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RT-free Nanopore direct RNA sequencing v1

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Protocol status: Working
We have used this protocol in the laboratory and it works for us. We have also used a version of it on the International Space Station for sequencing human poly(A) RNA.

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Abstract

Reverse Transcription-free (RT-free) poly(A) RNA protocol for Nanopore Direct RNA Sequencing

This protocol was developed as a collaboration between NASA Houston, Oxford Nanopore Technologies, and UC Santa Cruz to support direct RNA Nanopore sequencing on the International Space Station (ISS).

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Materials

This protocol uses the reagents as recommended from the SQK-RNA002 kit from Oxford Nanopore Technologies.

1 Preparing input RNA:

Record the quality, quantity and size of the input RNA.
Guide criteria:

- Average fragment size: > 500 bases
- Mass by Qubit RNA HS assay: 500 ng
- Please ensure there are no detergents or surfactants in the buffer

Transfer 500 ng of RNA to a 0.2 mL PCR tube.

Adjust the volume to 8 µL with nuclease-free water and proceed by adding buffer as shown in the table in Step 2 (below).

2 Heat and cool RNA: 8m 30s

	Reagent	of [Stock]	Add	to [Final]
	Poly(A)+ RNA	variable	8 µl	500 ng or 1.1 pmoles
	TE + NaCl buffer (90 mM TRIS pH 6.5, 9 mM EDTA, 450 mM NaCl)	9x	1 µl	1x
	TOTAL		9.0 µl	

- Mix the RNA and buffer by pipetting and spin down.
- Place the tube in a thermal cycler with the following program: 65 °C for 00:01:00, followed by cooling at 0.1 °C / second to 20 °C. Approximate time for this step is 00:07:30
- Transfer RNA to a 1.5 mL DNA LoBind Eppendorf tube and proceed by adding to the tube the additional reagents shown in step 3 (below).

3 RT Adapter (RTA) ligation: 10m

	Reagent	of [Stock]	Add	to [Final]
	RNA from Step 2	500 ng	9.0 µl	**500 ng or 1.1 pmoles**
	RT Adapter (RTA)		1 µl	

	RNA Calibration Strand (RCS) (Optional)	50 ng/µl	0.5 µl	25 ng
	5x Quick Ligation Reaction Buffer (NEB)	5x	3.0 µl	1x
	T4 DNA Ligase High Concentration (NEB)	2000 U/µl	1.5 µl	200 U/µl
	TOTAL		**15.0 µl**	

Note

- If not using RCS, substitute with 0.5 µL of NSF water.
- The high concentration T4 DNA ligase (NEB) is essential for the ligation steps to work at optimal efficiency.

- Mix by pipetting and spin down.
- Incubate the reaction for 00:10:00 at room temperature.

3.1 Cleaning up the ligation reaction using Agencourt RNAClean SPRI beads:

3.2 Add 27 µL (1.8X) of resuspended RNAClean XP beads to the ligation reaction and mix by pipetting.

3.3 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

Note

The tube can be put on a rack at ambient temperature for 00:05:00 in the absence of a hula mixer.

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

3.5 Keep the tube on magnet and wash the beads with 150 µL of Wash Buffer (WSB) without disturbing the pellet as described below:
- Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a pellet.
- Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet.

3.6 Remove the WSB using a pipette, and discard.

3.7 Spin down and place the tube back on the magnet. Pipette off any residual WSB.

3.8 Remove the tube from the magnetic rack and resuspend pellet in 20 µL Nuclease-free water.

3.9 Incubate for 00:05:00 at room temperature. 5m

3.10 Pellet beads on magnet until the eluate is clear and colorless.

3.11 Transfer eluate to a clean 1.5 mL DNA LoBind Eppendorf tube and proceed by adding to the tube the additional reagents shown in step 4.

4 Nanopore sequencing adapter (RMX) ligation:

	Reagent	of [Stock]	Add	to [Final]
	RNA from Step 3		20 µl	
	RNA adapter mix (RMX)		6 µl	
	5x Quick Ligation Reaction Buffer (NEB)	5x	8 µl	1x
	Nuclease-free water		3 µl	
	T4 DNA Ligase High Concentration (NEB)	2000 U/µl	3 µl	150 U/µl
	TOTAL		**40 µl**	

Note

The high concentration T4 DNA ligase (NEB) is essential for the ligation steps to work at optimal efficiency.

- Mix by pipetting and spin down.
- Incubate the reaction for 10 minutes at room temperature.

4.1 Cleaning up the ligation reaction using Agencourt RNAClean SPRI beads:

4.2 Add 40 µL (1X) of resuspended RNAClean XP beads to the ligation reaction and mix by pipetting.

4.3 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

Note

The tube can be put on a rack at ambient temperature for 00:05:00 in the absence of a hula mixer.

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

4.5 Add 150 µL of the Wash Buffer (WSB) to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant.

4.6 Repeat Step 4.5 a total of 2 washes.

4.7 Remove the tube from the magnetic rack and resuspend pellet in 21 µL Elution Buffer. Incubate for 00:10:00 at room temperature. 10m

4.8 Pellet beads on magnet until the eluate is clear and colorless.

4.9 Transfer elutate to a clean 1.5 mL Eppendorf DNA LoBind tube.

4.10 Use 1 µL of library to quantify using Qubit RNA HS assay.

Note

This is your final library.

5 Dilute library for loading:

Note

This protocol does not cover flowcell preparation. Please follow the current recommended protocol from Oxford Nanopore Technologies for preparing flowcell (priming and loading) and using software for the sequencing run.

	Reagent	of [Stock]	Add	to [Final]
	RNA library from Step 4		20 µl	
	Nuclease-free water		80 µl	
	RRB	2x	100 µl	1x
	TOTAL		**200 µl**	

- Mix the reagents above a clean 1.5 mL Eppendorf DNA LoBind tube and spin down.
- The prepared library is ready to be loaded onto the flowcell.