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Version 6

Nanopore amplicon sequencing V.6

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Protocol status: Working

We use this protocol and it's working



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Abstract

This protocol describes the step-by-step procedures for performing nanopore amplicon sequencing using the latest Kit 14 chemistry. The method is versatile and can be applied to a wide range of sequence-based analyses, including human microbiome profiling and biodiversity assessment using environmental DNA (eDNA metabarcoding).

Materials

Reagents and kits

- DNA sample
- Tailed PCR primers (target-specific primers with anchor sequences at the 5' ends)
- PrimeSTAR Max DNA Polymerase Ver.2 (TaKaRa, R047A)
- PCR Barcoding Expansion 1–12 (Oxford Nanopore Technologies, EXP-PBC001) or PCR Barcoding Expansion 1–96 (Oxford Nanopore Technologies, EXP-PBC096)
- ProNex Size-Selective Purification System (Promega, NG2001)
- 80% ethanol
- QuantiFluor ONE dsDNA System (Promega, E4871)
- NEBNext Ultra II End Repair/dA-tailing Module (New England Biolabs, E7546S)
- Ligation Sequencing Kit V14 (Oxford Nanopore Technologies, SQK-LSK114)
- Salt-T4 DNA Ligase (New England Biolabs, M0467S)
- UltraPure BSA (50 mg/mL) (Thermo Fisher Scientific, AM2616)
- Flow Cell Wash Kit (Oxford Nanopore Technologies, EXP-WSH004)

Equipments

- Thermal cycler
- Gel electrophoresis apparatus
- Magnetic separation rack
- Microcentrifuge
- Vortex mixer
- Quantus Fluorometer (Promega, E6150)
- MinION Mk1D (Oxford Nanopore Technologies, MIN-101D)
- MinION Flow Cell R10.4.1 (Oxford Nanopore Technologies, FLO-MIN114)
- Apple Silicon Mac



Protocol materials

⊗ NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

⊗ PCR Barcoding Expansion 1–12 **Oxford Nanopore Technologies Catalog #EXP-PBC001**

⊗ PCR Barcoding Expansion 1–96 **Oxford Nanopore Technologies Catalog #EXP-PBC096**

⊗ PrimeSTAR Max DNA Polymerase Ver.2 **Takara Bio Inc. Catalog #R047A**

⊗ QuantiFluor ONE dsDNA System **Promega Catalog #E4871**

⊗ Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #SQK-LSK114**

⊗ Salt-T4 DNA Ligase **New England Biolabs Catalog #M0467S**

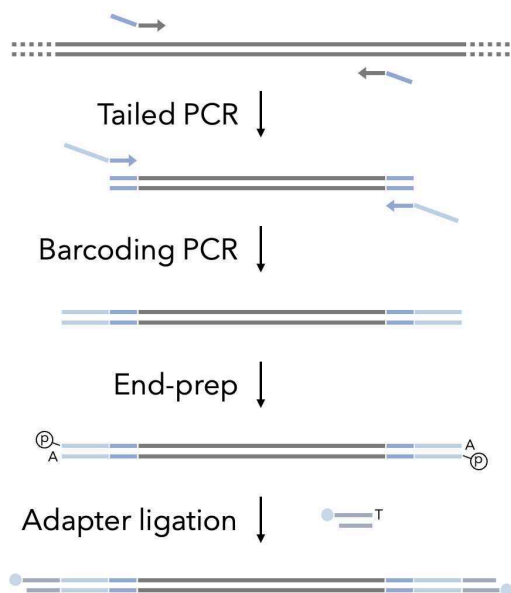
⊗ Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #EXP-WSH004**

⊗ ProNex Size-Selective Purification System **Promega Catalog #NG2001**

⊗ UltraPure BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #AM2616**

Workflow

1



In the first-round PCR reaction, the region of interest is amplified using specific primers flanked by anchor sequences. The tailed PCR products are then subjected to a second round of PCR to incorporate barcode sequences for multiplexing. The barcoded amplicons are pooled and end-prepped, allowing for the ligation of sequencing adapters.

First-round PCR with tailed primers

2 Prepare the PCR master mix.

A	B	C
Component	Volume	Final conc.
Template DNA	x μL	
5 μM FW/RV primer mix	1.5–2.5 μL	0.3–0.5 μM each
PrimeSTAR Max Ver.2 Premix (2X)	12.5 μL	1X
Water	up to 25 μL	

 PrimeSTAR Max DNA Polymerase Ver.2 **Takara Bio Inc.** Catalog #R047A

Tailed primers (user-supplied)

A	B
Primer	Sequence
Forward (FW)	5'-TTTCTGTTGGTGCTGATATTGC - target-specific sequence -3'
Reverse (RV)	5'-ACTTGCCTGTCGCTCTATCTTC - target-specific sequence -3'

The 5' anchor sequences serve as priming sites for barcoded primers used in the 2nd PCR.

Note

The following tailed primers are used for amplifying the V1–V9 region of the bacterial 16S rRNA gene. 16S rRNA gene-specific sequences are in bold letters.

- 27F*:
5'-TTTCTGTTGGTGCTGATATTGC **AGRGTTYGATYMTGGCTCAG**-3'
- 1492R:
5'-ACTTGCCTGTCGCTCTATCTTC **CRGHTACCTTGTTACGACTT**-3'

*We observed that the 27F universal primer described in Protocol Version 5 exhibits reduced amplification efficiency and specificity, likely due to its increased degeneracy. To avoid these issues, the primer used in the previous version of the protocol, as described above, is recommended.

3 Perform PCR.

A	B	C	D
Step	Temperature	Time	Cycles
Denaturation	98°C	10 sec	25–35
Annealing	57°C	15 sec	
Extension	68°C	5–10 sec/kb*	
Hold	4°C	∞	1

*e.g. Use an extension time of 10 seconds per cycle to amplify the near-full length (V1–V9 regions) of bacterial 16S rRNA genes (approximately 1.5 kbp), using the high-speed PrimeSTAR DNA polymerase.



- Analyze 1–2 μL of the PCR products by gel electrophoresis to verify successful amplification.

PCR clean-up (Optional)

- Allow the ProNex beads to equilibrate to room temperature.

Note

The first-round PCR products may need to be purified before proceeding to the second-round of PCR. This additional step removes reaction contaminants, which would be beneficial for the downstream analysis.



ProNex Size-Selective Purification System **Promega Catalog #NG2001**

- Add ProNex beads to the first-round PCR products and mix by pipetting.

A	B
Component	Volume
First-round PCR products	20 μL
ProNex Chemistry	20 μL

Note

The ratio of ProNex beads to sample varies depending on the desired DNA size cutoff range. The condition shown above will enrich DNA fragments larger than 1 kbp using a 1:1 ratio of ProNex beads to sample.

As an alternative to ProNex beads, AMPure XP can also be used. The ratio of AMPure beads to the sample should be adjusted accordingly.

- Incubate at Room temperature for 00:05:00 .
- Place the tube on a magnetic rack for 00:02:00 .



Equipment

NGS MagnaStand v.3 8Ch

NAME

Magnetic rack (0.2 mL tube)

TYPE

FastGene

BRAND

FG-SSMAG3

SKU

9 Pipette off the supernatant.

10 Wash the beads once with 80% ethanol using the following procedure:

10.1 Keeping on the magnetic rack, add  200 μ L of 80% ethanol without disturbing the bead pellet.

10.2 Discard the supernatant.

11 Spin down the tube and place it back on the magnetic rack.

12 Pipette off any residual ethanol.

13 Remove the tube from the magnetic rack and allow the sample to air-dry for ~2 min.

14 Resuspend the beads in  20 μ L of water.

15 Incubate at  Room temperature for  00:05:00 .



- 16 Place the tube on the magnetic rack for  00:02:00 .
- 17 Transfer the eluate to a new tube.

Second-round PCR with barcoded primers

- 18 Prepare the PCR master mix.

A	B
Component	Volume
First-round PCR products	1.0 μ L
PCR Barcode (BC)*	0.5 μ L
PrimeSTAR Max Ver.2 Premix (2X)	12.5 μ L
Water	11 μ L
Total	25 μ L

*One of barcoded primers (10 μ M BC) supplied in the PCR Barcoding Expansion (EXP-PBC001 or EXP-PBC096).



PCR Barcoding Expansion 1–12 **Oxford Nanopore Technologies Catalog** #EXP-PBC001



PCR Barcoding Expansion 1–96 **Oxford Nanopore Technologies Catalog** #EXP-PBC096

- 19 Perform PCR to incorporate barcode sequences.

A	B	C	D
Step	Temperature	Time	Cycles
Denaturation	98°C	10 sec	5–10
Annealing	57°C	15 sec	
Extension	68°C	5–10 sec/kb*	
Hold	4°C	∞	1

*e.g. Use an extension time of 10 seconds per cycle to amplify the near-full length (V1–V9 regions) of bacterial 16S rRNA genes (approximately 1.5 kbp), using the high-speed



PrimeSTAR DNA polymerase.

- 20 Analyze 1–2 μL of the PCR products by gel electrophoresis.

PCR clean-up

14m

- 21 Allow the ProNex beads to equilibrate to room temperature.

- 22 Pool the barcoded amplicons into a single tube.

Note

As an example, when working with 10 barcodes, pool 10 μL from each sample to obtain a final volume of 100 μL ; alternatively, the samples can be pooled in desired ratios, and the total volume can be adjusted flexibly depending on the experimental requirements.

Each sample may also be cleaned up separately prior to pooling. After quantifying the DNA, the samples can be pooled in the desired ratios to balance input amounts and achieve uniform sequencing data, and subsequently subjected to the End-prep (step 41).

- 23 Add ProNex beads to the pooled sample and mix by pipetting.

	A	B
	Component	Volume
	Pooled sample	100 μL
	ProNex Chemistry	100 μL

This is an example protocol based on an input sample volume of 100 μL .

Note

The ratio of ProNex beads to sample varies depending on the desired DNA size cutoff range. The condition shown above will enrich DNA fragments larger than 1 kbp using a 1:1 ratio of ProNex beads to sample.

As an alternative to ProNex beads, AMPure XP can also be used. The ratio of AMPure beads to the sample should be adjusted accordingly.



24 Incubate at Room temperature for 00:05:00 .

5m

25 Place the tube on a magnetic rack for 00:02:00 .

2m

26 Pipette off the supernatant.

27 Wash the beads with 80% ethanol as follows:

27.1 Keeping on the magnetic rack, add 200 μ L of 80% ethanol without disturbing the bead pellet.

27.2 Discard the supernatant.

28 Repeat steps 27.1 and 27.2 for a total of two washes.

29 Spin down the tube and place it back on the magnetic rack.

30 Pipette off any residual ethanol.

31 Remove the tube from the magnetic rack and allow the sample to air-dry for ~2 min.

32 Resuspend the beads in 26 μ L of water.

33 Incubate at Room temperature for 00:05:00 .

5m

34 Place the tube on the magnetic rack for 00:02:00 .

2m

35 Transfer the eluate to a new tube.



DNA quantification

- 36 Warm QuantiFluor ONE dsDNA dye to Room temperature .
- QuantiFluor ONE dsDNA System **Promega Catalog #E4871**
- 37 Add 1 μL of eluted sample to 200 μL of QuantiFluor ONE dsDNA dye in 0.5 mL tube.
- 38 Mix thoroughly by vortexing.
- 39 Incubate at Room temperature for 00:05:00 , protected from light. 5m
- 40 Measure fluorescence using the Quantus Fluorometer to quantify DNA concentration.

Equipment

Quantus Fluorometer

NAME

Promega

BRAND

E6150

SKU

End-prep

- 41 Prepare 200 fmol of pooled DNA (from step 35) in 25 μL of water.

Note

For full-length 16S rRNA gene sequencing, 200 fmol of 1.5 kbp amplicons correspond to approximately 200 ng in weight.

- 42 Mix the following components.



A	B
Component	Volume
Pooled amplicon DNA	25 µL
NEBNext Ultra II End Prep Reaction Buffer	3.5 µL
NEBNext Ultra II End Prep Enzyme Mix	1.5 µL
Total	30 µL



NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England**
Biolabs Catalog #E7546S

- 43 Incubate the reaction using a thermal cycler.

A	B
Temperature	Time
20°C	5 min
65°C	5 min
4°C	∞

Note

Amplicons are converted into 5'-phosphorylated and 3'-dA-tailed DNA, enabling ligation of Nanopore sequencing adapters.

Clean-up

14m

- 44 Allow the ProNex beads to equilibrate to room temperature.

- 45 Add ProNex beads to the end-prep reaction and mix by pipetting.

A	B
Component	Volume
End-prepped DNA	30 µL



A	B
ProNex Chemistry	30 µL

Note

The ratio of beads to sample volume should be adjusted according to the desired DNA fragment size. As an alternative to ProNex beads, AMPure XP can also be used.

46 Incubate at Room temperature for 00:05:00 .

5m

47 Place the tube on a magnetic rack for 00:02:00 .

2m

48 Pipette off the supernatant.

49 Wash the beads with 80% ethanol as follows:

49.1 Keeping on the magnetic rack, add 200 µL of 80% ethanol without disturbing the bead pellet.

49.2 Discard the supernatant.

50 Repeat steps 49.1 and 49.2 for a total of two washes.

51 Spin down the tube and place it back on the magnetic rack.

52 Pipette off any residual ethanol.

53 Remove the tube from the magnetic rack and allow the sample to air-dry for ~2 min.



- 54 Resuspend the beads in  30 μL of water.
- 55 Incubate at  Room temperature for  00:05:00 .
- 56 Place the tube on the magnetic rack for  00:02:00 .
- 57 Transfer the eluate to a new tube.

5m

2m

Adapter ligation

10m

- 58 Mix the following components.

A	B
Component	Volume
End-prepped DNA	30 μL
Ligation Buffer (LNB)*	12.5 μL
Ligation Adapter (LA)	2.5 μL
Salt-T4 DNA Ligase	5 μL
Total	50 μL

The Ligation Buffer (LNB) and Ligation Adapter (LA) are included in the Ligation Sequencing Kit V14.

*LNB is viscous and should be thoroughly mixed by pipetting.



Ligation Sequencing Kit V14 **Oxford Nanopore Technologies** Catalog #SQK-LSK114



Salt-T4 DNA Ligase **New England Biolabs** Catalog #M0467S

- 59 Incubate at  Room temperature for  00:10:00 .

10m

Clean-up

12m

- 60 Allow the ProNex beads to equilibrate to room temperature.



- 61 Add ProNex beads to the adapter-ligated sample and mix by pipetting.

A	B
Component	Volume
Adapter-ligated DNA	50 µL
ProNex Chemistry	50 µL

Note

The ratio of beads to sample volume should be adjusted according to the desired DNA fragment size. As an alternative to ProNex beads, AMPure XP can also be used.

- 62 Incubate at Room temperature for 00:05:00 .

5m

- 63 Place the tube on a magnetic rack for 00:02:00 .

- 64 Pipette off the supernatant.

- 65 Wash the beads with either Short Fragment Buffer (SFB) or Long Fragment Buffer (LFB) as follows:

Note

The Short Fragment Buffer (SFB) and Long Fragment Buffer (LFB) are included in the Ligation Sequencing Kit V14.

For full-length 16S rRNA gene amplicons (approximately 1.5 kbp), use SFB to retain DNA fragments of all sizes. LFB should be used to enrich for DNA fragments larger than 3 kbp.

- 65.1 Remove the tube from the magnetic rack and resuspend the beads in 100 µL of either SFB or LFB.



65.2 Place the tube on the magnetic rack for  00:02:00 .

2m

65.3 Discard the supernatant.

66 Repeat steps 65.1–65.3 for a total of two washes.

67 Spin down the tube and place it back on the magnetic rack.

68 Pipette off any residual wash buffer.

69 Remove the tube from the magnetic rack and allow the sample to air-dry for ~2 min.

70 Resuspend the beads in  15 μ L of Elution Buffer (EB).

Note

The Elution Buffer (EB) is included in the Ligation Sequencing Kit V14.

71 Incubate at  Room temperature for  00:05:00 .

5m

72 Place the tube on the magnetic rack for  00:02:00 .

73 Transfer the eluate to a new tube.

DNA quantification

74 Warm QuantiFluor ONE dsDNA dye to  Room temperature .

- 75 Add  1 μL of eluted sample to  200 μL of QuantiFluor ONE dsDNA dye in 0.5 mL tube.
- 76 Mix thoroughly by vortexing.
- 77 Incubate at  Room temperature for  00:05:00 , protected from light.
- 78 Measure fluorescence using the Quantus Fluorometer to quantify DNA concentration.

Flow cell check

- 79 Connect the MinION sequencer to the host computer and start the MinKNOW software.

Equipment

MinION Mk1D	NAME
Sequencer	TYPE
Oxford Nanopore Technologies	BRAND
MIN-101D	SKU

- 80 Open the MinION lid and insert a flow cell (R10.4.1) under the clip.



Equipment

MinION Flow Cell (R10.4.1)

NAME

Oxford Nanopore Technologies

BRAND

FLO-MIN114

SKU

- 81 Perform flow cell check.
- 82 Check the number of active pores available for the experiment.
- 83 Remove the flow cell from the MinION device and return it to the tray.

Flow cell priming

5m

- 84 Prepare the flow cell priming mix.

A	B
Component	Volume
Flow Cell Tether (FCT)	30 μ L
50 mg/mL Bovine Serum Albumin (BSA)	5 μ L
Flow Cell Flush (FCF)	1,170 μ L
Total	1,205 μ L

The Flow Cell Tether (FCT) and Flow Cell Flush (FCF) are included in the Ligation Sequencing Kit V14.

⊗ UltraPure BSA (50 mg/mL) **Thermo Fisher Scientific** Catalog #AM2616

- 85 Open the priming port cover of the flow cell.



86 If any air bubbles are present under the cover, remove them using the following procedure:

86.1 Set the volume of P1000 micropipette to 200 μL .

86.2 Insert the pipette tip into the priming port.

86.3 Turn the wheel of the pipette slowly to increase the volume and draw back 20–30 μL of the buffer.

Note

Care must be taken not to remove too much, ensuring that the sensor array of the flow cell remains covered by the buffer.

87 Load the priming mix into the flow cell through the priming port as follows:

Note

Avoid introducing air.

87.1 Using a P1000 micropipette, aspirate  800 μL of the priming mix.

87.2 Insert the pipette tip into the priming port.

87.3 Slowly turn the pipette wheel down to load the priming mix into the flow cell.

88 Wait for  00:05:00 .

5m

89 Lift the SpotON sample port cover upwards to uncover the sample port on the flow cell.



- 90 Load the remaining  200 μL of the priming mix into the **priming port** (caution: not the SpotON sample port) by slowly turning the pipette wheel down to dispense the mix and complete the flow cell priming.

Sample loading

- 91 For 1–10 kbp amplicons, prepare 35–50 fmol of DNA library (from step 73) in  12 μL of Elution Buffer (EB).

Note

For full-length 16S rRNA gene sequencing, 35–50 fmol of 1.5 kbp amplicons correspond to approximately 35–50 ng in weight.

- 92 Prepare the sequencing library for loading.

A	B
Component	Volume
DNA library	12 μL
Sequencing Buffer (SB)	37.5 μL
Library Beads (LIB)*	25.5 μL
Total	75 μL

The Sequencing Buffer (SB) and Library Beads (LIB) are included in the Ligation Sequencing Kit V14.

*Since LIB settle quickly, the bead suspension should be mixed immediately before adding it to the loading mixture.

- 93 Gently mix the sequencing library by pipetting just prior to loading.

- 94 Load  75 μL of the sequencing library into the flow cell via the SpotON sample port in a dropwise fashion.

Note

Using a P100 or P200 micropipette, let each drop flow into the sample port before adding the next one.



- 95 Gently lower the SpotON sample port cover back, ensuring the bung enters the sample port.
- 96 Close the priming port
- 97 Insert the flow cell into the MinION device.
- 98 Place the light shield onto the flow cell.
- 99 Close the MinION lid.

Nanopore sequencing

- 100 Set up a sequencing run on the MinKNOW software.

A	B
Parameter	Setting
Flow cell type	FLO-MIN114 (R10.4.1)
Kit	Ligation Sequencing Kit SQK-LSK114
Expansion pack	PCR Barcoding Expansion 1–12 EXP-PBC001
Basecalling	On
Basecalling model	Fast basecalling
Barcoding	On
Trim barcodes	On
Barcode both ends	On
Q score filtering	8 (default value)
Min/Max read length*	Optional

Above are typical examples of run parameters for real-time basecalling on the MinION Mk1D, connected to an Apple Silicon Mac.



*For full-length 16S rRNA gene sequencing, we set the minimum read length to 1,300 bases and the maximum read length to 1,800 bases for a passing read.

101 Start a sequencing run.

Flow cell washing and storage for reuse

1h 5m

102 Stop the sequencing run.

103 Remove the flow cell from the MinION device and place it in the tray.

104 Using a P1000 micropipette, aspirate the fluid from the waste channel through the waste port.

Note

Ensure that both the priming port and SpotON sample port are closed.

105 Prepare the flow cell wash mix and gently mix by pipetting.

A	B
Component	Volume
Wash Mix (WMX)*	2 µL
Wash Diluent (DIL)	398 µL
Total	400 µL

*WMX contains a nuclease, DNase I, which digests the DNA library.

 Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #EXP-WSH004**

106 Open the priming port cover of the flow cell.

107 [Optional] If necessary, remove air bubbles under the cover by following the procedure in step 86.



108 Load the wash mix into the flow cell via the priming port as follows:

Note

Avoid introducing air.

108.1 Using a P1000 micropipette, aspirate  200 µL of the wash mix.

108.2 Insert the pipette tip into the priming port.

108.3 Slowly turn the pipette wheel down to load the wash mix into the flow cell.

108.4 Wait for  00:05:00 .

5m

108.5 Load the remaining  200 µL of the wash mix into the priming port by following the procedure in steps 108.1–108.3.

109 Close the priming port.

110 Wait for  01:00:00 to digest the residual DNA on the flow cell.

1h

111 Open the priming port cover of the flow cell.

112 [Optional] If necessary, remove air bubbles under the cover by following the procedure in step 86.

113 Using a P1000 micropipette, load  500 µL of Storage Buffer (S) into the flow cell via the priming port by slowly turning the pipette wheel down.

Note

Avoid introducing air.



114 Close the priming port.

115 Using a P1000 micropipette, aspirate the fluid from the waste channel through the waste port.

Note

Ensure that both the priming port and SpotON sample port are closed.

116 Store the flow cell at  4 °C for subsequent use.