Amplicon sequencing on the MinION platform V.3

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ABSTRACT

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Materials

1
- KAPA2G Robust HotStart ReadyMix (2X) (KAPA BIOSYSTEMS, KK5701)
- Inner primer (forward): 5'-TTTCTGTTGGTGCTGATATTGC - target-specific sequence -3' (User-supplied)
- Inner primer (reverse): 5'-ACTTGCCTGTCGCTCTATCTTC - target-specific sequence -3' (User-supplied)
- PCR Barcoding Kit (Oxford Nanopore Technologies, SQK-PBK004)
- AMPure XP (Beckman Coulter, A63880)
- 70% ethanol
- Elution buffer: 10 mM Tris-HCl pH 8.0 with 50 mM NaCl
- QuantiFluor ONE dsDNA System (Promega, E4871)
- Flow cell R9.4 (Oxford Nanopore Technologies, FLO-MIN106D)
- Flow Cell Priming Kit (Oxford Nanopore Technologies, EXP-FLP001)

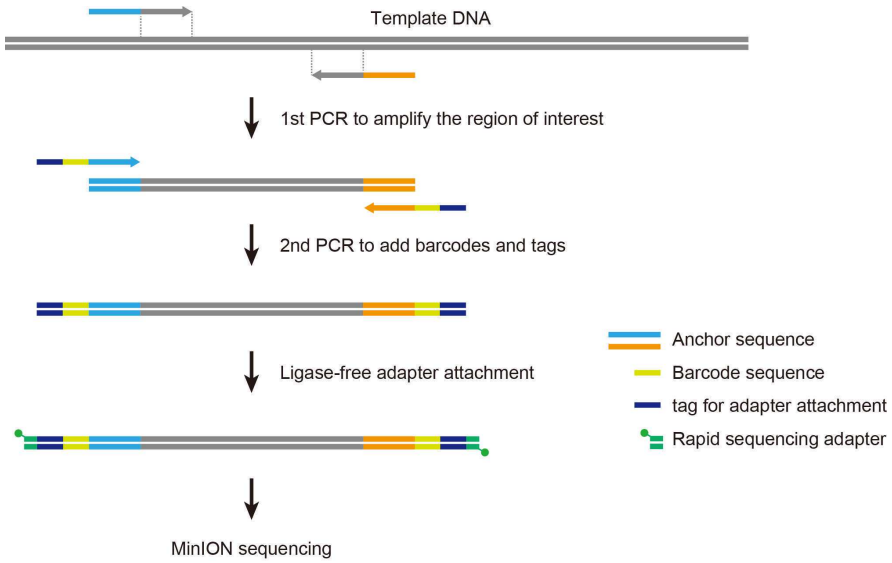
Equipment

2
- Thermal cycler
- Gel electrophoresis device

- Magnetic rack
- Quantus Fluorometer (Promega, E6150)
- MinION sequencer (Oxford Nanopore Technologies)

Workflow

3



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

4 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	12.5 µl	1X
Water	12 - x µl	
Total	25 µl	

5 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 sequence of bacterial 16S rRNA genes (approximately 1,500 bp). The following inner primers are used, with 16S rRNA gene-specific sequences in bold letters.

forward: 5'-TTTCTGTTGGTGCTGATATTGC **AGRGTTYGATYMTGGCTCAG**-3'

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reverse: 5'-ACTTGCCTGTCGCTCTATCTTC **CGGYTACCTTGTTACGACTT**-3'

6 Analyze the PCR product (2-5 µl) with gel electrophoresis to verify successful amplification.

2nd PCR with barcoded outer primers

7 Prepare the PCR master mix.

A	B
Component	**Volume**
1st PCR products	1.0 µl
LWB 01-12	0.5 µl
2X KAPA2G	12.5 µl
Water	11 µl
Total	25 µl

LWB 01-12: barcoded outer primers supplied in the PCR Barcoding Kit (SQK-PBK004)

8 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 1500 bp).

9 [Optional] Analyze the PCR product (1 µl) with gel electrophoresis.

PCR cleanup

10 Resuspend the AMPure XP beads by vortexing.

11 For selecting fragments of over 500 bp, add 10 µl of AMPure XP to 20 µl of PCR product (0.5x ratio).

12 Mix by pipetting and incubate for 5 min at room temperature.

Place the tube on a magnetic rack for 2 min to magnetically separate out the beads.

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13

14 Pipette off the supernatant.

15 Keeping on the magnetic rack, add 200 µl of 70% ethanol without disturbing the bead pellet, and discard the supernatant.

16 Repeat step 15 (wash the beads twice in total).

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

18 Pipette off any residual ethanol.

19 Air-dry for 1 min.
*Do not over dry the magnetic beads.

20 Remove the tube from the magnetic rack and resuspend the beads in 10 µl of elution buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl).

21 Incubate for 2 min at room temperature.

22 Place the tube on a magnetic rack for 2 min.

23 Transfer the eluate to a new tube.

DNA quantification

24 Warm QuantiFluor ONE dsDNA dye to room temperature.

25 Add 1 µl of eluted sample to 200 µl of QuantiFluor ONE dsDNA dye in 0.5 ml tube.

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26 Mix thoroughly by vortexing.

27 Incubate for 5 min at room temperature, protected from light.

28 Measure fluorescence using the Quantus Fluorometer for quantifying DNA concentrations.

29 [Optional] Analyze the sample with gel electrophoresis.

Sequencing library preparation

30 Pool all barcoded amplicons to a total of 50-100 fmoles in 10 µl of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.
*For full-length 16S rRNA gene amplicons (approximately 1,500 bp), 50-100 fmoles of DNA equates to ~50-100 ng.

31 Add 1 µl of RAP and mix gently by pipetting.
*RAP is supplied in the PCR Barcoding Kit (SQK-PBK004).

32 Incubate for 5 min at room temperature.

33 Store the library on ice until ready to load.

Flow cell check

34 Open the MinION lid and insert the flow cell under the clip.

35 Perform flow cell QC.

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

Sample loading

37 Prepare flow cell priming mix by adding 30 µl of Flush Tether (FLT) to the tube of Flush Buffer (FB).
*FLT and FB are supplied in the Flow Cell Priming Kit (EXP-FLP001).

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38 After opening the priming port, load 800 µl of the priming mix into the flow cell via the priming port and wait for 5 min.

39 Prepare the sequencing library for loading.

A	B
Component	**Volume**
Library	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 (SQK-PBK004).

40 Lift the spotON sample port cover and load 200 µl of the priming mix into the flow cell via **the priming port** (NOT the sample port).

41 Mix the prepared library gently by pipetting just prior to loading.

42 Load the library (75 µl) into the flow cell via the SpotON sample port in a dropwise fashion.

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

44 Start the sequencing run.

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