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Library preparation protocol to sequence full length 16S rRNA gene in Nanopore MinION sequencer

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol was optimised from an existing protocol to achieve maximum pore occupancy in a MinION flow cell. This protocol was performed using Ligation sequencing kit (ONT) in combination with native barcoding kit (ONT). Using this protocol, I was able to achieve ~12 Gbp of data from one MinION run from samples with very low input concentration.

Guidelines

- Make sure the reagents are always placed on ice except for the ones that are stable at room temperature
- Mix the contents by vortexing for few seconds prior to using them
- Spin down if there are any precipitates in the reagent.

Materials

Equipments and Reagents

- Platinum™ SuperFi™ PCR Master Mix
- Native Barcoding Kit 1D (EXP-NBD103)
- Ligation Sequencing Kit 1D (SQK-LSK108)
- Thermal Cycler at 20oC and 60oC
- Microcentrifuge
- Vortex mixer
- 96 well Megnetic rack or equivalent for Stripe PCR tubes
- Megnetic rack for Eppendorf tubes (1.5ml - 2ml)
- Heating block at 37oC or equivalent waterbath
- Pipettes P2, P10, P20, P200, P1000 and their corresponding tips
- Multichannel pipette P1-10, P200 and their corresponding tips
- NEBNext End repair / dA-tailing Module (E7546)
- NEB Blunt/TA Ligase Master Mix (M0367)
- Agencourt AMPure XP beads
- Freshly prepared 70% ethanol in nuclease free water
- 1.5ml Eppendorf DNA LoBind tubes
- 0.2 ml stripe PCR tubes
- 0.5 ml thin wall PCR tubes (for Qubit)
- nuclease free water
- NEB Next Quick Ligation Module
- Invitrogen Quibit and corresponding reagents

Before start

Run MinION QC to check the number of pores present before adding the library

PCR amplification

1 1

For a 100 µl PCR reaction, use the following setup

	Reagents	Volume (µl) for 100 µl reaction
	2X Platinum SuperFi PCR Master Mix	50 µl
	Water, nuclease-free	upto 100 µl
	Template DNA	5 µl (11.5 ng)
	10 µM forward primer	5
	10 µM reverse primer	5 µl

2 The PCR conditions are:

initial denaturation at 98°C for 30 sec
28 cycles of

- 98°C for 10 sec
- 55°C for 15 sec

- 72°C for 40 sec

with final extension at 72°C for 5 min

PCR product cleanup

3 Add 1 volume (100ul) of resuspended beads into each PCR reactions and mix by flicking the tubes

4 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature

5 Prepare 20 ml of fresh 70% ethanol in nuclease-free water.

6 Spin down the sample and pellet on a magnet. Keep the tube on the 96 well magnet rack, and pipette off the supernatant.

7 Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet using the multichannel pipette and 70% ethanol in a trough. Remove the 70% ethanol using a pipette and discard. Repeat.

8 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry for 3 -5 mins

9 Remove the tube from the magnetic rack and resuspend pellet in 15 µl nuclease-free water. Incubate for 2 minutes at room temperature.

10 Pellet beads on magnet until the eluate is clear and colourless.

11 Run 3 µl of the cleaned up DNA product in 1% agarose gel to ensure the cleaned product is free from primers.

12 Run 1 µl of DNA in qubit using high sensitivity assay kit and 1 µl in nanodrop to ensure the cleaned up DNA is free from contamination from RNA and/or protein.

13 Based on the qubit reading, take 500 ng of cleaned up DNA in 25 µl of nuclease free water.

End prep

14 Add the reagents below in the same order in a fresh 0.8 ml PCR strip tube

	Reagents	Volume
	500 ng input DNA	25 µl
	Ultra II End-prep reaction buffer	3.5 µl
	Ultra II End-prep enzyme mix	1.5 µl
	Total	30 µl

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15 Mix gently by flicking the tube, and spin down

16 Incubate for 10 minutes at 20 °C and 30 minutes at 65 °C using the thermal cycler with a lid temperature of 70°C.

17 Add 1 volume (30 µl) of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube.

18 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature

19 Spin down the sample and pellet on a 96 well magnet rack. Keep the tube on the magnet, and pipette off the supernatant individually

20 Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet using the multichannel pipette and 70% ethanol in a trough. Remove the 70% ethanol using a pipette and discard. Repeat.

21 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry for 3 -5 mins at room temperature.

22 Remove the tube from the magnetic rack and resuspend pellet in 15 µl nuclease-free water by flicking the tubes. Incubate for 2 minutes at room temperature.

23 Pellet beads on magnet until the eluate is clear and colourless.

24 Quantify 1 µl of end-prepped DNA using Qubit high sensitivity assay kit

25 For next stage, we need 80 ng per sample and hence, dilute the sample concentration to 80 ng to make a final volume of 9 µl in a new strip tube

Adding barcodes

26 Adding the following reagents in the same order as below

	Reagents	Volume
	80 ng end prep DNA	9 µl
	Native Barcode	1 µl
	Blunt/TA Ligase Master Mix	10 µl
	Total	20 µl

27 Mix gently by flicking the tube, and spin down

28 Incubate the reaction for 10 minutes at room temperature

29 Incubate the reaction for 10 minutes at room temperature. Add 20 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.

30 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature

31 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

32 Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet using the multichannel pipette and 70% ethanol in a trough. Remove the 70% ethanol using a pipette and discard. Repeat.

33 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry for 3 - 5 minutes at room temperature.

34 Remove the tube from the magnetic rack and resuspend pellet in 12 µl nuclease-free water. Incubate for 2 minutes at room temperature

35 Pellet beads on magnet until the eluate is clear and colourless.

36 Quantify 1 µl of barcoded DNA in Qubit high sensitivity assay.

37 Based on the qubit reading, add 36.4 ng of each barcoded sample in a 1.5 ml eppendorf tube to get a final concentration of more than 400 ng. If the volume exceeds 50 µl, perform 2.5 x volume AMPure XP bead cleanup with final elution volume of 52 µl of nuclease free water.

38 Quantify 1 µl of barcoded DNA in Qubit high sensitivity assay to concentration is greater than 400 ng.

Adapter ligation

39 Thaw the reagents in ice

40 Add the reagents in following order and flick between addition of each reagents

	Reagent	Volume
	pooled barcoded DNA (	50 µl

	> 400 ng)	
	Barcode Adapter Mix (BAM)	20 µl
	NEBNext Quick Ligation Reaction Buffer (5X)	20 µl
	Quick T4 DNA Ligase	10 µl
	Total	100 µl

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41 Incubate the reaction for 10 minutes at room temperature.

42 Prepare the AMPure XP beads for use; resuspend by vortexing. Prepare another 1ml of fresh 70% Ethanol. Prepare all the tubes and megnetic rack ready.

43 Add 40 µl of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by flicking.

44 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature. Take out the flowcell from the fridge to let it recover to room temperature.

45 Place on magnetic rack, allow beads to pellet and pipette off supernatant.

46 Add 140 µl of the Adapter Bead Binding buffer to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. Repeat.

47 Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer by flicking. Incubate for 10 minutes at room temperature.

48 Pellet beads on magnet until the eluate is clear and colourless, Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. Store on ice.

49 Quantify 1 µl of adapter ligated DNA in Qubit high sensitivity assay to ensure concentration is close to 200 ng

Priming flowcell

50 prepare the priming mix as follows in a 2 ml Eppendorf tube:

	Reagent	Volume
	Nuclease-free water	576 µl
	RPF	624 µl
	Total	1200 µl

51 Take out the flowcell from the box, flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.

52 After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls):

Set a P1000 pipette to 200 µl;

Insert the tip into the priming port;

Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.

Visually check that there is continuous buffer from the priming port across the sensor array.

53 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.

Pipette ~830 ul of the priming mix, before putting the tip on the priming port, slightly drop a little of the liquid to cover the port and attach the tip to the port. Slowly pipette the priming mix out but DO NOT pipette out everything. Remaining the last few (~20ul) of priming mix in the tip and take off the tip straightway. This is to make sure no air goes into the port.

Loading library

54 Thoroughly mix the contents of the RBF and LLB tubes by pipetting, Prepare the library for loading as follows:

	Reagent	Volume
	RBF	35.0 µl
	LLB	25.5 µl
	Nuclease-free water	2.5 µl
	DNA library	12 µl
	Total	75.0 µl

55 Complete the flow cell priming:

1.Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

2. Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles. The same idea, pipette ~230ul of the priming mix and be careful as the previous priming step.

56 Mix the prepared library gently by pipetting up and down just prior to loading.

57 Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

58 After the last drop was absorbed, quickly replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

59 Run the MinION sequencer