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Nanopore amplicon sequencing V.4

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Protocol status: Working

We use this protocol and it's working



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Abstract

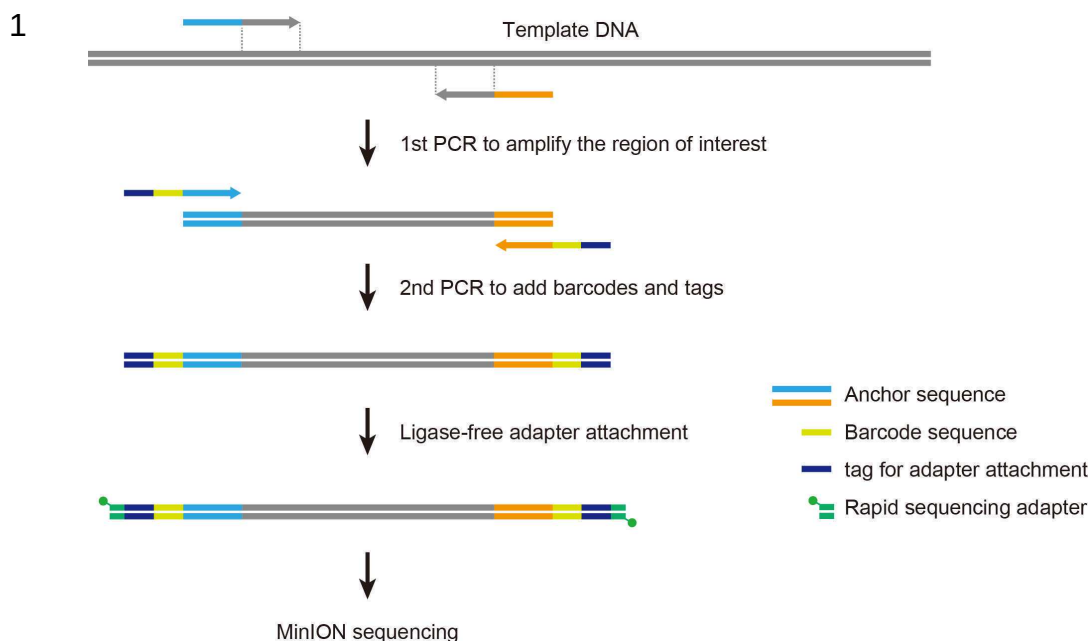
The two-step PCR method allows us to perform nanopore amplicon sequencing with a user-defined inner primer set combined with barcoded outer primers provided by Oxford Nanopore Technologies, taking advantage of rapid adapter attachment chemistry. This method can be applied to a wide range of sequence-based analyses, including microbiome profiling and the identification of genetic variations in targeted loci.

Materials

Reagents/Kits

- KAPA2G Robust HotStart ReadyMix (2X) (Kapa Biosystems, KK5701)
- PCR-grade water
- PCR Barcoding Kit (Oxford Nanopore Technologies, SQK-PBK004)
- E-Gel EX Agarose Gels, 1% (Thermo Fisher Scientific, G402021)
- E-Gel 1 Kb Plus Express DNA Ladder (Thermo Fisher Scientific, 10488091)
- Agencourt AMPure XP (Beckman Coulter, A63880)
- Freshly prepared 70% ethanol
- TN buffer: 10 mM Tris-HCl pH 8.0, 50 mM NaCl
- QuantiFluor ONE dsDNA System (Promega, E4871)
- Flow cell R9.4.1 (Oxford Nanopore Technologies, FLO-MIN106D)
- Flow Cell Priming Kit (Oxford Nanopore Technologies, EXP-FLP002)
- Flow Cell Wash Kit (Oxford Nanopore Technologies, EXP-WSH004)

Workflow



Two-step PCR approach for nanopore amplicon library preparation.

In the first PCR reaction, the region of interest is amplified using specific primers flanked by anchor sequences. The anchor sequences allow for a second round of PCR with Oxford Nanopore's barcoded outer primers with rapid adapter attachment chemistry. The second PCR with reduced cycle numbers generates barcoded amplicons with modified 5' ends for simplified post-PCR adapter attachment.

1st PCR with inner primers

2 Prepare the PCR master mix.

	A	B	C
	Component	Volume	Final conc.
	Template DNA	x μ L	
	10 μ M FW/RV primer mix	0.5 μ L	0.2 μ M each
	2X KAPA2G Robust HS ReadyMix	12.5 μ L	1X
	Water	12 - x μ L	
	Total	25 μ L	



**Inner 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 outer primers used in the 2nd PCR.

Note

The following inner 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 **AGRGTTYGATYMTGGCTCAG**-3'
- 1492R:
5'-ACTTGCCTGTCGCTCTATCTTC **CGGYTACCTTGTTACGACTT**-3'

3 Perform PCR.

A	B	C	D
Step	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	15 sec	25-35
Annealing	55°C	15 sec	
Extension	72°C	30 sec	
Hold	4°C	∞	1

The above is an example for amplifying the near-full length (V1–V9) sequence of bacterial 16S rRNA genes (approximately 1,500 bp).



Equipment

Veriti 96-Well Thermal Cycler	NAME
Applied Biosystems	BRAND
4375786	SKU

- 4 Analyze  2 μ L of the PCR products by gel electrophoresis to verify successful amplification.



Equipment

E-Gel Power Snap Electrophoresis Device	NAME
Thermo Fisher Scientific	BRAND
G8100	SKU

Equipment

E-Gel Power Snap Camera	NAME
Thermo Fisher Scientific	BRAND
G8200	SKU



2nd PCR with barcoded outer primers

5 Prepare the PCR master mix.



A	B
Component	Volume
1st PCR products	1.0 μ L
BP01–12	0.5 μ L
2X KAPA2G Robust HS ReadyMix	12.5 μ L
Water	11 μ L
Total	25 μ L

BP01–12: barcoded outer primers supplied in the PCR Barcoding Kit.

Note

The 1st PCR products may need to be purified using AMPure XP beads before the second round of PCR. This additional step removes reaction contaminants, including primer dimers, which would be beneficial for downstream analysis.

6 Perform PCR.



A	B	C	D
Step	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	15 sec	8–10
Annealing	62°C	15 sec	
Extension	72°C	30 sec	
Hold	4°C	∞	1

The above is an example for barcoding bacterial 16S rRNA gene amplicons (approximately 1600 bp).

Equipment

Veriti 96-Well Thermal Cycler	NAME
Applied Biosystems	BRAND
4375786	SKU

7 Analyze  1 μ L of the PCR products by gel electrophoresis.



Equipment

E-Gel Power Snap Electrophoresis Device	NAME
Thermo Fisher Scientific	BRAND
G8100	SKU

Equipment

E-Gel Power Snap Camera	NAME
Thermo Fisher Scientific	BRAND
G8200	SKU

PCR cleanup



8 Resuspend the AMPure XP beads by vortexing.



9 Add AMPure XP beads to the sample and mix by pipetting.



A	B
Component	Volume
2nd PCR products	24 μ L
AMPure XP	12 μ L

Note

To select DNA fragments of over 500 bp, add 0.5 μ L AMPure XP per 1 μ L of sample (0.5x ratio).

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

5m



11 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

12 Pipette off the supernatant.





13 Wash the beads with 70% ethanol as follows (1/2).



13.1 Keeping on the magnetic rack, add  200 μL of 70% ethanol without disturbing the bead pellet.

13.2 Discard the supernatant.

14 Wash the beads with 70% ethanol as follows (2/2).



14.1 Keeping on the magnetic rack, add  200 μL of 70% ethanol without disturbing the bead pellet.

14.2 Discard the supernatant.

15 Spin down and place the tube back in the magnetic rack.



16 Pipette off any residual ethanol.



17 Remove the tube from the magnetic rack and resuspend the beads in  10 μL of TN buffer.



Note

TN buffer: 10 mM Tris-HCl pH 8.0, 50 mM NaCl

18 Incubate at  Room temperature for  00:02:00 .

2m



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

2m



20 Transfer the eluate to a new tube.





20.1 [Optional] Analyze  1 μ L of the purified sample by gel electrophoresis to confirm the recovery.



DNA quantification

21 Warm QuantiFluor ONE dsDNA dye to  Room temperature .

22 Add  1 μ L of eluted sample to  200 μ L of QuantiFluor ONE dsDNA dye in 0.5 mL tube.



23 Mix thoroughly by vortexing.



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

5m



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



Equipment

Quantus Fluorometer

NAME

Promega

BRAND

E6150

SKU

Sequencing library preparation

26 Pool all barcoded amplicons to a total of  50-100 fmoles in  10 μ L of TN buffer.





A	B	C
Component	Volume	DNA
Sample #01 (25 ng/ μL)	1.0 μL	25 ng
Sample #02 (25 ng/ μL)	1.0 μL	25 ng
Sample #03 (25 ng/ μL)	1.0 μL	25 ng
Sample #04 (25 ng/ μL)	1.0 μL	25 ng
TN buffer	6.0 μL	-
Total	10 μL	100 ng

In the above example, four barcoded 16S rRNA gene amplicons (~1600 bp) are pooled together in equal proportions.

Note

For full-length 16S rRNA gene amplicons (approximately 1,600 bp), 50–100 fmoles of dsDNA equates to ~50–100 ng.

- 27 Add  1 μL of Rapid Adapter (RAP) and mix gently by pipetting.



Note

RAP is supplied in the PCR Barcoding Kit.

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

5m



- 29 Store the library  On ice until ready to load.

Flow cell check

- 30 Open the MinION lid and insert the flow cell under the clip.



Equipment

MinION Mk1C

NAME

Oxford Nanopore Technologies

BRAND

M1CBasicSP

SKU

- 31 Perform flow cell check.
- 32 Check the number of active pores available for the experiment.

Sample loading

- 33 Prepare flow cell priming mix and vortex thoroughly.



A	B
Component	Volume
Flush Tether (FLT)	30 μ L
Flush Buffer (FB)	1.17 mL
Total	1.2 mL

FLT and FB are supplied in the Flow Cell Priming Kit. FB is provided in tubes, pre-aliquoted with 1.17 mL.

- 34 Open the priming port cover of the flow cell.
- 35 Remove air bubbles under the cover as follows (if any).



35.1 Set the volume of P1000 micropipette to 200 µL.

35.2 Insert the tip into the priming port.

35.3 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.

36 Load  800 µL of the priming mix (from Step 33) into the flow cell via the priming port.

Note

Use P1000 micropipette. Avoid introducing air.

37 Wait for  00:05:00 .

5m

38 Prepare the sequencing library for loading.

A	B
Component	Volume
Library (from Step 29)	11 µl
Water	4.5 µl
Sequencing Buffer (SQB)	34 µl
Loading Beads (LB)	25.5 µl
Total	75 µl

SQB and LB are supplied in the PCR Barcoding Kit.

Note

Mix the LB suspension well before adding it to the loading mixture.

39 Lift the SpotON sample port cover of the flow cell.

40 Load  200 μL of the priming mix (from Step 33) into the flow cell via **the priming port** (caution: not the SpotON sample port).



Note

Use P1000 micropipette. Avoid introducing air.

41 Gently mix the sequencing library ( 75 μL , prepared in Step 38) by pipetting just prior to loading.



42 Load the library into the flow cell via the SpotON sample port in a dropwise fashion.



Note

Use P100 or P200 micropipette. Let each drop flow into the port before adding the next one.

43 Replace the SpotON sample port cover and close the priming port.

Nanopore sequencing

44 Start sequencing run.



A	B
Parameter	Setting
Flow cell type	FLO-MIN106 (R9.4.1)
Kit	PCR Barcoding Kit SQK-PBK004
Basecalling	On
Basecalling configuration	Fast basecalling
Barcoding	On
Trim barcodes	On
Barcode both ends	Off
Mid-read barcode filtering	On
Q score filtering	8 (default value)

Typical examples of run parameters with real-time basecalling on the MinION Mk1C.

Flushing a flow cell

1h

45 Stop sequencing run.

46 Prepare flow cell wash mix and gently mix by pipetting.



A	B
Component	Volume
Wash Mix (WMX)	2 μ L
Wash Diluent (DIL)	398 μ L
Total	400 μ L

WMX and DIL are supplied in the Flow Cell Wash Kit. WMX contains DNase I.

47 Remove fluid in the waste channel via the waste port.



**Note**

Ensure that both the priming port and SpotON sample port are closed.
Use P1000 micropipette.

48 Open the priming port cover of the flow cell.

49 [Optional] If necessary, remove air bubbles under the cover by following the procedure in Step 35.

50 Load  400 μL of the wash mix (from Step 46) into the flow cell via the priming port.

Note

Use P1000 micropipette. Avoid introducing air.

51 Close the priming port.

52 Wait for  01:00:00 to digest remaining DNA on the flow cell.

53 Remove fluid in the waste channel via the waste port.

Note

Ensure that both the priming port and SpotON sample port are closed.
Use P1000 micropipette.

54 Open the priming port cover of the flow cell.

55 [Optional] If necessary, remove air bubbles under the cover by following the procedure in Step 35.

56 Load  500 μL of Storage Buffer (S) into the flow cell via the priming port.

**Note**

Buffer S is supplied in the Flow Cell Wash Kit.
Use P1000 micropipette. Avoid introducing air.

57 Close the priming port.

58 Remove fluid in the waste channel via the waste port.

Note

Ensure that both the priming port and SpotON sample port are closed.
Use P1000 micropipette.

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

