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Sequencing the Virome of Wild Eastern Cottontail Rabbits using Oxford Nanopore Technologies (ONT) V.1

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Portable Genome Sequen… BIT 595 PGS

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We are still developing and optimizing this protocol

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Disclaimer

Protocols are linked.

Abstract

This protocol can be used to perform multiplex sequencing of a virome using Oxford Nanopore Technologies (ONT).

Forty RNA samples, including blood (n = 10), fecal (n = 10), epithelial (n = 10) and mucosal (n = 10) collected using cotton swabs from 10 wild Eastern Cottontail rabbits and stored at -80 °C. The 40 samples were combined into four pools each composed of ten samples of the same type. Sequencing was performed using the cDNA-PCR kit from

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Materials

RNA Extraction Using the TRIzol™ Protocol by Invitrogen

- 100–200 µl of pre-processed sample (blood, fecal, epithelial, and mucosal separately)
- TRIzol (Invitrogen)
- Phase Lock GelTM tubes (VWR) - optional
- RNase-free glycogen or GlycoBlue™ Coprecipitant (ThermoFisher)- optional
- 75% freshly-prepared ethanol
- Isopropanol
- Nuclease-free water or TE buffer
- 1.5 ml Eppendorf DNA LoBind tubes
- 1.5 ml Eppendorf tubes

Polyadenylation of Native RNA

- *E. coli* Poly(A) Polymerase (NEB: M0276L)
- 50 mM RNase-free EDTA (Invitrogen: **AM9260G**)
- Nuclease-free or DEPC-treated water, or 10 mM Tris-HCL, pH7.5
- Freshly-prepared 70% ethanol
- Agencourt RNAClean XP beads (Beckman Coulter: **A63987**)
- Nuclease-free 1.5 ml microcentrifuge tubes
- Heat block set to 37°C
- Centrifuge fitted for microcentrifuge tubes
- Vortex mixer
- Magnetic rack
- RNA HS Qubit kit (Invitrogen: **10320093**)
- Qubit fluorometer (Invitrogen: **16223001**)
- Hula mixer

Library Preparation with Oxford Nanopores Technologies (ONT)

- 10 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 500 ng total RNA
- cDNA-PCR Sequencing Kit V14 (SQK-PCS114)
- R10.4.1 flow cells (FLO-PRO114M)
- Flow Cell Wash Kit (EXP-WSH004)
- RNA Control Expansion (EXP-RCS001)
- Rapid Adapter Auxiliary V14 (EXP-RAA114)
- Sequencing Auxiliary Vials V14 (EXP-AUX003)
- Flow Cell Priming Kit V14 (EXP-FLP004)
- GridION **GridION Mk1 IT Requirements**

Pre-processing of Samples: Blood, Fecal, Epithelial, and Mucosal (Salival)

10s

1 **Blood, Fecal, and Epithelial Sample Pre-processing**

1.1 Place each swab in a 1.5-mL centrifuge tube with 500 µL of phosphate buffered saline (PBS). PBS is an isotonic solution that prevents the rupture of cells or shriveling of cells due to osmosis and is a non-toxic formulation for the cells.

1.2 Vortex the samples for 00:05:00 at room temperature, then centrifuge for 00:05:00 at 10000 rpm. Collect 400 µL in a fresh 1.5 ml Eppendorf DNA LoBind tube.

10m

2 **Mucosal (Salival) Sample Pre-Processing (Dilution)**

2.1 Dilute 100 µL of the sample in 300 µL of Saline Solution [M] 0.8 Mass Percent in a fresh 1.5 ml Eppendorf DNA LoBind tube. Normal saline solution can serve as RNA preservative, eliminating the need for snap-freezing the sample.

2.2 Vortex mix 00:00:10 s .

10s

RNA Extraction Using the TRIzol™ Protocol by Invitrogen

3 **Critical Step**: Ensure you start with at least 3X more TRIzol™ than the volume of your starting material. Extract 250 µL of each sample in a 1.5 ml Eppendorf DNA LoBind tube.

4 Follow the standard **TRIzol™ protocol**, using a Phase Lock GelTM tube to trap the organic phase. Add 1 µg RNase-free glycogen to the first isopropanol precipitation. Then use standard 1.5 ml tubes for the pelleting steps.

4.1 **Optional Step**: GlycoBlue Coprecipitant can be used to make the pellet visible.

5 Elute the RNA in 100 µL nuclease-free water or TE buffer. (Yields 10-20 ng of RNA)

Assessing input RNA

6 Oxford Nanopore Technologies (ONT) protocols recommend an input quantity in mass. The Direct RNA Sequencing Kit (SQK-RNA004) from ONT is used to prepare RNA for nanopore sequencing from an input of as low as 300 ng poly(A)+ RNA or 1 µg of total RNA (additional optimization may be required for total RNA). Therefore, it is important to assess the input RNA to determine if it is of high enough quality and amount to be used in direct RNA sequencing. The following protocol is described by ONT for **assessing input RNA**:

6.1

Polyadenylating with *E. coli* poly(A) polymerase

7 In a 1.5 ml microcentrifuge tube, set up the 3' polyadenylation reaction as follows:

Reagent	Voulme	Final
Non-polyadenylated RNA	X µl	≤ 10 µg
10X *E. coli* poly(A) polymerase buffer	2 µl	1X
ATP (10 mM)	2 µl	1 mM
Nuclease-free water	15-X µl	
E. coli poly(A) polymerase (5 U/µl)	1 µl	5U
TOTAL	**20 µl**	

7.1 Incubate the reaction mixture at 37 °C for 00:01:00 . 1m

7.2 Stop the reaction by adding 5 µL of 50 mM EDTA (to a final concentration of 10 mM EDTA). The final volume will be 25 µL

7.3 **Note**: An incubation time of 30–90 seconds to add a 3' adenosine homopolymer (poly(A) tail) of approximately 50-100 nucleotides in length. An increase in incubation time will result in

longer poly(A) tails. The maximum incubation time is 5 minutes and longer timings may result in a lower total sequencing yield.

7.4 **Optional Step**: It is recommended that EDTA be removed before starting any sequencing kit protocols.

8 Add 45 µL of RNase-free SPRI beads to the reaction.

9 Incubate on a Hula Mixer for 00:05:00 at room temperature. 5m

10 Spin down the sample and pellet on a magnet.

11 . Keep the tube on the magnet and pipette off and discard the supernatant.

12 Keep the tube on the magnet and wash the beads with 200 µl of freshly-prepared 70% ethanol. Carefully turn the tube 180° twice in the rack to wash pelleted beads. Pippette off and discard the supernatant.

13 Repeat the previous step for a total of two washes.

14 Briefly spin down and place the tube back on the magnet.

14.1 Pipette off any residual ethanol. Allow to dry for 00:00:30, but do not dry the pellet to the point of cracking. 30s

15 Remove the tube from the magnetic rack and resuspend the pellet in 12 µL of nuclease-free water. Incubate on ice for 00:05:00. 5m

16 Pellet the beads on a magnet until the eluate is clear and colourless.

17 Remove and retain 12 µL of eluate containing the 3'-polyadenylated RNA in a clean 1.5 ml microcentrifuge tube.

18 Remove 1 µL of final eluate and quantify the final concentration using HS RNA Qubit kit.

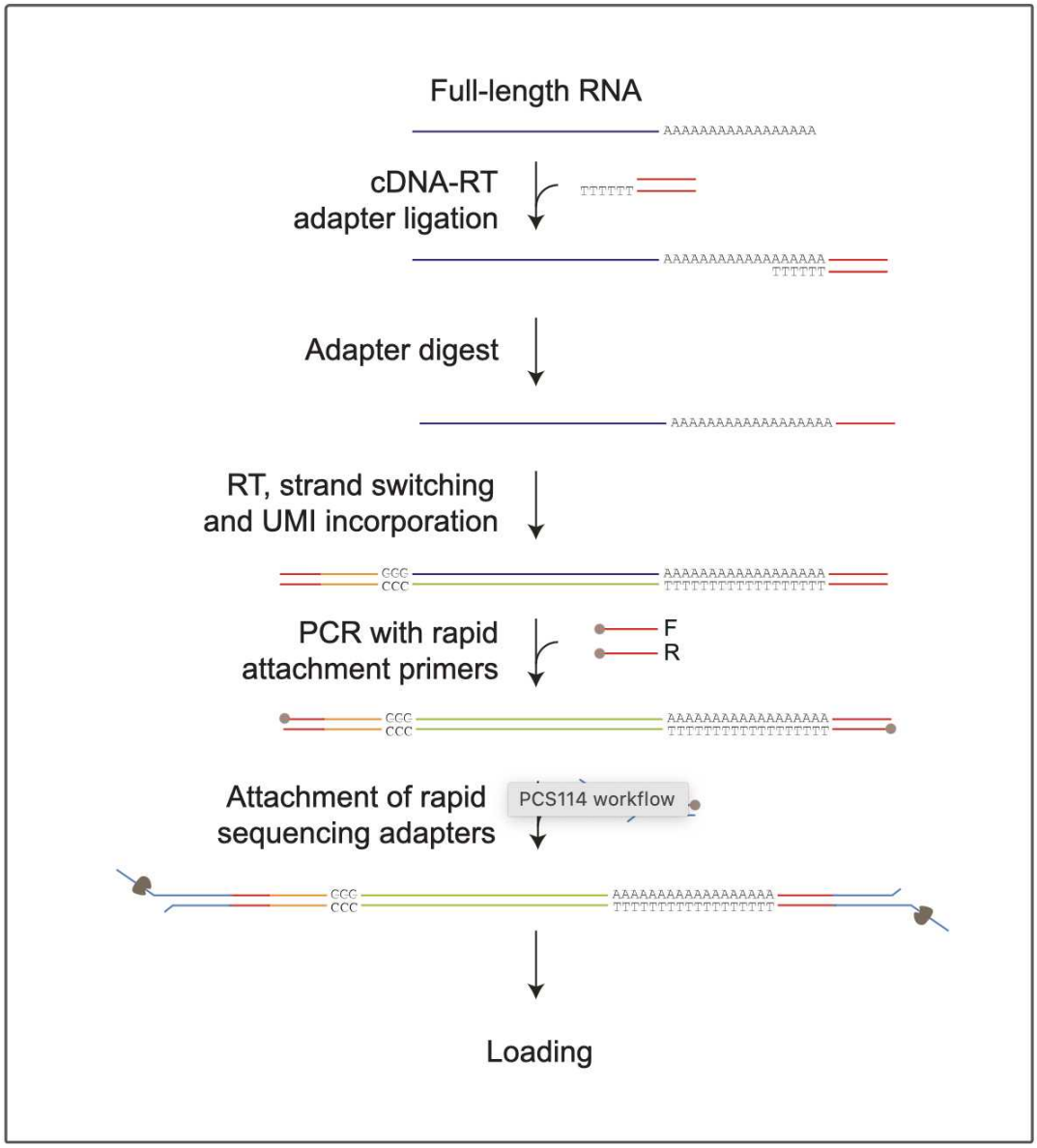
18.1 **Optional step**: Dilute the final eluate to 100 ng/µl and quantify 1 µL using Agilent 6000 Nano RNA kit, using total RNA or mRNA setting depending on the sample.

19 Store the final product at -80 °C or proceed immediately with the library preparation, keeping your sample on ice.

Library Preparation with the Direct RNA Sequencing Kit (SQK-RNA004)

20 Use the Direct RNA Sequencing (SQK-RNA004)Protocol from Oxford Nanopore Technologies (ONT). The table below is an overview of the steps required in the library preparation, including timings and stopping points.

Library preparation step	Process	Time	Stop option
Reverse transcription and strand-switching	Prepare full-length cDNA from Poly(A)+ RNA (or total RNA)	170 minutes	-20°C overnight
Selecting for full-length transcripts by PCR	Amplify the cDNA by PCR using rapid attachment primers during the PCR step	40 minutes	4°C short-term storage or for repeated use, such as re-loading your flow cell. -80°C for single-use long-term storage.
Adapter ligation	Attach the sequencing adapters to the to the PCR products.	5 minutes	We strongly recommend sequencing your library as soon as it is adapted.
Priming and loading the flow cell	Prime the flow cell and load the prepared cDNA library for sequencing	5 minutes	


Full-length RNA
AAAAAAAAAAAAAAAA
cDNA-RT
adapter ligation
TTTTTT
AAAAAAAAAAAAAAAAAA
TTTTTT
Adapter digest
AAAAAAAAAAAAAAAAAA
RT, strand switching
and UMI incorporation
CCC
CCC
AAAAAAAAAAAAAAAAAA
TTTTTTTTTTTTTTTTTT
PCR with rapid
attachment primers
F
R
CCC
CCC
AAAAAAAAAAAAAAAAAA
TTTTTTTTTTTTTTTTTT
Attachment of rapid
sequencing adapters
PCS114 workflow
CCC
CCC
AAAAAAAAAAAAAAAAAA
TTTTTTTTTTTTTTTTTT
Loading

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