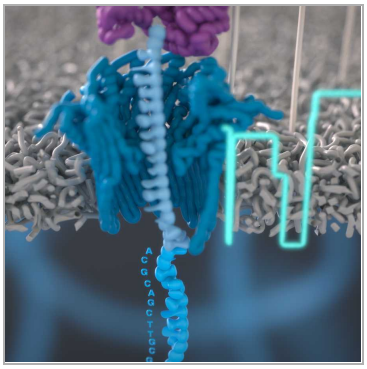

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OPEN ACCESS

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Protocol status: In development
We are still developing and optimizing this protocol

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Nanopore Rapid PCR Barcoding for Genomic Samples

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ABSTRACT

This protocol is for Rapid sequencing of DNA using PCR Barcoding (SQK-RPB004) and has been adapted from Oxford Nanopore Technologies.

MATERIALS

Equipment:
-Microfuge
-Timer
-Thermal Cycler
-Pipette and tips P2, P10, P20, P100, P200, P1000.

Consumables:
-1.5 ml Eppendorf DNA LoBind tubes
-0.2 ml thin-walled PCR tubes
-Nuclease-free water
-Agencourt AMPure XP Beads
-LongAmp Taq 2X Master Mix
-Fresh 70% ethanol in nuclease-free water
-10 mM Tris-HCl pH 8.0 with 50 mM NaCl

Materials:
-1-5 ng high molecular weight genomic DNA
-Rapid PCR Barcoding Kit
-Flow Cell Priming Kit

BEFORE START INSTRUCTIONS

Overall time is about 30 to 40 minutes.

COLLECT THESE:
Equipment:
-Microfuge
-Timer
-Thermal Cycler
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Materials:
-1-5 ng high molecular weight genomic DNA
-Rapid PCR Barcoding Kit
-Flow Cell Priming Kit

Checklist

1 Gather Materials, Consumables and Equipment.
These are listed in the Materials tab also

Equipment:
-Microfuge
-Timer
-Thermal Cycler
-Pipette and tips: P2, P10, P20, P100, P200, P1000

Consumables:
-1.5 ml Eppendorf DNA LoBind Tubes
-0.2 ml thin-walled PCR tubes
-Nuclease-free water
-Agencourt AMPure XP Beads
-LongAmp Taq 2x Master Mix
-Freshly prepped 70% ethanol in Nuclease-free water
-10 mM Tris-HCl pH 8.0 with 50 mM NaCl

Materials:

-1-5 ng high molecular weight Genomic DNA

-Rapid PCR Barcoding Kit

-Flow Cell Priming Kit

Library Preparation

22m 30s

2 Thaw and prepare the reagents as follows:

-Barcodes (RLB 01-12A) at Room Temp

-Fragmentation Mix (FRM) on ice

-Rapid Adaptor (RAP) on ice

2.1 Prepare the DNA in Nuclease-free water:

-Transfer 1-5 ng genomic DNA into a DNA LoBind tube

-Adjust volume to 3 µL with nuclease-free water

-Mix thoroughly by flicking (Avoid unwanted shearing)

-Spin down briefly in Microfuge

2.2 In a thin 0.2 ml thin-walled PCR tube, Mix the following:

- 3 µL 3 1-5 ng template DNA
- 1 µL 1 Fragmentation Mix (FRM)

Mix Gently by flicking tube and spin down.

2.3 Incubate tube in a thermal cycler at 30C for 1 minute. 00:01:00 30C 2m

Then for 1 minute at 80C 00:01:00 80C

Briefly put tube on ice to cool down

2.4 Set up a PCR reaction as follows in 0.2 ml thin-walled PCR tube:

- 20 µL 2 Nuclease-free water
- 4 µL Tagmented DNA
- 1 µL RLB (01,12A, at 10µM)
- 25 µL LongAmp Taq 2X Master Mix

Mix gently by flicking tube and spin down

3 **Amplifying using following cycling conditions:** 15m 30s

-Initial Denaturation 00:03:00 95C (1 Cycle)

-Denaturation 00:00:15 95C (14 Cycles)

-Annealing 00:00:15 56C (14 Cycles)

-Extension 00:06:00 65C (14 Cycles)
-Final extension 00:06:00 65C (1 Cycle)
-HOLD AT 4C

3.1 Transfer sample to clean 1.5ml Eppendorf DNA LoBind tube and Resuspend AMPure XP Beads by vortexing

-Add 30 µL of resuspended AMPure XP Beads to reaction, mix by Pipetting

3.2 Incubate on Hula Mixer 00:05:00 Room Temp 5m

Prepare 500 µL of fresh 70% ethanol in Nuclease-Free water during incubation

3.3 Take tube from Hula Mixer and put on a magnet. Once solution looks clear pipette off supernatant

While on magnet wash beads with 200 µL of 70% ethanol.

> Note
>
> Take care to not disturb pellet when washing beads with the 70% ethanol

Remove ethanol using a pipette and discard.

3.4 Repeat above washing and removal step.

3.5 Spin down and place tube on magnetic rack. Pipette off any residual ethanol in the tube and keep the top open to allow the pellet to dry for about 30 seconds.

> Note
>
> DO NOT DRY PELLET TO POINT OF CRACKING. DO NOT EXCEED 30 SECONDS.

3.6 Take tube out of magnetic rack and resuspend pellet in 10 µL of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl. 2m

Incubate 00:02:00 at Room Temp

3.7 Put tube back on magnetic rack and wait until elute is clear and colorless. Once clear, remove and retain 10 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. Dispose the pelleted beads and previous tube.

> **Note**
>
> You may want to spend down the tube after getting 10 µL of eluate and put back on magnetic rack to get the possible remaining eluate out to use in the next step of quantifying on Qubit Fluorometer. You can also use remaining eluate if present after retaining the initial 10 µl.

4 Quantify 1 µL of eluted sample using a Qubit Fluorometer.

5 Pool all barcoded libraries in desired ratios to a total of 50-100 fmoles in 10 µL of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.

> **Note**
>
> PLEASE BE SURE TO POOL ALL BARCODED LIBRARIES TOGETHER BEFORE PROCEEDING TO NEXT STEP.

5.1 Add 1 µL of RAP to barcoded DNA. Mix by gently flicking tube and spin down.

5.2 Incubate reaction for 00:05:00 at Room Temp 5m

> **Note**
>
> After incubating store this library on ice until ready to load into MinION flow cell.

Priming and Loading the SpotON Flow Cell

5m

6

Thaw the following at Room Temperature:
-Sequencing Buffer (SQB)
-Loading Beads (LB)
-Flush Tether (FLT)
-One tube of Flush Tether (FB)

Once thawed, mix reagents by vortexing then spin down at room temperature.

> **Note**
>
> Sequencing Tether (SQT) tube will NOT be used in protocol.

6.1 Prepare the flow cell priming mix:
- add 30 µL of thawed and mixed Flush Tether (FLT) directly to tube of thawed and mixed Flush Buffer (FB).
- Mix by vortexing at room temperature.

6.2 Open the MinION device lid and slide the flow cell under the clip. Slide the priming port cover clockwise to open the priming port.

> **Note**
>
> Be careful when drawing back buffer from flow cell. DO NOT remove more than 20-30 µL, and make sure that the array of pores are covered by buffer at all times. DO NOT introduce air bubbles into array; this can irreversibly damage pores.

6.3 After opening priming port, check for small air bubbles under the cover. Draw back small volume to remove any bubbles (a few µl): 5m
-Set a P1000 to 200 µL
- Insert tip into priming port
- Turn wheel on pipette until dial shows 220-230 µL (This allows you to draw back 20-30 µL), or until you can see a SMALL amount of buffer entering pipette tip.

> **Note**
>
> Visually check that there is continuous buffer from the priming port across the sensor array

- Load 800 µL of the priming mix into the flow cell via the priming port. Avoid introduction of air bubbles. Wait 00:05:00 . During this 5 minutes, proceed to the next sub-step

6.4 Thoroughly mix the contents of the Loading Beads (LB) tubes by vortexing.

> **Note**
>
> These loading beads (SB) will need to be mixed in immediately before use.

In a new tube, prepare the library for loading as follows:
- 34 µL of Sequencing Buffer (SQB)
- 25.5 µL Loading Beads (LB), mixed IMMEDIATELY before use.
- 4.5 µL Nuclease-free water
- 11 µL DNA library

6.5 Complete the flow cell priming:
-Gently lift the SpotON sample port cover to make the SpotOn sample port accessible.
-Load 200 µL of priming mix into flow cell via the priming port (NOT the SpotON sample port), avoid introduction of air bubbles

6.6 Mix prepared library gently by pipetting up and down prior to loading

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

Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.