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Multiplexing microbial gDNA with SQK-RBK004 rapid kit

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Purpose

This protocol is intended to guide users in creating genomic libraries for MinION & GridION sequencing, using Oxford Nanopore's SQK-RBK004 rapid kit with high molecular weight microbial gDNA.

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This is a modified protocol intended to reduce DNA shearing. An unmodified SQK-RBK004 protocol from the Oxford Nanopore Community can be found here. (Note: An Oxford Nanopore Community account is required.)

Equipment

MinION Mk1B (or Mk1C)

Windows or Ubuntu laptop with 1TB SSD & MinKNOW installed (not required with Mk1C)

Pipettes: P1000, P200, P10

SPRI magnetic plate

Thermocycler (or 2 heat blocks with appropriate fitting)

Fridge/freezer

Plate spinner

Qubit fluorometer

QC of the MinION flow cell to quantify active nanopores:
·Let the flow cell reach room temperature (~30 minutes)
·Open MinKnow & select 'Flow Cell Check'
·Click Start on the bottom right of the screen
·Upon completion, record number of active pores

Have gDNA ready.

UV a MIDI plate (can re-use plate as long as clean wells are used).
Warm SPRI beads (AMPure or Mag-Bind) slightly.
Thaw Barcodes and RAP.
Prepare Ethanol (80%).
Get ice.

Initial DNA Quantification

1 Use Qubit fluorometer (or equivalent) to determine concentration of gDNA in samples.

For all samples with values less than 40 ng/µL, perform SPRI bead concentration as indicated in the following section.

SPRI bead concentration

2 Use a pipette to estimate the total volume of gDNA. Record this value

3 In the MIDI plate, aliquot gDNA to SPRI beads at a 1:1 ratio. Gently pipette-mix 10 times, then leave the sample at room temperature for 2 minutes.

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4 Place MIDI plate with samples on the magnetic plate for 2 minutes, then remove the supernatant ensuring the SPRI+DNA pellet is not disturbed.

5 Perform 2x ethanol washes as with Illumina prep (on the magnet add 200 µL of 80% ethanol, immediately remove & repeat).

Once the second ethanol wash has been removed, incubate the plate for a maximum of 5 minutes to air dry.

DO NOT OVER DRY.

6 Take the MIDI plate off the magnet and add between 15-30 µL of molecular grade water.

Gently pipette mix 10 times.

7 Place MIDI plate back on the magnet.

With the plate on the magnet, remove 2µL for Qubit measurement using the BR kit.

8 Adjust concentrations to 53.3 ng/µL (acceptable limits between 40-60 ng/µL) and begin rapid barcoding.

Rapid Barcoding

9 In 8-strip PCR tubes, aliquot 7.5 µL of gDNA at 53.3ng/µL.

10 To each aliquot, add 2.5 µL of fragmentation mix barcode. Use a different barcode for each sample.

11 Mix gently by flicking the tube, and spin down.

12 In a suitable thermal cycler or heat-blocks, incubate the tube at 30 °C for 1 minute and then at 80 °C for 1 minute.

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13 Briefly put the tube on ice to cool it down.

14 In a 500 µL reaction tube, pool all barcoded samples in your desired ratios.

This will yield a total volume of 120µL when preparing 12 isolates.

15 Aliquot a 1:1 ratio of SPRI beads to the pool of barcoded samples.

This will be 120µL of SPRI beads if preparing 12 isolates.

16 Gently pipette-mix 10 times, then leave the sample at room temperature for 2 minutes.

17 Rotate the reaction tube containing the barcoded pool for 5 minutes.

18 Place the reaction tube containing barcoded pool against the magnet for 5 minutes

Alternatively, an additional clean well in the MIDI plate can be used for this step.

19 If the mix is not clear at 5 minutes, wait until it is clear to move on to the next step.

20 Remove the supernatant – and place into a new 500µL reaction tube. Store this until protocol is complete.

21 Repeat the 2x ethanol wash steps as in step 5.

22 Off the magnet, add 15 µL of molecular grade water, gently pipette mix 10 times, and incubate at RT for 2 minutes.

23 Qubit 2 µL, which should leave 10 µL comfortably. Record the value.

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Qubit results should be between 50ng/µL to 150ng/µL – as this means the total load volume is between 500ng and 1500 µL on the flow cell.

24 Transfer 10 µL to a 500 µL reaction tube and add 1 µL of RAP.

Mix gently by flicking the tube and spin down.

25 Incubate the reaction for up to 30 minutes at room temperature, and then place on ice until you are ready to prepare the final library mixture and load into the flow cell.

26 **Prepare reagents for the next section by thawing:**

·Sequencing Buffer (SQB)
·Loading beads (LB)
·Flush tether (FLT)
·One tube of flush buffer (FB)

Place these on ice once thawed.

Preparing Reagents for Priming & Loading of the Pooled Library

27 Immediately before priming the flow cell:

·Mix SQB and FLB by vortexing, spin down and place back on ice.

·Mix FLT via pipetting, spin down and place back on ice.

·Set a P1000 pipette to 200µL

Priming the Flow Cell

28 A useful video for beginners can be found here: https://www.youtube.com/watch?v=CC11Jlydqrc

Take care to avoid introducing any air during pipetting, as the array of pores must never be exposed to air.

29 Flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.

30 Attach a 1250 µL tip to the P1000 pipette, and insert the tip into the flow cell's priming port.

Turn the pipette's volume-adjustment wheel until you can see a small volume of buffer entering the pipette tip, or the dial shows 220-230 µL. Removing more than 20-30 µL of buffer

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risks damaging the pores in the array.

31 Visually confirm there is continuous buffer from the priming port across the sensor array.

32 Prepare the flow cell priming mix:

·Add 30µL of thawed FLT directly to the thawed and mixed FLB.

·Mix by pipetting up and down carefully.

33 Load 800µl of the flow cell priming mix into the flow cell via the priming port.

Wait for 5 minutes. In this 5 minute step prepare the DNA with loading beads mixture.

Loading the Pooled Library

34 Prepare the DNA with loading beads mixture:

34 µL SQB

25.5 µL LB –**pipette-mix the loading beads before removing**

4.5 µL – molecular grade water

11 µL DNA library

75µl total

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

36 Avoiding the introduction of air bubbles, load an additional 200 µL of priming mix into the flow cell **via the priming port** (not the SpotON sample port).

You load this 200µL in the same place as you did when adding 800µL of priming mix, however this time with the SpotON port open (you will see some fluid moving up & down by the SpotON port).

37 Mix the final 75µL DNA library mixture by pipetting & load to the flow cell via the SpotON sample port in a dropwise fashion.

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38 Close all ports.

Close the lid of the MinION device.

MinKNOW

39 To open the MinKNOW GUI, double–click the MinKNOW icon located on the Windows Desktop or Ubuntu Launcher.

Wait for the MinKNOW GUI to load, select the local MinION, and click Connect.

Follow on-screen instructions, selecting SQK-RBK004 as the kit. Confirm your other settings and select Start.

40 Upon sequencing completion, upload fast5 data to a GPU partition for basecalling with the most recent version of ONT's Guppy basecalling software.

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