Nanopore amplicon sequencing V.4

Yoshiyuki Matsuo[1]

[1]Kansai Medical University

4

May 25, 2022

1

dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

Yoshiyuki Matsuo

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.

DOI

dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

https://doi.org/10.1186/s12920-022-01218-8

Yoshiyuki Matsuo 2022. Nanopore amplicon sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4 Yoshiyuki Matsuo

amplicon sequencing, long read, nanopore, MinION

protocol ,

May 19, 2022

May 25, 2022

62894

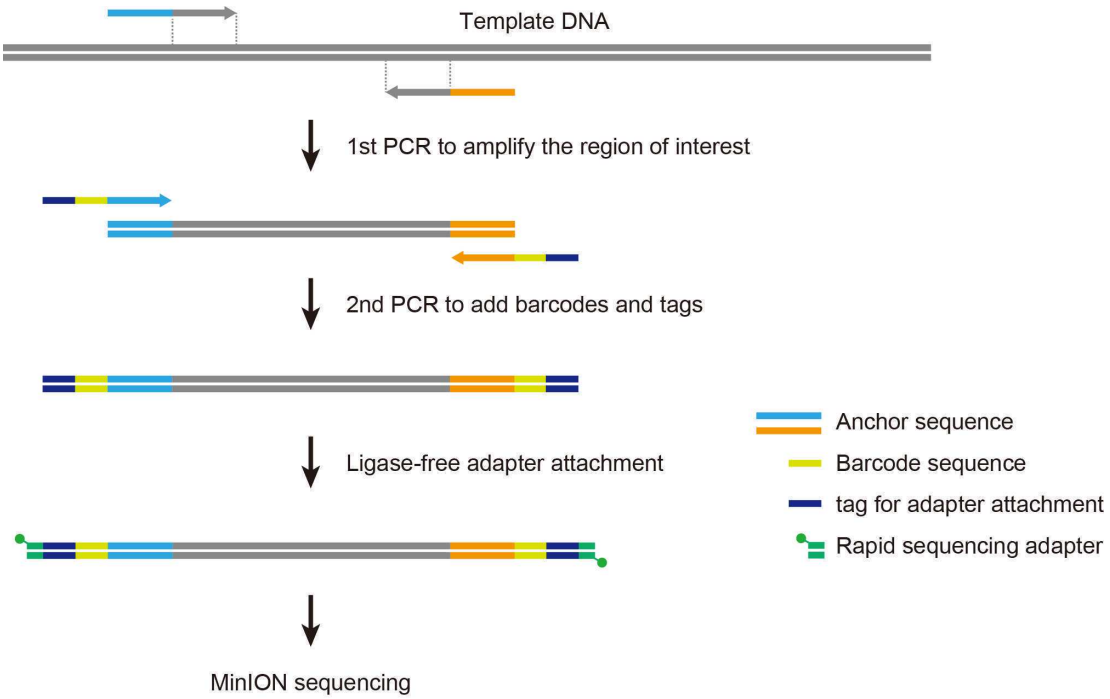
Reagents/Kits

- 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

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

1



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 µL	
10 µM FW/RV primer mix	0.5 µL	0.2 µM each
2X KAPA2G Robust HS ReadyMix	12.5 µL	1X
Water	12 - x µL	
Total	25 µL	

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.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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).

Veriti 96-Well Thermal Cycler

Applied Biosystems **4375786**

4

Analyze **2 µL** of the PCR products by gel electrophoresis to verify successful amplification.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

E-Gel Power Snap Electrophoresis Device

Thermo Fisher Scientific **G8100**

E-Gel Power Snap Camera

Thermo Fisher Scientific **G8200**

2nd PCR with barcoded outer primers

5 

Prepare the PCR master mix.

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

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

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.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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).

Veriti 96-Well Thermal Cycler

Applied Biosystems **4375786**

7

Analyze **1 µL** of the PCR products by gel electrophoresis.

E-Gel Power Snap Electrophoresis Device

Thermo Fisher Scientific **G8100**

E-Gel Power Snap Camera

Thermo Fisher Scientific **G8200**

PCR cleanup

8

Resuspend the AMPure XP beads by vortexing.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

9

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

A	B
Component	**Volume**
2nd PCR products	24 µL
AMPure XP	12 µL

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

10 

5m

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

11 

2m

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

NGS MagnaStand v.3 8Ch
Magnetic rack (0.2 mL tube)
FastGene **FG-SSMAG3**

12 

Pipette off the supernatant.

13 

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

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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.

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

18 

2m

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

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

19 2m

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

20

Transfer the eluate to a new tube.

20.1

[Optional] Analyze **1 µL** 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 µL** of eluted sample to **200 µL** of QuantiFluor ONE dsDNA dye in 0.5 mL tube.

23

Mix thoroughly by vortexing.

24 5m

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

25

Measure fluorescence using the Quantus Fluorometer to quantify DNA concentration.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

Quantus Fluorometer

Promega **E6150**

Sequencing library preparation

26

Pool all barcoded amplicons to a total of **50-100 fmoles** in **10 µL** 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.

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.

RAP is supplied in the PCR Barcoding Kit.

28

5m

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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

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.

MinION Mk1C

Oxford Nanopore Technologies	**M1CBasicSP**

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 µL
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.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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.

> 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.

> Use P1000 micropipette. Avoid introducing air.

37 5m

Wait for **00:05:00** .

38

Prepare the sequencing library for loading.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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.

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).

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.

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

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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 µL
Wash Diluent (DIL)	398 µL
Total	400 µL

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

47 

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

Remove fluid in the waste channel via the waste port.

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.

Use P1000 micropipette. Avoid introducing air.

51 Close the priming port.

52 1h

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

53

Remove fluid in the waste channel via the waste port.

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

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4

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.

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.

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.

Citation: Yoshiyuki Matsuo Nanopore amplicon sequencing https://dx.doi.org/10.17504/protocols.io.8epv5zrodv1b/v4