Feb 23, 2024

Barcoded and targeted cDNA library preparation for Oxford Nanopore Technologies sequencing

Nature Communications

DOI
https://dx.doi.org/10.17504/protocols.io.kqdg3xzwzg25/v1

Rosemary Bamford[1], Szi Kay Leung[1], Aaron Jeffries[1], Jonathan Mill[2]

[1]Department of Clinical and Biomedical Sciences, University of Exeter; [2]University of Exeter

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Rosemary A Bamford
University of Exeter

DOI: https://dx.doi.org/10.17504/protocols.io.kqdg3xzwzg25/v1

External link: https://doi.org/10.1038/s41467-024-50486-8

Protocol Citation: Rosemary Bamford, Szi Kay Leung, Aaron Jeffries, Jonathan Mill 2024. Barcoded and targeted cDNA library preparation for Oxford Nanopore Technologies sequencing. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.kqdg3xzwzg25/v1

Manuscript citation:
Leung, S.K., Bamford, R.A., Jeffries, A.R. *et al.* Long-read transcript sequencing identifies differential isoform expression in the entorhinal cortex in a transgenic model of tau pathology. *Nat Commun* **15**, 6458 (2024). https://doi.org/10.1038/s41467-024-50486-8

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

Created: February 07, 2024

Last Modified: February 23, 2024

Protocol Integer ID: 94844

Keywords: low input samples for ont transcriptome library preparation, ont transcriptome library preparation, cdna enrichment, enrichment of cdna molecule, library preparation for oxford nanopore technology, sufficient material for cdna enrichment, low input cdna synthesis, pacbio cdna capture, sequencing library preparation, idt hybridisation probe, using idt hybridisation probe, adding oxford nanopore technology, compatible barcodes during reverse transcription, cdna molecule, using idt xgen lockdown probe, oxford nanopore technology, idt xgen lockdown probe, cdna pcr amplification, reverse transcription, rna, ont library preparation, targeted enrichment, ont ligation, optional step before ont library preparation, sequencing

Funders Acknowledgements:
MRC Research Grant
Grant ID: MR/R005176/1
MRC Clinical Research Infrastructure Grant
Grant ID: MR/M008924/1
Simons Foundation for Autism Research (SFARI)
Grant ID: 809383
Simons Foundation for Autism Research (SFARI)
Grant ID: 573312

Abstract

The NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module (NEB) was adapted for the purpose of adding Oxford Nanopore Technologies (ONT) compatible barcodes during reverse transcription of RNA. This is a useful process for multiplexing low input samples for ONT transcriptome library preparation.
An optional step before ONT library preparation is the targeted enrichment of cDNA molecules using IDT hybridisation probes. Here we provide a protocol for this process based on the 'PacBio cDNA capture using IDT xGen Lockdown Probes' protocol.
Using this approach, up to 100 samples can be barcoded with individual ONT barcodes via reverse transcription. Samples can then be pooled together and a cDNA PCR amplification performed. This allows sufficient material for cDNA enrichment and/or ONT ligation sequencing library preparation.

Materials

NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina (E6420S)
Nuclease-free water
dNTPs (NEB, N0447)
TSO-ONT primer (IDT, 100 uM)
ProNex beads (Promega, NG2001)
0.2 ml PCR tubes (low bind)
PCR primers including blockers (IDT, bespoke) - detail including sequences included in protocol
xGen Lockdown hybridisation + wash kit (IDT, 1080577)
xGen Lockdown panels/probes (IDT, bespoke)
Takara LA Taq DNA polymerase hot start (Takara, RR042A)
Ampure XP beads (Fisher Scientific, 10136224)
ONT ligation library kit (Oxford Nanopore Technologies, SQK-LSK114)
HS D5000 screentape (Agilent, 5067-5592)
Qubit dsDNA HS kit (Fisher Scientific, 10616763)

Before start

Order the bespoke IDT primers and dilute to the correct concentration.

Bespoke barcoding of cDNA

1 This protocol is adapted from that provided with NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module (NEB, E6420S). Input and component values have been modified from the original kit protocol.

2 **Primer annealing for first strand synthesis**

Prepare in a PCR tube on ice. A reaction mix of up to 7 µL of total RNA (75 ng) is added to 1 µL [M] 2 millimolar (mM) TSO-ONT primer, 1 µL [M] 10 millimolar (mM) dNTPs (NEB) and made up to 9 µL with nuclease-free H_2O. Gently invert a few times and spin briefly.

Note

Pipetting and flicking the tube may shear RNA/cDNA so try to avoid this for long read sequencing. Gently invert the tube(s) instead and then briefly spin down.

Note

The TSO-ONT primers follow this pattern (TSO primer sequence-ONT barcode sequence-oligo$(dT)_{30}$). The sequences of ONT barcodes are available from the ONT community. Primers can be ordered from Integrated DNA Technologies (IDT) (idtdna.com). Here is an example containing ONT barcode 01:

5' AAGCAGTGGTATCAACGCAGAGTAC-AAGAAAGTTGTCGGTGTCTTTGTG-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN 3'

3 Incubate the mixture in a thermocycler for 00:05:00 at 70 °C with the heated lid at 105 °C , then snap cool on ice for 00:01:00 . 6m

4 **Reverse Transcription (RT) and Template Switching**

Meanwhile, prepare the reverse transcription mix which consists of 2.5 µL NEBNext Single Cell RT buffer (lilac); 0.5 µL NEBNext Template Switching Oligo (lilac); 1 µL NEBNext Single Cell RT enzyme mix (lilac) and 1.5 µL nuclease-free water. Mix by gentle inversion and spin briefly. Add 5.5 µL of reverse transcription mix to each sample. Gently invert a few times and spin briefly.

Note

Briefly vortex the NEBNext Single Cell RT buffer prior to use

5 Place the mix in a thermocycler (heated lid at 105 °C): 1h 40m

42 °C for 01:30:00

70 °C for 00:10:00

hold at 4 °C .

Note

Safe stopping point: samples can be stored overnight at 4 °C or -20 °C

6 **cDNA amplification by PCR**

Prepare the cDNA amplification mix which consists of 25 µL NEBNext Single Cell cDNA PCR Master Mix (orange); 1 µL NEBNext Single Cell cDNA PCR Primer (orange); 0.25 µL NEBNext Cell Lysis Buffer (10X) (white) and 13.75 µL nuclease-free water. Mix by gentle inversion and spin briefly. Add 40 µL of cDNA amplification mix to each sample. Gently invert a few times and spin briefly.

7 PCR cycling conditions: 21m 25s

Heated lid set to 105 °C

Initial denaturation:

98 °C for 00:00:45

For 14 cycles:

98 °C for 00:00:10

65 °C for 00:12:30 ,

72 °C for 00:03:00 ,

Final extension:

72 °C for 00:05:00

hold at 4 °C

Note

The number of cycles and extension time have been increased to prioritise the amplification of longer cDNA fragments. This can be optimised for your own samples.

Note

Safe stopping point: samples can be stored overnight at 4 °C or -20 °C

8 **Cleanup of Amplified cDNA** 40m

Bring Promega Pronex beads to Room temperature for 00:30:00 prior to use. Spin tubes down briefly. 0.85X Promega Pronex beads are added. Gently invert a few times and spin briefly. Incubate at Room temperature on Hula mixer for 00:05:00 . Briefly spin tubes and place on magnet. Leave beads to settle for 00:05:00 . Perform 2× 80% EtOH washes. Amplified cDNA is eluted in 27 µL 1X TE.

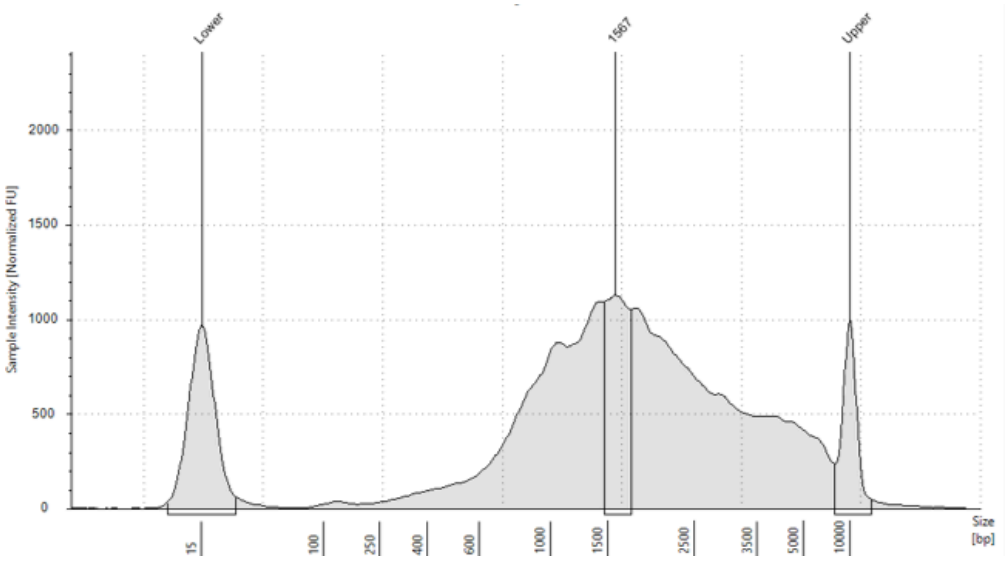
Note

Eluted cDNA can then be equimolar pooled for ONT library preparation directly or taken forward for enrichment.

9 Dilute sample 1:5 and run an Agilent HS D5000 screentape

Expected result

A broad peak centred around typical transcript size of 1.5-2 kB.



Total RNA is used to synthesise cDNA and amplified using 14 cycles.

cDNA enrichment

10 We performed an adapted version of the 'PacBio cDNA Capture Using IDT xGen Lockdown Probes' protocol (**Other Documentation - PacBio**) and xGen Lockdown Panel.

Note

We used the xGen Custom Hyb Panel Design Tool (https://eu.idtdna.com/pages/tools/xgen-hyb-panel-design-tool) to design 120-mer hybridisation probes against genes of interest. One probe was designed per exon unless the exon was > 1 kB when an extra probe was included.

11 Barcoded cDNA is equimolar pooled for a total of 1 µg cDNA per capture reaction.

Prepare in a 0.2 mL PCR tube.

Note

The PCR tube needs to have a hole in the cap (use an 18-20 gauge or smaller needle).

11.1 1 µL of TSO blocker and 1 µL of polyT blocker (poly(dT)$_{30}$) oligonucleotides (both at [M] 1 millimolar (mM)) were added to the pooled cDNA.

Note

TSO blocker oligo: 5' AAGCAGTGGTATCAACGCAGAGTAC 3'
PolyT blocker oligo: 5' TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/3InvdT 3'

11.2 Dry the mixture using a DNA vacuum concentrator (set temperature to 45 °C). Do not overdry.

11.3 Add IDT 2X hybridisation buffer (8.5 µL), hybridisation buffer enhancer (2.7 µL) and nuclease free water (1.8 µL) to the dried-down sample. Replace the lid (do not cover the hole with tape). Mix the reaction by gently tapping the tube, followed by a quick spin.

Note

If you plan to add more than one probe panel then do not add the nuclease free water at this stage.

11.4 Place the mixture on a 95 °C thermocycler for 00:10:00 to denature the cDNA. 10m

11.5 After allowing the mixture to cool briefly for 00:02:00 at Room temperature . 2m
4 µL of xGen Lockdown Panel is added for a total volume of 17 µL . Probes should never be added while at 95 °C .

Note

Thaw panels at Room temperature .

If you are adding more than one panel then add 3 µL of each panel.

11.6 After 00:05:00 at Room temperature, briefly spin the tubes and incubate in a thermocycler at 65 °C overnight (lid temperature 100 °C). 5m

12 Prepare the wash buffers and Dynabeads M-270 Streptavidin beads provided in the xGen Lockdown Hybridisation and Wash kit and use as per instructions below. The xGen Lockdown Hybridisation and Wash kit is optimal for 3 months at -20 °C and poor yields may occur if the kit has expired.

12.1 **Prepare wash buffers** 15m

	Buffer Stock	Stock Conc.	Vol. Buffer	Vol. Water	Total Volume	Final Conc.
	Wash buffer I	10X	40 µL	360 µL	400 µL	1X
	Wash buffer II	10X	20 µL	180 µL	200 µL	1X
	Wash buffer III	10X	20 µL	180 µL	200 µL	1X
	Stringent wash buffer	10X	50 µL	450 µL	500 µL	1X
	Bead wash buffer	2X	250 µL	250 µL	500 µL	1X

These volumes are for a single sample - scale up for multiple samples.

Preheat the following wash buffers to 65 °C in a heat block or water bath for at least 00:15:00 :

200 µL of 1X wash buffer I

400 µL of 1X stringent wash buffer

Other reagents can be kept at Room temperature

12.2 **Prepare the capture beads** 35m 15s

1. Allow the Dynabeads M-270 Streptavidin to warm to room temperature for 00:30:00 prior to use.
2. Mix the beads thoroughly by vortexing for 00:00:15

3. For a single sample, aliquot 100 µL beads into a 1.5 mL LoBind tube. Scale up volume for multiple samples.
4. Place the LoBind tube in a magnetic rack. When the supernatant is clear, remove and discard the supernatant being careful not to disturb the beads. Any remaining traces of liquid will be removed with subsequent wash steps. Give it 00:05:00 to settle. The Dynabeads are "filmy" and slow to collect to the side of the tube.
5. While the LoBind tube is in the magnetic rack, add 200 µL of 1X bead wash buffer. For multiple samples, wash with 200 µL x X samples.
6. Remove the tube from the magnetic rack and vortex until the beads are in solution.
7. Quickly spin and place the LoBind tube back in the magnetic rack to collect the beads to the side of the tube. Once clear, remove and discard the liquid.
8. Repeat steps 5-7 for a total of two washes.
9. Resuspend by vortexing the beads in 100 µL of 1X bead wash buffer. For multiple samples, scale up accordingly.
10. Place the tube in the magnetic rack to collect beads to the side of the tube. Once clear, remove and discard the supernatant.
11. The washed beads are now ready to bind the captured DNA. Proceed immediately to the next step. Do not allow the capture beads to dry. Small amounts of residual bead wash buffer will not interfere with binding of DNA to the capture beads.

12.3 **Bind cDNA to the capture beads** 55m

Transfer the 17 µL hybridised probe/sample mixture to the washed capture beads. Mix by tapping the tube until the sample is homogenous.
Incubate in a heat block set to 65 °C for 00:45:00 or transfer the mix to a PCR tube and incubate in a thermocycler (heated lid set to 75 °C). Hand mix periodically by gently tapping the tube to keep the beads in suspension, every 00:10:00 .

12.4 **Wash the captured cDNA** 16m

1. The 1X wash buffer I and 1X stringent wash buffer should have been pre-heated to 65 °C .
2. After the above incubation, remove the tube from the heat block and add 100 µL pre-heated 1X wash buffer I.
3. Mix thoroughly by tapping the tube until the sample is homogenous.
4. If using a PCR tube, transfer the sample to a 1.5 mL LoBind tube (careful of bubbles).
5. Place the tube in a magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear.

6. Remove the tube from the magnetic rack and add 200 µL of 1X stringent wash buffer heated to 65 °C . Mix by tapping the tube until the sample is homogenous. Work quickly so that the temperature does not drop.
7. Incubate at 65 °C for 00:05:00 .
8. Repeat steps 5-7 for a total of two washes using 1X stringent wash buffer heated to 65 °C .
9. Place the tubes in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear.
10. Add 200 µL of Room temperature 1X wash buffer I. Hand mix by gently tapping the tube, followed by a quick spin.
11. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. It will clear quickly but allow 00:01:00 .
12. Add 200 µL of Room temperature 1X wash buffer II. Hand mix by gently tapping the tube, followed by a quick spin.
13. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. Allow 00:05:00 .
14. Add 200 µL of Room temperature 1X wash buffer III. Hand mix by gently tapping the tube. Quick spin.
15. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. Allow 00:05:00 .
16. Remove the tubes from the magnetic rack and add 50 µL of 1x TE.
17. Store the beads plus captured samples at -20 °C or proceed to the next step. It is not necessary to separate the beads from the eluted DNA.

13 **Amplification of captured DNA sample**

	Component	Volume
	Water	104.5 µL
	10x LA PCR buffer	20 µL
	2.5 mM dNTPs	16 µL
	12 uM TSO amp primer	8.3 µL
	Takara LA Taq DNA polymerase	1.2 µL
	Captured library	50 µL

	Component	Volume
	Total	200 µL

PCR reaction mix

Note

TSO amp primer: 5' AAGCAGTGGTATCAACGCAGAGT 3'

Split the PCR mix into two tubes, 100 µL each.

Amplify using the following PCR protocol:

	Step	Temperature	Time
	1	95 °C	2 mins
	2	95 °C	20 s
	3	68 °C	10 mins
	4	Repeat steps 2-3 for a total of 11 cycles	
	5	72 °C	10 mins
	6	4 °C	hold

After amplification, pool the 100 µL reactions.

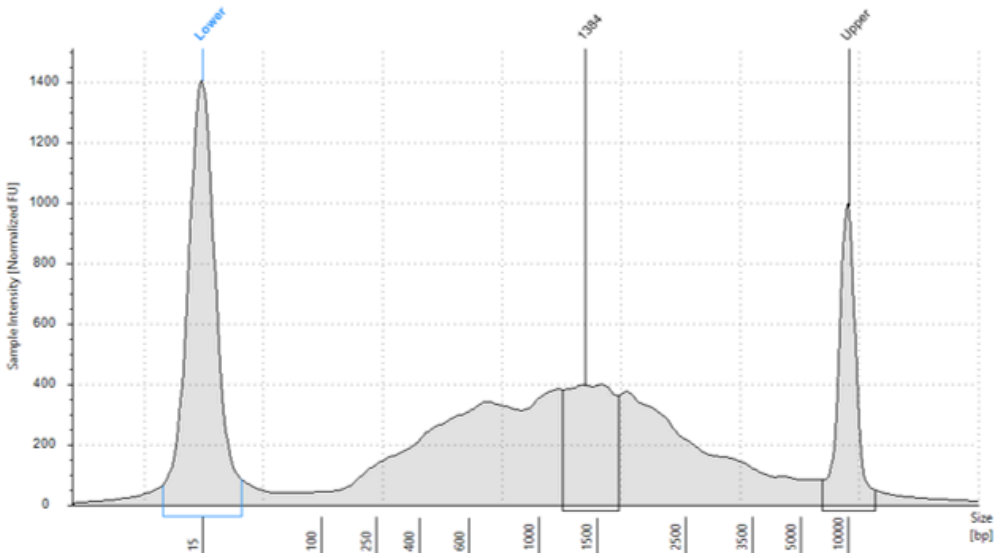
14 **Post amplification clean up** 45m

Bring AMPure beads to Room temperature for 00:30:00 prior to use.
Spin tubes down briefly. 1X AMPure beads are added. Gently invert a few times and spin briefly. Incubate at Room temperature for 00:10:00 . Briefly spin tubes and place on magnet. Leave beads to settle for 00:05:00 . Perform 2× 80% EtOH washes. Amplified cDNA is eluted in 27 µL 1X TE.

15 Quantification was determined using the Qubit DNA High sensitivity assay (Invitrogen, UK) (1:5 dilution) and HS D5000 screentape (Agilent, UK) (1:5 dilution).

Expected result

A broad peak centred around typical transcript size of 1.5-2 kB and a concentration of between 3-15 ng/uL. Lengths may vary depending on the lengths of targeted fragments.



Enriched cDNA amplified using 11 cycles.

Library preparation and sequencing

16 Library preparation was performed using ONT's ligation kit (SQK-LSK114). Follow supplier protocol, which also includes details for sequencing. Recommended library input is 100-200 fmol.