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UltraMarathonRT® Direct RNA Sequencing Protocol

Introduction

UltraMarathonRT® (uMRT) is an ultra-processive group II intron encoded reverse transcriptase with intrinsic helicase activity that can copy RNA of any length or complexity at ambient temperatures (20-30°C). In this protocol, we describe the step-by-step procedure to perform Direct RNA Sequencing with UltraMarathonRT using the Oxford Nanopore Technology (ONT) Direct RNA sequencing SQK-RNA004 kit.

Reagents included in kit

1) UltraMarathonRT® (20 U/µL)
2) 2x RT Reaction Buffer
3) dNTP mix (10 mM each)
4) RNA Conditioner
5) 5x Ligation Buffer Q
6) DNA Ligase (2,000,000 U/mL)
7) RNase Inhibitor (40 U/µL)
8) Quenching Buffer
9) Nuclease-free Water

Additional Materials required (but not included in this kit)

1) Oxford Nanopore Technologies (ONT) **SQK-RNA004** or **SQK-RNA004-XL** Direct RNA Sequencing kit.
2) ONT MinION/GridION RNA Flow Cells (**FLO-MIN004RA**) or PromethION RNA Flow Cells (**FLO-PRO004RA**).
3) Agencourt RNAClean XP beads (Beckman Coulter®, A63987).
4) Qubit™ dsDNA HS Assay Kit (ThermoFisher, Q32851).
5) Qubit™ Assay Tubes (Invitrogen, Q32856)
6) 70% Ethanol (freshly prepared).
7) 0.2 ml thin-walled DNase/RNase-free PCR tubes.

Equipment needed

1) ONT MinION/GridION or PromethION sequencing device.
2) Single-channel pipette and filter pipette tips.
3) Magnetic separation rack/stand.
4) Heating block with heated lid or thermal cycler.
5) Qubit™ fluorometer (or equivalent for QC check).
6) Table-top centrifuge.
7) Vortex mixer.
8) Ice bucket with ice.

Before starting the procedure

The RNA library generated by this protocol **must be sequenced immediately and cannot be stored for later use.** Therefore, the following steps are recommended to prevent unnecessary delay between final library preparation and sequencing.

1) Download and install the ONT MinKnow software

To be sure that you're ready to sequence immediately after library preparation, use the following guide to properly install the MinKNOW software: MinKNOW Installation

2) Download the protocol for loading of the ONT Flow Cells

Because the procedure of flow cell priming and loading is slightly different for the two types of ONT flow cells compatible with this kit (FLO-MIN004RA & FLO-PRO004RA), we strongly recommend you follow the ONT suggested protocols for proper flow cell handling. They can be found here: FLO-MIN004RA (MinION) and FLO-PRO004RA (PromethION)

3) RNAConnect Reagents

- Thaw 2x RT Reaction Buffer, 5x Ligation Buffer Q, dNTP's, RNA Conditioning Reagent and Quenching Buffer at room temperature. Ensure all reagents are thoroughly mixed by vortexing, then spin down and place on ice.
- Place UltraMarathonRT, DNA Ligase, and RNase Inhibitor on ice.
  Note: *The 5x Ligation Buffer Q is very viscous. Moving it to room temperature 5-10 minutes before use will help ensure accurate pipetting.*

4) ONT SQK-RNA004 reagents

- Thaw the Wash Buffer (WSB) and RNA Elution Buffer (REB) at room temperature, thoroughly mix by vortexing, then spin down and place on ice.
- Thaw the RT Adapter (RTA) and RNA Ligation Adapter (RLA) on ice, gently flick the tube several times to mix then spin down to collect content and keep on ice.
- If using the RNA CS (RCS) provided by ONT: Thaw the RCS on ice, gently flick the tube several times to mix, then spin down and place on ice. Dilute the RCS according to the following ONT recommendations, gently tap to mix, then place on ice:

Reagent	Volume (µL)
RNA CS	1
Nuclease-free water	2
Total	**3**

5) Other Reagents

Allow the Agencourt RNAClean XP beads to equilibrate to room temperature.

Library Preparation

A. RNA treatment with RNA Conditioning Reagent and RTA Annealing

1) Combine the following reagents in a new 0.2 mL nuclease-free PCR tube. Gently flick the tube at least 10 times to mix, then spin down.

Reagent	Volume (µL)
1 µg total RNA or 300 ng poly (A)+ RNA	1
RNA Conditioning Reagent	1
Water	2
Total	**4**

Note: *If using RNA CS, replace 0.5 µL of water with 0.5 µL of diluted RNA CS.*

2) Incubate at 95°C for 30 seconds, then hold at 4°C or snap chill on ice.
3) Add 1 µL of **ONT RT Adapter (RTA)** to the 4 µL conditioned RNA sample and gently flick the tube at least 10 times to mix, then spin down.
4) Incubate at 55°C for 1 minute, then hold at 4°C or snap chill on ice.

B. RT Adapter (RTA) Ligation

1) Add the following components to the 5 µL annealed sample in the following order:

Reagent	Volume (µL)
Annealed RNA (from step A.4)	5
5x Ligation Buffer Q	3
Nuclease Free Water	4.5
Murine RNase Inhibitor	1
DNA Ligase	1.5
Total	**15**

2) Mix thoroughly with gentle pipetting at least 10 times and incubate for 10 minutes at room temperature to perform ligation.

C. Ligation Cleanup and Reverse Transcription

1) Resuspend the stock of Agencourt RNAClean XP beads by vortexing.
2) Add 27 µL (1.8x) of resuspended Agencourt RNAClean XP beads to the RTA ligation reaction and gently flick the tube until thoroughly mixed.
3) Incubate for 5 minutes at room temperature.
4) Prepare 200 µl of fresh 70% ethanol in nuclease-free water.
5) After 5-minute incubation, spin down the sample and pellet the beads on a magnetic rack. Pipette off the supernatant when clear and colorless (~3-5 minutes).
6) While keeping the tube on magnet, wash the beads with 150 µL of freshly prepared 70% ethanol, wait for 30 seconds, then remove the 70% ethanol using a pipette and discard.
7) Collect any residual ethanol with a quick spin down, then place the tube back on the magnetic rack. Keep the beads on magnet while pipetting off any residual ethanol.
8) Remove the tube from the magnetic rack and resuspend the pellet in 15 µL of nuclease-free water, then gently flick the tube until thoroughly mixed.
9) Incubate for 5 minutes at room temperature.
10) During the 5-minute incubation, prepare the reverse transcription (RT) reaction by combining the following reagents in a clean 0.2 mL nuclease-free PCR tube:

Reagent	Volume (µL)
2x RT Reaction Buffer	20
UltraMarathonRT	2
dNTP mix	2
Murine RNase Inhibitor	1
Total	**25**

11) Gently flick the tube until thoroughly mixed, then spin down and place on ice.
12) After the 5-minute incubation from step C.9 is complete, spin down sample and pellet the beads on a magnetic rack until the solution is clear and colorless (~2 minutes).
13) Transfer the 15 µL of eluate into the 25 µL RT reaction, then gently flick the tube until thoroughly mixed and spin down.
14) Incubate at 30°C for 30 minutes to perform RT.
Note: *Set heated lid to 40°C to prevent evaporation.*
15) Add 4.5 µL of **Quenching Buffer** to the 40 µL RT reaction, then gently flick the tube until thoroughly mixed and spin down.
16) Incubate at 70°C for 5 minutes to inactivate enzyme, then immediately continue to step D.
Note: *Set heated lid to 80°C to prevent evaporation.*

D. RT Cleanup

1) Resuspend the stock of Agencourt RNAClean XP beads by vortexing.
2) Add 80 µL (1.8x) of resuspended Agencourt RNAClean XP beads to the RT reaction and gently flick the tube until thoroughly mixed.
3) Incubate for 5 minutes at room temperature.
4) Prepare 400 µL of fresh 70% ethanol in nuclease-free water.
5) After 5-minute incubation, spin down sample and pellet beads on a magnetic rack. Pipette off the supernatant when clear and colorless (~3-5 minutes).
6) Keeping the tube on magnet, wash the beads with 150 µL of freshly prepared 70% ethanol, as described below:
    a) Add 150 µL of freshly prepared 70% ethanol to the tube and ensure the beads are pelleted on one side of the tube.
    b) Keeping the magnetic rack on the benchtop, rotate the tube by 180°. Wait for the beads to migrate towards the magnet and form a pellet.

c) Rotate the tube 180° again (back to the starting position) and wait for the beads to pellet again.
Note: *If beads stick to the walls of the tube, keep the tube on the magnetic rack and dislodge them using a p200 pipette and tip. Beads will quickly migrate to the magnet once dislodged.*

7) Carefully remove the 70% ethanol using a pipette and discard.
8) Repeat steps 6 & 7 for a total of two washes.
9) Collect any residual ethanol with a quick spin down, then place the tube back on the magnetic rack. Keep the beads on magnet while pipetting off any residual ethanol.
10) Remove the tube from the magnetic rack and resuspend the pellet in 23 µL of nuclease-free water, then gently flick the tube until thoroughly mixed.
11) Incubate for 5 minutes at room temperature.
12) Spin down the sample and pellet the beads on a magnetic rack until the solution is clear and colorless (~ 2 minutes).
13) Remove and retain 23 µL of eluate into a clean 0.2 mL nuclease-free PCR tube.

At this stage the RT-RNA sample can be stored at -80°C for later use. Please note, this is the only pause point in this protocol.

E. RNA Ligation Adapter (RLA) Addition

Thaw ONT Sequencing Buffer (SB), Library Solution (LIS), Flow Cell Flush (FCF) and RNA Flush-Tether (RFT) at room temperature. Thoroughly mix by vortexing then spin down and place on ice.

If using a new ONT flow cell stored in 4°C, move to room temperature to equilibrate at this point.

Place the ONT Wash Buffer (WSB) and RNA Elution Buffer (REB) at room temperature before starting RLA ligation.

1) Combine the reagents in the following order:

Reagent	Volume (µL)
Clean RT-RNA Sample (from step D.13)	23
5x Ligation Buffer Q	8
RNA Ligation Adapter (RLA)	6
DNA Ligase	3
Total	**40**

2) Mix thoroughly with gentle pipetting at least 10 times and incubate for 10 minutes at room temperature to perform ligation.
3) Resuspend the stock of Agencourt RNAClean XP beads by vortexing.
4) Add 16 µL (0.4x) of resuspended Agencourt RNAClean XP beads to the RLA ligation reaction and gently flick the tube until thoroughly mixed.
5) Incubate for 5 minutes at room temperature.
6) Spin down sample and pellet beads on a magnetic rack. Pipette off the supernatant when clear and colorless (~3-5 minutes).
7) Remove tube from rack and add 150 µL of the Wash Buffer (WSB) to the beads. Gently flick the tube until thoroughly mixed, then spin down and pellet beads on a magnetic rack. Pipette off the supernatant when clear and colorless (~2 minutes).
8) Repeat step 7 for a total of two washes.
9) Collect any residual WSB with a quick spin down, then place the tube back on the magnetic rack. Keep the beads on magnet while pipetting off any residual WSB.
10) Remove the tube from the magnetic rack and resuspend the pellet in 13 µL RNA Elution Buffer (REB), then gently flick the tube until thoroughly mixed.
11) Incubate for 10 minutes at room temperature. Gently flick the tube several times after ~ 5 minutes to ensure the beads stay suspended in solution.
Note: *It's recommended to perform a "flow cell check" at this point and be sure your pore count falls within the acceptable range stated by ONT.* *Flow Cell Check Instructions*
12) Spin down and pellet the beads on a magnetic rack until the solution is clear and colorless (~1-2 minutes).
13) Remove and retain 13 µL of eluate into a clean 0.2 mL nuclease-free PCR tube.
14) Quantify 1 µL of the eluate using the Qubit DNA HS assay.
Note: *You should expect a final yield of ~ 50-70 ng when starting with 1 µg of total RNA.*

The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.

The RNA library must be sequenced immediately and cannot be stored for later use.

F. Loading the ONT Flow Cell and Performing Sequencing

1) Because the procedure of flow cell priming and loading is slightly different for the two ONT flow cells compatible with this kit (FLO-MIN004RA & FLO-PRO004RA), please follow the ONT suggested protocols for proper flow cell handling and data acquisition.
    a) They can be found here: FLO-MIN004RA (MinION) and FLO-PRO004RA (PromethION).