sample using a Qubit fluorometer.

4.6. Adapter ligation

a. Prepare the Ligation Adapter, Ligation Buffer of the ONT ligation kit, and the Quick T4 ligase.

4.6.a.1. Thaw the Ligation adapter and Quick T4 ligase on ice. Thaw the Ligation Buffer at room temperature

4.6.a.2. Mix well by flicking or invert the tubes except the Ligation Buffer, mix the Ligation Buffer by pipetting. **Do not vortex any tube.**
4.6.a.3. Spin down the tubes before opening.

b. Add 12.5 µl Ligation buffer (LNB) to the 30 µl DNA of step 4.5.
c. Add 2.5 µl Ligation Adapter.
d. Add 5 µl Quick T4 ligase.
e. Mix well by pipetting with a sufficient volume (for example 20 µl).
f. Incubate at room temperature 10 minutes.

4.7 Bead purification 2:

a. Vortex the beads thoroughly to resuspend.
b. Add 30 µL beads solution to the tube from step 4.6 and mix well by pipetting carefully up and down 5 times.
c. Incubate for 5 minutes, off magnet.
d. Prepare wash buffer mix by mixing 40 µL Short Fragment Buffer (SFB) with 120 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 80 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 <u>without leaving traces of liquid.</u> (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.**

4.8. Elution 2:

a. Off magnet: Add 7 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 7 µL eluate to a new tube.
f. Quantify 1 µL of the eluted sample using a Qubit fluorometer.

4.9. Prepare flow cell and library:

a. In a new tube add 117µl Flow cell flush with 3µl Flow cell tether and mix by pipetting.
b. Prime 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.**
c. Prepare the library by mixing 15µl sequencing buffer, 10µl library beads, 1-5 µl DNA to load 10-50fmol (12-55ng for 16S amplicon + adapters), and adjust volume to 30 µl with Elution Buffer.
d. Add the library to the flow cell.

e. Start sequence run and use the following settings:
- 4.9.e.1. Select LSK-114 (or future ligation kit) and PCB-096 as barcode expansion pack.
- 4.9.e.2. Set minimal read-length to 1000bp.
- 4.9.e.3. For best results, basecall with super-high accuracy model and set barcoding score to 70.
- 4.9.e.4. If fast intermediate analysis is required, set POD5 and FASTQ reads per file to 100.

f. 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.

Data Analysis

Sequencing is performed on any Oxford Nanopore Technologies (ONT) platform using MinKNOW software that can demultiplex the samples. After demultiplexing, the data could be processed by *in-house* or open-source pipelines like EMU and Kraken2. Adapter, barcode, and primer sequences should be removed prior analysis using for example NanoFilt by removing 120bp on both ends (other length removal is needed when barcodes are removed by MinKNOW).

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, 40 PCR cycles could be used but will result in a higher percentage of background 16S reads.

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 the 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-lengths 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. Furthermore, the correct mix of SFB:LFB (1:3) should be used as it will impact the size selection.

Appendix A: Adjusted LSK-114 sequence protocol for MinION flow cell

Check the nanopore website for the latest protocols suiting your needs and sequence kits. Below an adjusted amplicon sequencing protocol for the MinION R10.4.1 flow cell using LSK-114 ligation sequence kit is described.

1.1. 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.

1.1.b.1. Thaw all reagents on ice.
1.1.b.2. Mix well by flicking or invert the tubes. **Do not vortex the Ultra II End Enzyme mix.**
1.1.b.3. Spin down the tubes before opening
1.1.b.4. 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.

1.2 Bead purification 1:

a. Vortex the beads thoroughly to resuspend.
b. Add 60 µL beads solution to the tube from step 1.1 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.

2x Ethanol wash

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.

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.

1.3 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.

1.4 Adapter ligation

a. Prepare the Ligation Adapter, Ligation Buffer of the ONT ligation kit, and the Quick T4 ligase.

1.4.a.1 Thaw the Ligation adapter and Quick T4 ligase on ice. Thaw the Ligation Buffer at room temperature
1.4.a.2 Mix well by flicking or invert the tubes except the Ligation Buffer, mix the Ligation Buffer by pipetting. **Do not vortex any tube.**
1.4.a.3 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.

1.5 Bead purification 2:

a. Vortex the beads thoroughly to resuspend.
b. Add 60 µL beads solution to the tube from step 1.4 and 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 <u>without leaving traces of liquid.</u> (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.

1.6 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.

1.7 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. Draw back 20µl 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:
  1.7.h.1 select LSK-114 (or future ligation kit) and PCB-096 as barcode expansion pack.
  1.7.h.2 Set minimal read-length to 1000bp.
  1.7.h.3 For best results, basecall with super-high accuracy model and set barcoding score to 70.
  1.7.h.4 If fast intermediate analysis is required, set POD5 and FASTQ reads per file to 100.
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.

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	2024-03-01

Legal Notice

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www.nimagen.com