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Protocol to use for direct RNA sequencing of plants with Oxford Nanopore Technologies

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We use this protocol and it's working

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Abstract

Transcriptomic analysis is a key component of understanding gene expression and regulatory mechanisms. Oxford Nanopore's direct RNA sequencing kit is optimized to sequence native RNA without converting RNA samples into cDNA. The kit has been developed to sequence strands ranging from 70 to 26,000 nucleotides. Nanopore Direct RNA Sequencing, DRS, has been used to analyze cellular mRNA, noncoding RNA, and RNA viruses. (Jain et al, 2022)

Oxford Nanopore Technologies, ONT, recommends that the RNA sample has an absorbance 260/280 ~2.0 and a 260/230 ~2.0-2.2.
If the RNA has a 260/280 less than ~2.0, it indicates the presence of DNA. A 260/280 less than ~1.8 presence of protein or phenol in the RNA sample.

Attachments

JPG

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The image is developed by the author, Nivedha Nataraj.

Materials

- Plant RNA Lysis Solution
- Wash Buffer WB 1 (concentrated)
- Wash Buffer 2 (concentrated)
- GeneJET RNA Purification Columns pre-assembled with Collection Tubes
- Collection Tubes, 2 mL
- Collection Tubes, 1.5 mL
- Ethanol (96-100%)
- DTT (ThermoScientific #R0861)
- 300 ng of poly(A) tailed RNA or 1 µg of total RNA in 8 µl
- RT Adapter (RTA)
- RNA CS (RCS)
- Wash Buffer (WSB)
- RNA Ligation Adapter (RLA)
- RNA Elution Buffer (REB)
- Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- SuperScript III Reverse Transcriptase, 5x First-strand buffer and 100 mM DTT (Thermo Fisher Scientific, cat # 18080044)
- *10 mM dNTP solution* (e.g. NEB, cat # N0447)
- NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit 1x dsDNA HS Assay Kit (ThermoFisher, Q33230)
- Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- *Hula mixer* (gentle rotator mixer)
- Thermal cycler
- Magnetic rack
- Qubit fluorometer (or equivalent for QC check)

Priming and Loading SpotON Flow Cell

- Library Solution (LIS)
- Sequencing Buffer (SB)RNA Flush Tether (RFT)
- Flow Cell Flush (FCF)
- MinION and GridION Flow Cell
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- 1.5 ml Eppendorf DNA LoBind tubes
- MinION or GridION device
- MinION and GridION Flow Cell Light Shield

RNA Extraction - ThermoScientific GeneJET Plant RNA Purification Mini Kit

1 Add 10 µL of 2M DTT for each 500 µL of Plant RNA Lysis Solution.

2 Add 500 µL of the Lysis+DTT Solution is added to a 1.5 mL Eppendorf Microcentrifuge tube.

3 Weigh 100 g of plant tissue into the microcentrifuge tub and vortex to mix.

4 Incubate the sample for 3 minutes at 56°C.

5 Centrifuge for 5 minutes at 20,000 xg (14,000 rpm).

6 Transfer the supernantant to a clean Eppendorf Microcentrifuge tube.

7 Add 250 µL of 96% ethanol.

8 Transfer the samples to a GeneJET Purification Column and centrifuge the samples for 1 minute at 12,000 xg (11,000 rpm). Discard the flow-through solution and reassemble the column.

9 Add 700 µL of Wash Buffer WB 1 to the purification column (ensure the wash buffer has been prepped to the manufacturer's specifications). Centrifuge for 1 minute at 12,000 xg (11,000 rpm). Discard the flow-through and collection tube,

10 Place the purification column into a clean 2 mL collection tube.

11 Add 500 µL of Wash Buffer WB 2 to the purification column. Centrifuge for 1 minute at 12,000 xg (11,000 rpm). Discard the flow-through. Reassemble the column and collection tube.

12 Repeat step 11 and re-spin the column for 1 minute at a maximum speed ≥20,000 xg (≥ 14,000 rpm). Discard the collection tube and flow through. Transfer the purification column to an RNase-free 1.5 mL collection tube.

13 To elute the RNA, add 50 µL of nuclease-free water to the center of the purification column membrane and centrifuge for 1 minute at 12,000 xg (11,000 rpm).

14 Discard the purification column. The purified RNA can be stored at -20°C until use.

Library Preparation - ONT Direct RNA Sequencing Kit

15 Before Starting Library Preparation: 1 µL of total RNA in 7µL of nuclease-free water to make 8 µL of total sample.

16 Spin down and RT Adapter (RTA, Direct RNA Sequencing Kit) and RNA Ligation Adapter (RLA, Direct RNA Sequencing Kit).

17 Thaw the Wash Buffer (WSB, Direct RNA Sequencing Kit) and RNA Elution Buffer (REB, Direct RNA Sequencing Kit) at room temperature and mix by vortexing before placing them on ice.

18 Dilute 1 µL of RNA sample using nuclease-free water (ThermoFisher, AM9937) in a 0.2 mL thin-walled PCR tube. Adjust the final volume to 8 µL.

19 To the 0.2 mL thin-walled PCR tube, add:

19.1

	A	B
	Reagent	Volume
	RNA sample	8.5 µL
	NEBNext Quick Ligation Reaction Buffer (NEB, B6058)	3 µL
	RNaseOUT™ (Life Technologies, 10777019)	1 µL
	RT Adapter (RTA, Direct RNA Sequencing Kit)	1 µL
	T4 DNA Ligase (NEB, M0202T/M)	1.5 µL
	Total	15 µL

20 Mix by pipetting and spin down.

21 Incubate at room temperature for 10 minutes.

22 In a clean 1.5 mL Eppendorf tube, combine:

22.1

	A	B
	Reagent	Volume
	Nuclease-free water (ThermoFisher, AM9937)	9 µL
	10 mM dNTPs (NEB, N0447)	2 µL
	5X First-strand buffer	8 µL
	DTT	4 µL
	Total	23 µL

23 Transfer the reverse transcriptase master mix to the 0.2 mL PCR tube with the adapter-ligated RNA.

24 Add 2 µL of the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044) to the reaction.

25 Place the tube in a thermal cycler and incubate at 50°C for 50 minutes and then 70°C for 10 minutes, and bring the sample to 4°C.

26 Transfer the sample to a clean 1.5 mL Eppendorf DNA LoBind tube.

27 Add 72 µL of resuspended Agencourt RNAClean XP beads to the reaction.

28 Incubate on a Hula mixer for 5 minutes at room temperature.

29 Spin down the sample and pellet on a magnet. Keep the tube on the magnet and pipette off the supernatant when the sample is clear.

30 Wash the beads with 150 µL of freshly prepared 70% ethanol. Carefully remove the 70% ethanol and discard.

31 Spin down the tube and place it back on the magnet. Remove any residual ethanol.

32 Remove the tube from the magnet and resuspend the pellet with 23 µL nuclease-free water. Incubate for 5 minutes at room temperature.

33 Pellet the beads on a magnet until the elute is clear.

34 Remove and retain the 23 µL of elute in a clean 1.5 mL Eppendorf DNA LoBind tube.

35 Store at -80°C for later use.

Addition of Adapters

36 In the sample tube the sample is stored in, add:

36.1

	A	B
	Reagent	Volume
	RT-RNA sample	23 µL
	NEBNext Quick Ligation Reaction Buffer (NEB, B6058)	8 µL
	RNA Ligation Adapter (RLA, Direct RNA Sequencing Kit)	3 µL
	T4 DNA Ligase (NEB, M0202T/M)	3 µL
	Total	40 µL

37 Incubate the reaction for 10 minutes at room temperature.

38 Add 16 µL of Agencourt RNAClean XP beads to the reaction and mix by pipetting.

39 Incubate on a hula mixer for 5 minutes at room temperature.

40 Spin down the sample and pellet on a magnet. Pipette off the supernatant.

41 Add 150 µL of the Wash Buffer (WSB, Direct RNA Sequencing Kit), resuspend the pellet, and return to the magnet.

42 Repeat step 41.

43 Spin the sample, replace it with the magnet, and pipette off the remaining Wash Buffer.

44 Add 13 µL of RNA Elution Buffer (REB, Direct RNA Sequencing Kit) and pellet the beads on a magnet for 5 minutes until the elute is clear.

45 Retain the 13 µL of elute in a clean 1.5 mL Eppendorf tube.

46 Quantity 1 µL of adapted RNA using the Qubit fluorometer DNA HS assay.

47 RNA must immediately be sequenced and cannot be stored.

Priming and Loading the SpotON Flow Cell

48 Combine the following reagents in a clean 1.5 mL Eppendorf DNA LoBind Tube. Mix by vortexing at room temperature.

48.1

	A	B
	Reagent	Volume per flow cell
	RNA Flush Tether (RFT)	30 µL
	Flow Cell Flush (FCF)	1,170 µL
	Total	1,200 µL

49 Open the GridION device lid and slide the flow cell under the clip. Press firmly to ensure correct thermal and electrical contact.

50 Slide the flow cell priming port cover clockwise to open and check for small air bubbles under the cover. Use a P1000 pipette that is set at 200 µL, insert it into the priming port, and turn the wheel to 220-230 µL to draw back 20-30 µL, or until a small volume of buffer enters the pipette tip.

51 Load 800 µL of the priming mix into the flow cell, ensuring to avoid any air bubbles. Wait for 5 minutes to prepare the library.

52 Prepare the library in a new 1.5 mL Eppendorf DNA LoBind tube by adding:

52.1

	A	B
	Reagent	Volume per flow cell
	Sequencing Buffer (SB)	37.5 µL
	Library Solution (LIS)	25.5 µL
	RNA library	12 µL
	Total	75 µL

53 Load 200 µL of the priming mix to complete the flow cell priming.

54 Mix the prepared library before loading, and add 75 µL of the prepared library into the flow cell dropwise.

55 Gently replace the sample port cover and place the light shield onto the flow cell.

56 Close the lid and set up a sequencing run.

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