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Nanopore amplicon sequencing V.5

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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

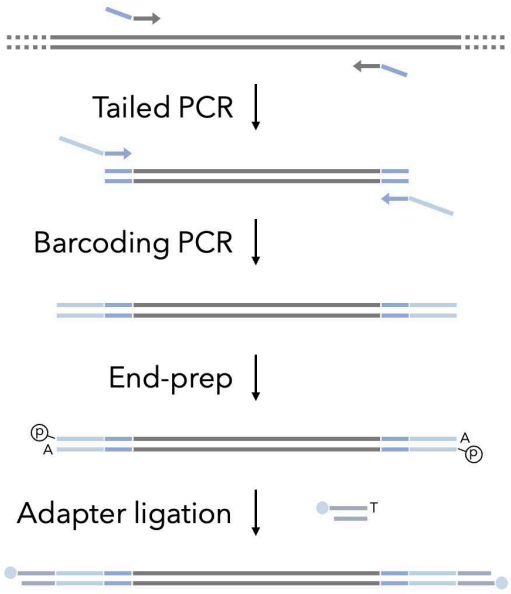
- Thermal cycler
- Gel electrophoresis apparatus
- Magnetic separation rack
- Microcentrifuge
- Vortex mixer
- Quantus Fluorometer (Promega, E6150)
- MinION Mk1B (Oxford Nanopore Technologies)
- MinION Flow Cell R10.4.1 (Oxford Nanopore Technologies, FLO-MIN114)
- Apple Silicon Mac (e.g., Mac Studio with M1 Max and MacBook Pro with M1 Pro)

Protocol materials

- PCR Barcoding Expansion 1–12 **Oxford Nanopore Technologies Catalog #**EXP-PBC001
- PCR Barcoding Expansion 1–96 **Oxford Nanopore Technologies Catalog #**EXP-PBC096
- ProNex Size-Selective Purification System **Promega Catalog #**NG2001
- QuantiFluor ONE dsDNA System **Promega Catalog #**E4871
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114
- Salt-T4 DNA Ligase **New England Biolabs Catalog #**M0467S
- UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #** AM2616
- Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #**EXP-WSH004
- PrimeSTAR Max DNA Polymerase Ver.2 **Takara Bio Inc. Catalog #**R047A

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 16S rRNA gene. 16S rRNA gene-specific sequences are in bold letters.

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

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.

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

Second-round PCR with barcoded primers

5 Prepare the PCR master mix.

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

*One of barcoded primers (10 µM 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

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, including primer dimers, which would be beneficial for the downstream analysis.

6 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.

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

PCR clean-up

14m

8 Allow the ProNex beads to equilibrate to room temperature.

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

9 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. The samples can be pooled in desired ratios, and the total volume can be flexible, depending on the experimental requirements.

Alternatively, each sample can be cleaned up separately prior to pooling. After quantifying the DNA, the samples can be pooled in the desired ratios and subsequently subjected to the End-prep (step 28).

10 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.

11 Incubate at Room temperature for 00:05:00 . 5m

12 Place the tube on a magnetic rack for 00:02:00 . 2m

Equipment

NGS MagnaStand v.3 8Ch	NAME
Magnetic rack (0.2 mL tube)	TYPE
FastGene	BRAND
FG-SSMAG3	SKU

13 Pipette off the supernatant.

14 Wash the beads with 80% ethanol as follows:

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

14.2 Discard the supernatant.

15 Repeat steps 14.1 and 14.2 for a total of two washes.

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

17 Pipette off any residual ethanol.

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

19 Resuspend the beads in 26 µL of water.

20 Incubate at Room temperature for 00:05:00 . 5m

21 Place the tube on the magnetic rack for 00:02:00 . 2m

22 Transfer the eluate to a new tube.

DNA quantification

23 Warm QuantiFluor ONE dsDNA dye to Room temperature .

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

24 Add 1 µL of eluted sample to 200 µL of QuantiFluor ONE dsDNA dye in 0.5 mL tube.

25 Mix thoroughly by vortexing.

26 Incubate at Room temperature for 00:05:00 , protected from light. 5m

27 Measure fluorescence using the Quantus Fluorometer to quantify DNA concentration.

Equipment

Quantus Fluorometer	NAME
Promega	BRAND
E6150	SKU

End-prep

28 Prepare 200 fmol of pooled DNA (from step 22) 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.

29 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

30 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

31 Allow the ProNex beads to equilibrate to room temperature.

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

	A	B
	Component	**Volume**
	End-prepped DNA	30 µL
	ProNex Chemistry	30 µL

Note

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

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

5m

34 Place the tube on a magnetic rack for 00:02:00 . 2m

35 Pipette off the supernatant.

36 Wash the beads with 80% ethanol as follows:

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

36.2 Discard the supernatant.

37 Repeat steps 36.1 and 36.2 for a total of two washes.

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

39 Pipette off any residual ethanol.

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

41 Resuspend the beads in 30 µL of water.

42 Incubate at Room temperature for 00:05:00 . 5m

43 Place the tube on the magnetic rack for 00:02:00 . 2m

44 Transfer the eluate to a new tube.

Adapter ligation

10m

45 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) 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

46 Incubate at Room temperature for 00:10:00 . 10m

Clean-up

12m

47 Allow the ProNex beads to equilibrate to room temperature.

48 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 accordingly. As an alternative to ProNex beads, AMPure XP can also be used.

49 Incubate at Room temperature for 00:05:00 . 5m

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

51 Pipette off the supernatant.

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

Note

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.

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

52.2 Place the tube on the magnetic rack for 00:02:00 . 2m

52.3 Discard the supernatant.

53 Repeat steps 52.1–52.3 for a total of two washes.

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

55 Pipette off any residual wash buffer.

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

57 Resuspend the beads in 15 µL of Elution Buffer (EB).

58 Incubate at Room temperature for 00:05:00 . 5m

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

60 Transfer the eluate to a new tube.

DNA quantification

61 Warm QuantiFluor ONE dsDNA dye to Room temperature .

62 Add 1 µL of eluted sample to 200 µL of QuantiFluor ONE dsDNA dye in 0.5 mL tube.

63 Mix thoroughly by vortexing.

64 Incubate at Room temperature for 00:05:00 , protected from light.

65 Measure fluorescence using the Quantus Fluorometer to quantify DNA concentration.

Flow cell check

66 Connect the MinION to the host computer and start the MinKNOW software.

Equipment	
MinION	NAME
Sequencer	TYPE
Oxford Nanopore Technologies	BRAND
MinION 1B / MinION 1C	SKU

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

Equipment	
MinION/GridION Flow Cell (R10.4.1)	NAME
Flow cell	TYPE
Oxford Nanopore Technologies	BRAND
FLO-MIN114	SKU
https://store.nanoporetech.com/flow-cell-r10-4-1.html	LINK

68 Perform flow cell check.

69 Check the number of active pores available for the experiment.

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

Flow cell priming

5m

71 Prepare the flow cell priming mix.

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

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

72 Open the priming port cover of the flow cell.

73 Remove air bubbles under the cover as follows (if any):

73.1 Set the volume of P1000 micropipette to 200 µL.

73.2 Insert the pipette tip into the priming port.

73.3 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, keeping the sensor array of the flow cell covered by the buffer.

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

Note

Avoid introducing air.

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

74.2 Insert the pipette tip into the priming port.

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

74.4 Wait for 00:05:00 . 5m

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

74.6 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

75 For 1–10 kbp amplicons, prepare 35–50 fmol of DNA library (from step 60) 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.

76 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

	A	B
	Total	75 µL

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

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

78 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.

79 Gently lower the SpotON sample port cover back, ensuring the bung enters the sample port.

80 Close the priming port

81 Insert the flow cell into the MinION device.

82 Place the light shield onto the flow cell.

83 Close the MinION lid.

Nanopore sequencing

84 Set up a sequencing run on the MinKNOW software.

	A	B
	Parameter	**Setting**

	A	B
	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 Mk1B, 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.

85 Start a sequencing run.

Flow cell washing and storage for reuse

1h 5m

86 Stop the sequencing run.

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

88 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.

89 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

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

90 Open the priming port cover of the flow cell.

91 [Optional] If necessary, remove air bubbles under the cover by following the procedure in step 73.

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

Note

Avoid introducing air.

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

92.2 Insert the pipette tip into the priming port.

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

92.4 Wait for 00:05:00 . 5m

92.5 Load the remaining 200 µL of the wash mix into the priming port by following the procedure in steps 92.1–92.3.

93 Close the priming port.

94 Wait for 01:00:00 to digest the residual DNA on the flow cell. 1h

95 Open the priming port cover of the flow cell.

96 [Optional] If necessary, remove air bubbles under the cover by following the procedure in step 73.

97 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.

98 Close the priming port.

99 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.

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