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Oxford Nanopore Technologies (ONT) direct RNA sequencing

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We use this protocol and it's working

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Abstract

Step-by-step protocol describing the workflow for ONT direct RNA sequencing including:

- RNA extraction
- mRNA poly(A) selection
- library preparation for ONT direct RNA sequencing
- ONT direct RNA data analysis



RNA extraction

2h

- 1 Isolate total RNA was isolated from tissue (~1.5 mm³ sections) using the RNeasy Mini Kit (Qiagen, Catalogue #74104) according to manufacturer's instructions. On-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen, #79254). 1h
- 2 Measure RNA concentration via QUBIT and assess RIN values via Bioanalyzer (e.g. Bioanalyzer High Sensitivity kit (Agilent)). Do not use less than 1.5 µg of RNA for the next step. RIN value should be >7. 1h

Poly(A) mRNA isolation

1h

- 3 Select poly(A) mRNA using the NEBNext High Input Poly(A) mRNA Isolation Module (NEB, Catalogue #E3370S) as follows:
 - 3.1 Wash 20 µl of High Input Oligo d(T)₂₅ Beads twice with 100 µl of RNA Binding Buffer (2X) using a magnetic rack to pellet the magnetic beads. These quantities are enough to process 1 sample. 5m
 - 3.2 After removing the second wash, add 50 µl of RNA Binding Buffer (2X) and resuspend the beads by pipetting up and down. 2m
 - 3.3 Combine the washed beads in 50 µl of RNA Binding Buffer (2X) to 50 µl of RNA from Step 1 (RNA extraction). If you have <50 µl of total RNA, add nuclease-free water to reach a final volume of 50 µl. 2m
 - 3.4 Incubate the RNA and beads on a thermal cycler at 65 °C for 5 minutes, then cool down to 4 °C (heated lid @ ≥ 75 °C). 7m
 - 3.5 Resuspend the beads slowly but thoroughly and then incubate the sample @ Room temperature for 10 minutes resuspending the beads once halfway through the incubation. 10m
 - 3.6 Pellet the sample on a magnetic rack for a few minutes (make sure the supernatant is clear and colourless!), then remove the supernatant. The beads now retain poly(A)-selected RNA. 2m
 - 3.7 Wash beads with 200 µl of Wash Buffer by mixing thoroughly. 1m
 - 3.8 Pellet the beads and remove the supernatant. 2m



- 3.9 Repeat Steps 3.7 and 3.8 once more. 3m
- 3.10 Add 50 µl of Tris Buffer by gently pipetting, and incubate the sample on a thermocycler @ 80 °C for 2 minutes, then cool to 25 °C with heated lid at ≥ 90 °C . This temporarily releases the mRNA from the beads for a second selection (see Step 3.11). 5m
- 3.11 Add an additional 50 µl of RNA Binding Buffer (2X) to re-bind the poly(A) RNA to beads and incubate for 10 minutes @ Room temperature to increase specificity of poly(A) selection. Resuspend the beads once halfway through the incubation. 10m
- 3.12 Pellet the beads on the magnetic rack, remove the supernatant. 2m
- 3.13 Wash the beads with 200 µl of Wash Buffer by adding the buffer, incubating for 2 minutes @ Room temperature , pelleting the beads and removing the supernatant. 3m
- 3.14 Release the selected mRNA from the beads by adding 17 µl of Tris Buffer, pipetting thoroughly, and incubating the sample for 2 minutes @ 80 °C , then cooling to 25 °C . 3m
- 3.15 Pellet the beads on the magnetic rack and collect the supernatant (poly(A)-selected mRNA) to a clean 0.2 PCR tube. 3m

ONT direct RNA sequencing library preparation

2h 40m

- 4 Prepare the library for long-read direct RNA sequencing following the latest version of the Direct RNA sequencing protocol provided by the manufacturer. The protocol can be found on the Oxford Nanopore Technologies website in association with the kit required for library preparation, the SQK-RNA004 ligation kit. It is important to retrieve this protocol from the manufacturer's website since it could vary slightly depending on the machine used for sequencing. 2h 30m
- 5 Insert the RNA flowcell (FLO-PRO004RA) into the sequencer (e.g. a PromethION 2 Solo, as we used), and prime it using 1 ml of priming mix (1170 µl of Flow Cell Flush [FCF] + 30 µl of RNA Flush Tether [RFT]) according to the manufacturer's instructions. 8m



- 6 Combine the long-read direct RNA sequencing library (32 μ l) from Step 4 mixed with 100 μ l of Sequencing Buffer (SB) and 68 μ l of Library Solution (LIS) and load the total 200 μ l on the flow cell for sequencing.

2m

ONT direct RNA data analysis

- 7 Basecall data using dorado (we used dorado version 0.7.1-CUDA-11.7.0) with model rna004_130bps_sup@v3.0.1 and --modified-bases-models using dorado's model rna004_130bps_sup@v3.0.1_m6A_GRACH@v1.
- 8 Map reads using minumap2 (-ax splice -uf -k14 -y) using the latest human reference genome (we used hg38).
- 9 Visualise the data using the sorted and indexed bam file (for us, samtools 1.16.1) on IgV (we used version 2.18.2).