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Version 1

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

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**Protocol status:** In development

**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 Gel™ 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  $\mu\text{L}$  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  $\mu\text{L}$  in a fresh 1.5 ml Eppendorf DNA LoBind tube.

10m

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

- 2.1 Dilute  100  $\mu\text{L}$  of the sample in  300  $\mu\text{L}$  of Saline Solution  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  $\mu\text{L}$  of each sample in a 1.5 ml Eppendorf DNA LoBind tube.



- 4 Follow the standard TRIzol™ protocol, using a Phase Lock Gel™ tube to trap the organic phase. Add  1  $\mu\text{g}$  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  $\mu\text{L}$  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  $\mu\text{g}$  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                                                 | Volume                             | Final                 |
|---------------------------------------------------------|------------------------------------|-----------------------|
| Non-polyadenylated RNA                                  | X $\mu\text{l}$                    | $\leq 10 \mu\text{g}$ |
| 10X <i>E. coli</i> poly(A) polymerase buffer            | 2 $\mu\text{l}$                    | 1X                    |
| ATP (10 mM)                                             | 2 $\mu\text{l}$                    | 1 mM                  |
| Nuclease-free water                                     | 15–X $\mu\text{l}$                 |                       |
| <i>E. coli</i> poly(A) polymerase (5 U/ $\mu\text{l}$ ) | 1 $\mu\text{l}$                    | 5U                    |
| <b>TOTAL</b>                                            | <b>20 <math>\mu\text{l}</math></b> |                       |

- 7.1 Incubate the reaction mixture at  37  $^{\circ}\text{C}$  for  00:01:00 .

1m



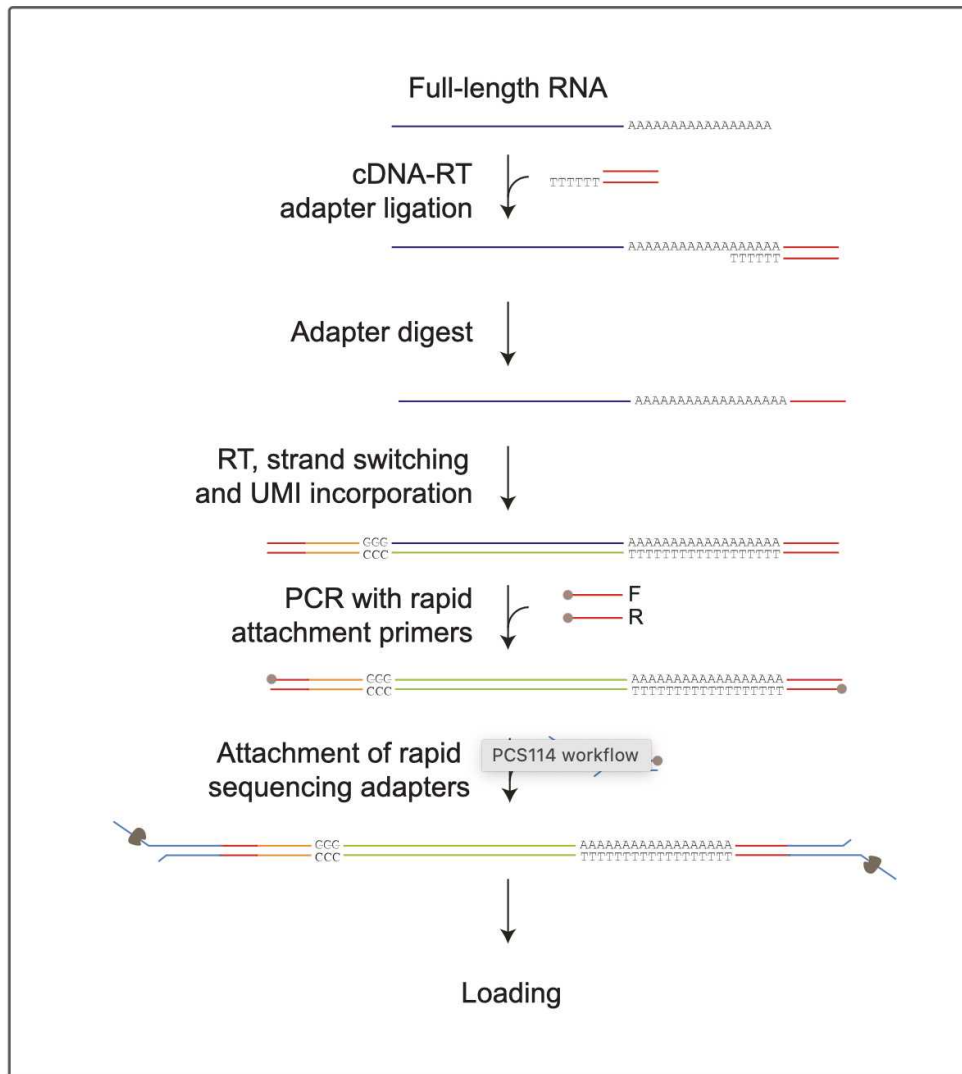
- 7.2 Stop the reaction by adding  5  $\mu\text{L}$  of 50 mM EDTA (to a final concentration of 10 mM EDTA). The final volume will be  25  $\mu\text{L}$
- 7.3 **Note:** An incubation time of 30–90 seconds to add a 3' adenosine homopolymer (poly(A) tail) of approximately 50–100 nucleotides in length. 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  $\mu\text{L}$  of RNase-free SPRI beads to the reaction.
- 9 Incubate on a Hula Mixer for  00:05:00 at room temperature. 
- 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  $\mu\text{L}$  of freshly-prepared 70% ethanol. Carefully turn the tube 180° twice in the rack to wash pelleted beads. Pipette 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. 
- 15 Remove the tube from the magnetic rack and resuspend the pellet in  12  $\mu\text{L}$  of  nuclease-free water. Incubate on ice for  00:05:00 .
- 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.<br>-80°C for single-use long-term storage. |
| Adapter ligation                             | Attach the sequencing adapters 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   |                                                                                                                           |





## Protocol references

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