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SMART-9N amplification enabling rapid nanopore sequencing of RNA viruses

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We use this protocol and it's working

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Abstract

This protocol describes a rapid Oxford Nanopore Technologies (ONT) workflow for untargeted sequencing of RNA viruses. It has been optimized for wastewater and nasal swabs processed using the Nucleic Acid Observatory (NAO) protocols **Concentration and nucleic acid extraction of viruses from wastewater influent V.3** and **Concentration and total RNA extraction of viruses from nasal swabs V.1**, respectively. It assumes that viral RNA extracts have undergone background depletion and does not detail those steps. In brief, RNA is reverse transcribed using SMART-9N amplification and prepared for sequencing with ONT's Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24). In our experience, this approach consistently yields sufficient material for ONT’s recommended inputs and recovers a diverse range of RNA viruses from both wastewater and nasal swabs, with long reads, high genome coverage, and reduced background noise.

Guidelines

RNA processing and handling: Please review **Protocol Note: Working with RNA Samples** before handling RNA samples.

Materials

Consumables:

- 100 µM RLB RT 9N primer (IDT - Sequence: 5′-TTTTTCGTGCGCCGCTTCAACNNNNNNNNN-3′)
- 100 µM RLB TSOmG template-switching oligo (IDT - Sequence: 5′-GCTAATCATTGCTTTTTCGTGCGCCGCTTCAACATmGmGmG-3′)
- LunaScript RT Master Mix Kit (NEB, E3025)
- Sequenase Version 2.0 DNA Polymerase (ABI, 70775Y200UN)
- Rapid PCR Barcoding Kit 24 V14 (ONT, SQK-RPB114.24)
- 2X LongAmp *Taq* Master Mix (NEB, M0287)
- Agencourt AMPure XP beads (Beckman Coulter^{TM}, A63881)
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- 100% ethanol
- 1.5 mL Eppendorf DNA LoBind tubes
- 0.2 mL thin-walled PCR tubes
- Qubit^{TM} dsDNA HS Assay Kit (ThermoFisher, Q32851)
- Qubit^{TM} Assay Tubes (Invitrogen, Q32856)

Equipment:

- Ice bucket with ice
- Microfuge
- Micropipettes (1000 uL, 200 uL, 20 uL, 2 uL) and holder
- Timer
- Vortex mixer
- Thermal cycler
- Magnetic separation racks: compatible with 0.2 mL tubes and 1.5 mL tubes
- Hula mixer (gentle rotator mixer)
- Qubit^{TM} fluorometer

Before start

Prepare a biosafety cabinet or dead air box for RNA processing and handling. Gather materials and reagents. Ensure proper PPE.

Stage 1. cDNA synthesis and clean-up

1h 16m

1 Prepare 12 µL of each viral RNA extract in a separate clean 0.2 mL tube. 1m

2 Add 2 µL of RLB RT 9N (100 µM) to each 0.2 mL tube containing viral RNA extract. 1m

3 Incubate the reaction at 65 °C for 00:05:00 , then transfer the samples to ice immediately. Keep the samples on ice for 00:02:00 . 7m

4 Add 2 µL of TSOmG (100 µM) followed by 4 µL of LunaScript RT Master Mix Kit (Primer-free) to each 0.2 mL tube for a total volume of 20 µL . 2m

5 Incubate in thermal cycler using the following protocol: 13m

	Step	Temperature	Time
	Primer Annealing	25°C	2 minutes
	cDNA Synthesis	55°C	10 minutes
	Heat Inactivation	95°C	1 minute

6 Prepare the following Sequenase master mix (adjusted to +10% volume) in a 1.5 mL tube, according to the total number of samples: 5m

	A	B	C
			Enter the number of samples:
	Reagents	Volume (µL) per sample	2
	5X Sequenase Buffer	2	4.4

	A	B	C
	Sequenase Dilution Buffer	0.9	1.98
	Sequenase	0.6	1.32
	NFW	7.7	16.94
	TOTAL	11.2	24.64

Thoroughly mix the reaction by gently pipetting and briefly spinning down.

7 Add 11.2 µL of Sequenase master mix to each sample. 1m

8 Incubate at 37 °C for 00:08:00 . 8m

9 Resuspend AMPure XP Beads (AXP) by vortexing. Add 25 µL (0.8X) of AXP beads to each sample and mix by flicking the tube. 2m

10 Incubate on a Hula mixer at Room temperature for 00:05:00 . 5m

11 Spin down and pellet on a magnet for 00:05:00 ; remove supernatant. 6m

12 Add 200 µL freshly prepared 80% ethanol (do not remove from magnet); incubate 00:00:30 , remove ethanol. Repeat. 2m

13 Dry spin and remove residual ethanol. Allow pellet to air dry for about 00:00:30 on the magnet, but do not dry the pellet to the point of cracking. 2m

14 Remove the tube from the magnet and resuspend pellet in 11 µL NFW. 1m

15 Incubate on a Hula mixer at Room temperature for 00:10:00 . 10m

16 Spin down, pellet on magnet, and transfer 10 µL of eluate to a clean 0.2 mL PCR tube. 5m

17 Use 2 µL of each sample to quantify with Qubit dsDNA HS assay. Use remaining 8 µL sample for Rapid Barcode PCR. 5m

Note

Take your cDNA sample(s) forward to Stage 2 of the protocol.
Optional: if you are not using your sample(s) immediately, store at -20°C.

Stage 2. Rapid Barcode PCR

2h 10m

18 Prepare 5 µL each of viral cDNA (diluted to a concentration of 1 ng/µL, for a total of 5 ng) in a separate clean 0.2 ml tube. 5m

Note

If the concentration of viral cDNA is <1 ng/µL, then the volume can be increased to reach a total of 5 ng. Additional volume beyond 5 µL must be subtracted from the 20 µL of NFW added in step 19.

19 To each tube, add 20 µL of NFW, 1 µL of Rapid Barcode Primer (RLB01-24, at 10 µM), and 25 µL 2X LongAmp *Taq* Master Mix. Mix by gently flicking the tube and spin down. 5m

20 Amplify using the following cycling conditions: 95 °C for 00:00:45 ; 25 cycles of 95 °C for 00:00:15 , 56 °C for 00:00:15 and 65 °C for 00:04:00 followed by a final extension step of 65 °C for 00:06:00 . Hold at 10 °C . 2h

Note

Take your PCR product(s) forward to the clean-up, quantification, and adapter attachment steps.
Optional: PCR amplification may be performed and held at the final hold temperature overnight.

Stage 3. Clean-up, quantification, and adapter attachment

47m

21 Quantify the sample tubes (PCR products from the previous step) using the Qubit dsDNA HS Assay Kit. 5m

22 In a new 1.5 mL Eppendorf DNA LoBind tube, pool all barcoded samples in equal ratios to a combined final concentration 800 ng. 5m

Note

For example: if 10 barcodes were used, add 80 ng from each sample.

23 Resuspend the AXP beads by vortexing. Add 0.6X volume of AXP beads to the pooled samples. 2m

24 Incubate on a Hula mixer at Room temperature for 00:05:00 . 5m

25 Spin down and pellet on a magnet for 00:05:00 ; remove supernatant. 6m

26 Add 500 µL freshly prepared 80% ethanol (do not remove from magnet); incubate 00:00:30 , remove ethanol. Repeat. 2m

27 Dry spin and remove residual ethanol. Allow pellet to air dry for about 00:00:30 on the magnet, but do not dry the pellet to the point of cracking. 2m

28 Remove the tube from the magnet and resuspend pellet in 14 µL Elution Buffer (EB). 1m

29 Incubate at Room temperature for 00:02:00 . 2m

30 Spin down, pellet on magnet, and transfer 13 µL of eluate to a clean 1.5 mL tube. 5m

31 Use 2 µL of eluate to quantify with Qubit dsDNA HS assay. Use remaining 11 µL for adapter attachment. 5m

Expected result

The eluate should be approximately 10–40 ng/µL.

32 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the Rapid Adapter (RA) dilution as described below and mix by pipetting: 1m

	Reagent	Volume
	Rapid Adapter (RA)	1.5 µL
	Adapter Buffer (ADB)	3.5 µL
	TOTAL	5 µL

33 Add 1 µL of diluted RA to the library pool. Mix gently by flicking the tube and spin down. 1m

34 Incubate at Room temperature RT for 00:05:00 . 5m

35 The prepared library pool is used for loading into a MinION/GridION/PromethION Flow Cell. Keep the pool on ice or at 4 °C until ready to load.

Protocol references

Rapid metagenomic sequencing for surveillance of bacterial, fungal and viral pathogens using SQK-RPB114.24