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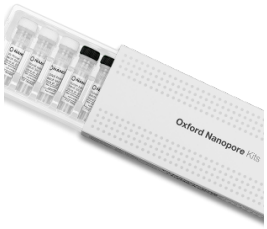
PCR-cDNA Barcoding (SQK-PCB109)



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Oxford Nanopore Technologies[1]
[1]nanoporetech.com

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P Costa

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We use this protocol and it's working

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Disclaimer

Abstract

Barcoding or multiplexing is useful when the amount of data required per sample is less than the total amount of data that can be generated from a single flow cell: it allows a user to pool multiple samples and sequence them together, making more efficient use of the flow cell.

The PCR-cDNA Barcoding Kit is used to prepare cDNA for nanopore sequencing for up to 12 samples, from an input of as low as 1 ng poly-A+ RNA. Users who do not have poly-A+ enriched RNA can use 50 ng of total RNA but additional optimisation may be required.

Taking full-length poly-A+ RNA, complementary strand synthesis and strand switching are performed using kit-supplied oligonucleotides. The kit contains 12 primer pairs which are used to generate and then amplify double-stranded cDNA by PCR amplification: each primer pair contains a barcode and 5′ tag which facilitates the ligase-free attachment of Rapid Sequencing Adapters. Amplified and barcoded samples are then pooled together, and Rapid Sequencing Adapters are added to the pooled mix.

Guidelines

This kit is highly recommended for users who:

- would like to identify and quantify full-length transcripts.
- want to explore isoforms, splice variants and fusion transcripts using full-length cDNAs have a low starting amount of RNA.
- would like to generate high amounts of cDNA data.
- wish to start from total RNA.
- wish to multiplex samples to reduce price per sample.

Materials

- 1 ng PolyA+ RNA (or ~50 ng total RNA)
- PCR-cDNA Barcoding kit (SQK-PCB109)
- Flow Cell Priming Kit (EXP-FLP002)

Troubleshooting

Safety warnings

P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.

None of the components are considered to be a significant hazard due to their small quantity. Get medical attention if any discomfort continues. Show Safety Data Sheet to the medical personnel.

Appropriate engineering controls: Provide adequate ventilation. Good general ventilation should be adequate to control worker exposure to airborne contaminants.

Eye/face protection: Eyewear complying with an approved standard should be worn if a risk assessment indicates eye contact is possible. Personal protective equipment for eye and face protection should comply with European Standard EN166. The following protection should be worn: Chemical splash goggles.

Hand protection Chemical-resistant, impervious gloves complying with an approved standard should be worn if a risk assessment indicates skin contact is possible. The most suitable glove should be chosen in consultation with the glove supplier/manufacturer, who can provide information about the breakthrough time of the glove material. To protect hands from chemicals, gloves should comply with European Standard EN374. Considering the data specified by the glove manufacturer, check during use that the gloves are retaining their protective properties and change them as soon as any deterioration is detected. Frequent changes are recommended.

Other skin and body protection: Appropriate footwear and additional protective clothing complying with an approved standard should be worn if a risk assessment indicates skin contamination is possible.

Hygiene measures Provide eyewash station and safety shower. Contaminated work clothing should not be allowed out of the workplace. Wash contaminated clothing before reuse. Clean equipment and the work area every day. Good personal hygiene procedures should be implemented. Wash at the end of each work shift and before eating, smoking and using the toilet. When using do not eat, drink or smoke.

Respiratory protection Respiratory protection complying with an approved standard should be worn if a risk assessment indicates inhalation of contaminants is possible. Provide adequate ventilation. Large Spillages: If ventilation is inadequate, suitable respiratory protection must be worn.

Environmental exposure controls: Not regarded as dangerous for the environment.

Before start

Before start checklist.

MATERIALS:

- 1 ng PolyA+ RNA (or ~50 ng total RNA)
- PCR-cDNA Barcoding kit (SQK-PCB109)
- Flow Cell Priming Kit (EXP-FLP002)

CONSUMABLES:

- Agencourt AMPure XP beads.
- 1.5 ml Eppendorf DNA LoBind tubes.
- 0.2 ml thin-walled PCR tubes.
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease- free water.
- 10 mM dNTP solution (e.g. NEB N0447)
- LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- RNaseOUTTM, 40 U/µl (Life Technologies, 10777019)
- Exonuclease I (NEB, M0293)

EQUIPMENT:

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes.
- Microfuge Vortex mixer.
- Thermal cycler.
- Ice bucket with ice Timer.
- Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf 022510509)
- Qubit fluorometer (or equivalent for QC check)
- Pipettes and pipette tips P2, P10, P20, P100, P200, P1000.

Reverse transcription and strand-switching

1h 40m

1 Prepare the following reaction in a 0.2 ml PCR tube:

1.1 x µl 1 ng PolyA+ RNA (or ~50 ng total RNA)

1.2 1 µl VN Primers (VNP), at 2 µM

1.3 1 µl 10 mM dNTPs

1.4 9-x µl RNase-free water.

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

3 Incubate at 65° C for 5 minutes and then snap cool on a pre-chilled freezer block.

4 In a separate tube, mix together the following: 4 µl 5x RT Buffer

4.1 1 µl RNaseOUT

4.2 1 µl Nuclease-free water

4.3 2 µl Strand-Switching Primer (SSP, at 10 µM)

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

6 Add the strand-switching buffer to the snap-cooled, annealed mRNA, mix by flicking the tube and spin down.

7 Incubate at 42° C for 2 minutes.

8 Add 1 µl of Maxima H Minus Reverse Transcriptase. The total volume is now 20 µl.

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

10 Incubate using the following protocol:
Reverse transcription and strand-switching 90 mins @ 42° C (1 cycle) Heat inactivation 5 mins @ 85° C (1 cycle)
Hold @ 4° C

Selecting for full-length transcripts by PCR and barcoding samples. 40m

11 The PCR steps outlined below adds barcodes to each reverse transcribed RNA (cDNA) sample. The Barcode Primers provided in the PCR-cDNA Barcoding kit (SQK-PCB109) are used to barcode/multiplex up to 12 individual samples on a single flow cell.

Note

IMPORTANT: Each PCR reaction uses 5 µl of reverse transcribed RNA sample (out of a total of 20 µl). Therefore, sufficient material is available to perform up to four PCR reactions per sample. Do NOT, however, use all 20 µl of reverse transcribed RNA (cDNA) in a single PCR reaction.

Note

IMPORTANT: This kit enables multiplexing of up to 12 samples. The default method allows you to perform one 50 µl PCR reaction per sample. If multiplexing two or three samples, however, two separate PCR reactions per sample should be performed; if running just one sample, four separate PCR reactions should be performed. These recommendations aim to ensure that enough PCR product is generated for optimal flow cell performance.

It is recommended that any remaining reverse transcription reaction is retained to allow for further PCR reactions if greater yield is required.

12 For each sample (up to 12), prepare the following reaction at RT:

12.1 5 µl Reverse-transcribed RNA sample

12.2 1.5 µl Barcode Primers (BP01-BP12)

12.3 18.5 µl Nuclease-free water

12.4 25 µl 2x LongAmp Taq Master Mix

13 Amplify using the following cycling conditions:

13.1 Initial denaturation 30 secs @ 95° C (1 cycle)

13.2 Denaturation 15 secs @ 95° C (11-18* cycles)

13.3 Annealing 15 secs @ 62° C (11-18* cycles)

13.4 Extension 50 secs per kb @ 65° C (11-18* cycles)

13.5 Final extension 6 mins @ 65° C (1 cycle)

13.6 Hold @ 4° C

14 Add 1 µl of NEB Exonuclease 1 (20 units) directly to each PCR tube. Mix by pipetting.

15 Incubate the reaction at 37° C for 15 min, followed by 80° C for 15 minutes.

16 Pool any PCR reactions containing the same barcoded sample in a clean 1.5 ml Eppendorf DNA LoBind tube.

17 Resuspend the AMPure XP beads by vortexing.

18 Add 0.8X equivalents of resuspended AMPure XP beads to the reaction and mix by pipetting.

19 Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.

20 Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.

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

22 Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

23 Repeat the previous step.

24 Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.

25 Remove the tube from the magnetic rack and resuspend pellet in 12 µl of Elution Buffer (EB). Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.

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

27 Remove and retain 12 µl of eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube.

Dispose of the pelleted beads

28 Analyse 1 µl of the amplified DNA for size, quantity and quality.

Note

IMPORTANT: Sometimes a high-molecular weight product is visible in the wells of the gel when the PCR products are run, instead of the expected smear. These libraries are typically associated with poor sequencing performance. We have found that repeating the PCR with fewer cycles can remedy this.

29 In a 1.5 ml Eppendorf DNA LoBind tube, pool together a total of 100 fmol of the amplified cDNA barcoded samples to a final volume of 11 µl in Elution Buffer (EB).

Please check the Mass to Molarity table in the protocol.

Adapter addition

7m

30 Add 1 µl of Rapid Adapter (RAP) to the amplified cDNA library.

31 Mix by pipetting and spin down.

32 Incubate the reaction for 5 minutes at RT.

33 Spin down briefly.

Priming and loading the SpotON flow cell.

15m

34 The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.

Note

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

35 Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before placing the tubes on ice as soon as thawing is complete.

36 Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing, spin down then return to ice.

37 Open the MinION Mk1B lid and slide the flow cell under the clip.

38 Slide the priming port cover clockwise to open the priming port.

Note

IMPORTANT: Take care when drawing back buffer from the 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. Introducing air bubbles into the array can irreversibly damage pores.

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

39.1 Set a P1000 pipette to 200 µl

39.2 Insert the tip into the priming port

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

40 Prepare the flow cell priming mix: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing.

41 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. During this time, prepare the library for loading by following the steps below.

42 Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

Note

IMPORTANT: The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

43 In a new tube, prepare the library for loading as follows:

43.1 37.5 µl Sequencing Buffer (SQB)

43.2 25.5 µl Loading Beads (LB), mixed immediately before use.

43.3 12 µl DNA library

44 Complete the flow cell priming:

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

44.2 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.

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

44.4 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.

44.5 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.

Ending the experiment

45 After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR

46 Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.

Note

IMPORTANT: If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.