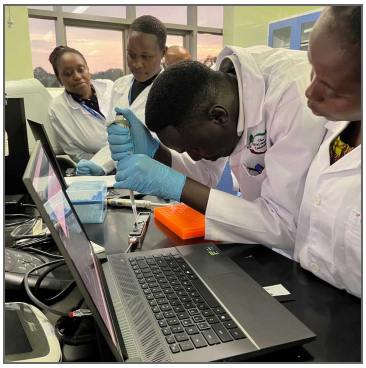

MAR 08, 2024

DOH Workshop Protocol Part 3: Library preparation for Rapid Sequencing DNA V14 Barcoding kit (SQK-RBK114.24) with Pronex modification

In 2 collections

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ABSTRACT

This protocol performs creation of Nanopore sequencing libraries for the MinION flow cell using the Rapid Barcoding Kit

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Protocol status: Working

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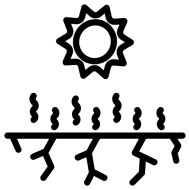
PROTOCOL integer ID: 96354

GUIDELINES

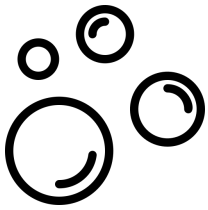
Important throughout the protocol:

Be gentle when pipetting, too much fast pipetting/ extended vortexing can shear the DNA which will result in poorer sequencing outcomes.



Do not let the beads dry after removal of supernatant. This can be avoided by keeping Eppendorf tubes closed if beads are not submerged.
Note: When bead pellet is moist, it appears shiny. As they start to dry, the shine reduces, and cracks start to form.

When working with beads ensure they are thoroughly mixed before using. This can be achieved by vortexing for at least 10 seconds before use and vortexing between use to prevent beads settling.



Be careful when pipetting into a Nanopore flow cell to avoid introducing bubbles into the channels. Bubbles can damage nanopore array. Practice pipetting on a used flow cell.

MATERIALS

Material

- gDNA (~ 200 ng - 300 ng of DNA, maximum 10 µL). If DNA is too concentrated, dilute with NFW to 10 µL .

Equipment

- P1000 pipette (Micropipette with 100 µL – 1000 µL range)
- P200 pipette (Micropipette with 20 µL – 200 µL range)
- P20 pipette (Micropipette with 2 µL – 20 µL range)
- P10 pipette (Micropipette with 0.5 µL – 10 µL range)
- PCR thermoblock (21 °C - 80 °C required) OR Bento Lab
- DNA fluorometer (Promega Quantus or Themofisher Qubit)

Consumables

- P1000 filter pipette tips (with 100 µL – 1000 µL range)
- P200 filter pipette tips (with 20 µL – 200 µL range)
- P20 filter pipette tips (with 2 µL – 20 µL range)
- P10 filter pipette tip (with 0.5 µL – 10 µL range)
- 0.2 ml thin-walled PCR tubes x Number of Samples
- Nuclease free water (NFW)
- 1.5 ml Eppendorf DNA LoBind tubes
- ProNex® Size-Selective Purification System

1. Pronex beads
2. Wash buffer

- Nanopore Rapid sequencing V14 - Amplicon sequencing (SQK-RBK114.24)

1. Rapid Adapter (RA)
2. Adapter Buffer (ADB)
3. AMPure XP Beads (AXP) (not used in this protocol)
4. Elution Buffer (EB)
5. Sequencing Buffer (SB)
6. Library Beads (LIB)
7. Flow Cell Flush (FCF)

8. Flow Cell Tether (FCT)
9. Rapid Barcodes x 24 (RB01-24)

- Ice bucket with Ice

BEFORE START INSTRUCTIONS

1. Calculate the dilution of your starting genomic DNA from the extraction step to make up 10 µL with a maximum concentration of 20 undetermined - 30 undetermined (200 ng - 300 ng total DNA). (For example, if your DNA from the previous protocol was 100 undetermined, you would only need 2 µL of your sample, diluted in 8 µL of NFW).
2. Assign each sample to a Barcode (1-24) and note this down.
3. Program the thermal cycler to incubate at 30 °C for 00:02:00 then 80 °C for another 00:02:00. Do not start it yet.

DOH Workshop Protocol Part 3

1h 9m 19s

1 Prepare one 0.2 ml thin-walled PCR tube for each sample from the previous step. Label the top of the tube with the barcode number.

2 In each 0.2 ml thin-walled PCR tubes:

2.1 Pipette an appropriate amount of your sample (1 µL - 10 µL) and add NFW (1 µL - 10 µL), if necessary, to get 200 ng - 300 ng of DNA in a 10 µL total volume.

2.2 1.5 µL of your chosen Rapid Barcode (RB01-24). (1 barcode per sample). 3s

Note

Spin barcodes down for 00:00:02 - 00:00:03 before use.

3 Mix by gently by stirring or pipetting until thoroughly mixed. If there are any bubbles present, spin down briefly (00:00:02 - 00:00:03) to ensure all liquid is at the bottom. 5s

Note

Barcodes will be a thicker liquid, visually check they have been mixed in.

Pipette gently, be careful not to shear DNA

4 Incubate the tubes in the thermal cycler (PCR machine) at 30 °C for 00:02:00 then 80 °C for another 00:02:00 . 4m

5 Briefly place the tubes On ice to cool.

6 Pool all your samples into a 1.5ml clean eppendorf DNA LoBind tube. Note down the total volume after pooling. It should be 11.5 µL multiplied by the number of samples.

Note

Pipette gently, be careful not to shear DNA

7 **Only 1 person is required to carry out the following steps.** 10s

Resuspend the Pronex beads by vortexing for 00:00:10 or longer.

8 Use a 1:1 ratio of sample to Pronex beads and mix into the sample by slowly pipetting 10 times.

Note

If sticky clumps of bead-bound DNA form, be careful not to take any beads either in the pipette tip or on the outside of the pipette tip.

Pipette gently, be careful not to shear DNA

9 Incubate the sample at Room temperature for 00:10:00 . 10m

10 **To do while waiting** 2m

Take out Elution Buffer (EB) to thaw On ice .

Place the sample on a magnetic stand for 00:02:00 until the solution becomes clear and the beads form a pellet on one side of the tube.

11 While leaving the tube on the magnet, carefully remove and discardsupernatant without disturbing the beads.

12 Wash 1: While still on the magnetic stand, carefully add 200 µL of Pronex wash buffer without flushing directly onto the pellet. If 200 µL is not enough to submerge the pellet, use more wash buffer.

13 Allow to incubate for 00:00:30 - 00:01:00 . 1m

14 While leaving the tube on the magnet, carefully remove and discard supernatant without disturbing the beads.

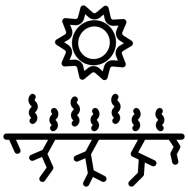
15 Wash 2: While still on the magnetic stand, carefully add 200 µL of Pronex wash buffer without flushing directly onto the pellet. If 200 µL is not enough to submerge the pellet, use more wash buffer.

16 Allow to incubate for 00:00:30 - 00:01:00 . 1m

17 While leaving the tube on the magnet, carefully remove and discard supernatant without disturbing the beads.

18 Allow the sample to air dry with lids open for 00:02:00 - 00:05:00 watching it until the pellet is no longer shiny. 7m

Note



Watch the pellet closely as it dries

19 Remove the tube from the magnetic rack and add 15 µL of elution buffer (included in the Nanopore Rapid Barcoding kit). Resuspend the beads by slowly pipetting or stirring with the pipette tip.

Note

- Be as gentle as possible while ensuring that pellet is resuspended.

Pipette gently, be careful not to shear DNA

20 Incubate the samples at Room temperature for 00:10:00 to elute the DNA. 10m

21 Return the tube to the magnetic stand for 00:01:00 until the solution becomes clear and the beads form a pellet. 1m

22 Store the Pronex beads in the fridge.

23 Transfer 11 µL of the eluate into a clean 1.5ml Eppendorf DNA LoBind tube.

Note

Pipette gently, be careful not to shear DNA

24 Take another 1 µL of the elute from the tube on the magnetic stand for quantification on a fluorometer (Qubit or Quantus). The remaining beads can be kept in a closed tube On ice, for re-elution, if necessary.

25 In a new 1.5 mL Eppendorf DNA LoBind tube, mix the following:

- 1.5 µL Rapid Adapter (RA)
- 3.5 µL Adapter Buffer (ADB)

26 Add 1 µL of this RA + ADB mixture to the DNA.

27 Mix gently by flicking and spin down briefly (00:00:02 - 00:00:03). 3s

28 Incubate this for 00:30:00 at Room temperature . 30m

29 **Preparing the flowcell**

Remove the following Nanopore Rapid Kit (RBK-114.24) items from the -20 °C freezer, spin down and store On ice .

- SB (Sequencing Buffer)
- LIB (Library Beads)
- FCT (Flow Cell Tether)
- FCF (Flow Cell Flush)
- Bovine Serum Albumin (BSA) at 50mg/ml

30 Prepare the flow cell Priming Mix in a fresh 1.5 mL Eppendorf DNA LoBind. Mix by inverting the tube.

- 1170 µL FCF
- 5 µL BSA (Bovine Serum Albumin)
- 30 µL FCT

31 Remove the flow cell you want to use and slide it under the metal clip in the Mk1B or Mk1C MinION. Press down firmly to ensure correct contact with the thermal and electrical connections.

- Mk1B: Plug in the MinION Mk1B to a laptop with Minknow software
- Mk1C: Turn on the MinION Mk1C.

32 Complete a flow cell check to assess the number of pores available on the flow cell.

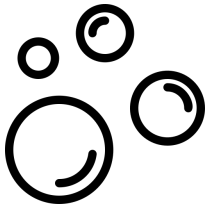
33 Rotate the flow cell priming port cover clockwise to open the priming port.

34 After opening the priming port there will be a small air bubble under the cover that needs to be removed.

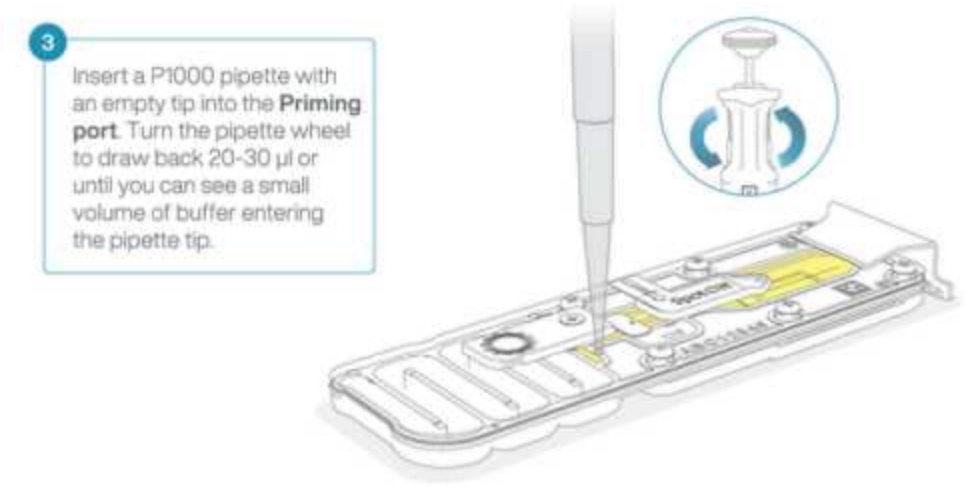
- Set a P1000 pipette to 200 µL
- Insert the tip into the priming port
- Turn the adjustment wheel slowly, pausing every few µls,until the pipette shows 220 µL - 230 µL to draw a total of 20 µL - 30 µL out of the priming port, or until you can see a small volume of liquid entering the pipette tip.

Note

- There may be a small delay before the liquid comes out of the port into the pipette tip. Do not draw out more than 30 µL .
- Check that there is a continuous flow of buffer from the priming port to the nanopore sensor array, and that no bubbles are present.

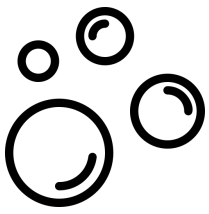


Be careful not to introduce bubbles while pipetting

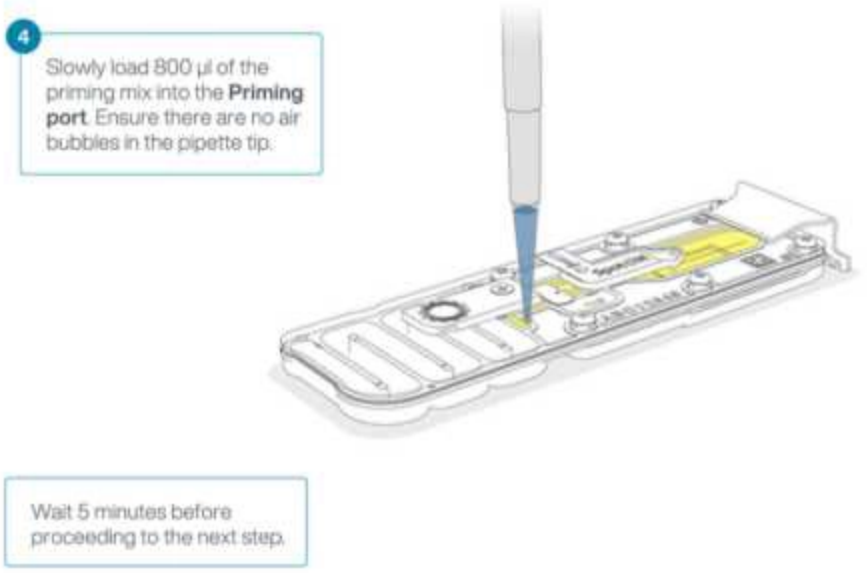


35 Load slowly 800 µL of the Priming Mix from Step 30 into the priming port, without introducing any bubbles. Leave for 00:05:00 and while waiting, proceed to the next step. 5m

Note



Be careful not to introduce bubbles while pipetting



36 Mix the LIB (Library Beads) thoroughly by pipetting.

37 In a new 1.5 ml LoBind tube, mix the following to prepare your library:

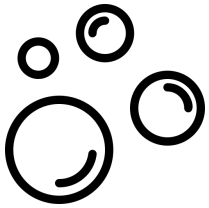
- 37.5 µL SB (Sequencing Buffer)
- 25.5 µL LIB (Library Beads), mixed immediately before use
- 12 µL of DNA library (your sample)

38 Gently open the SpotON sample port cover

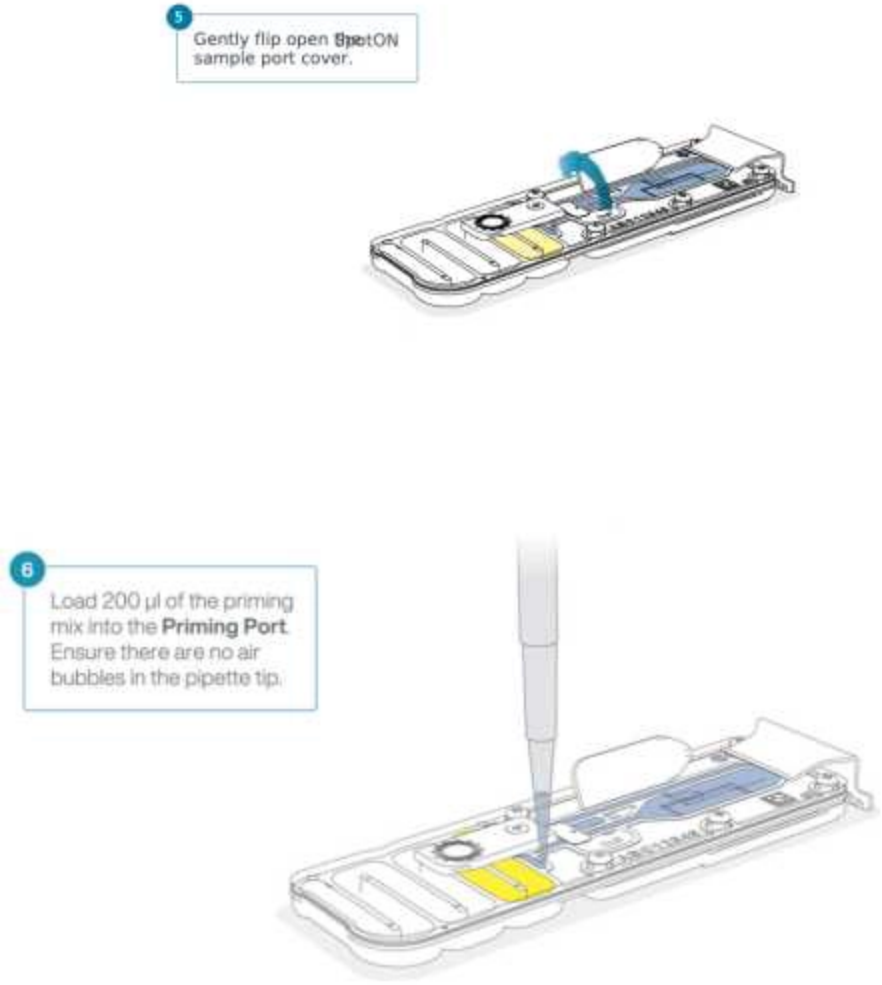
39

Load slowly 200 µL of the Priming Mix into the priming port (**not the SpotON sample port**). Again, avoid introducing any air bubbles.

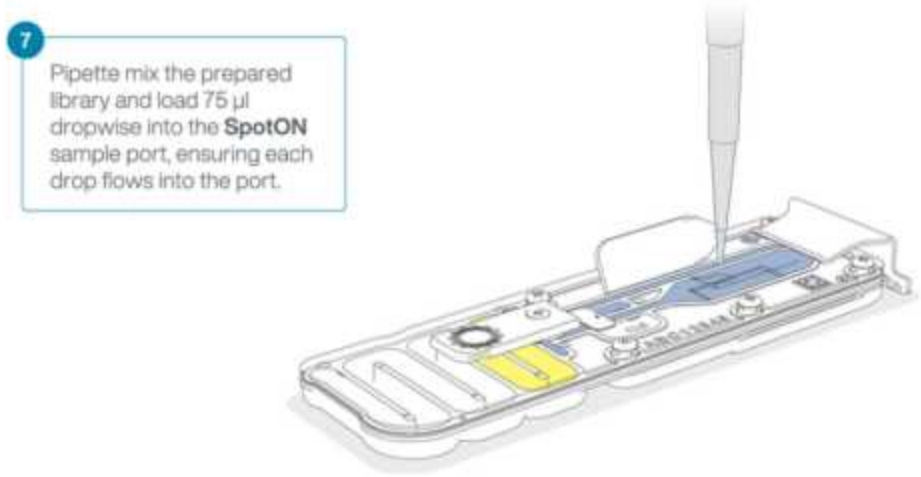
Note



Be careful not to introduce bubbles while pipetting



40 Mix the prepared library by gently pipetting just prior to loading drop by drop 75 µL of the prepared library from Step 37 onto the SpotON sample port. Let each drop flow into the port before adding the next drop.



41 Replace the SpotON sample port cover and gently press down to ensure the bung is in the port.

42 Rotate the Priming port cover back to close the Priming port.

43 Quickly cover the lid of the MinION to protect from light.

44 Start the run on the Minknow software interface.