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# RCA-NGS for RNA viruses with ONT V14 chemistry Forked from RCA-NGS for RNA viruses

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#### **ABSTRACT**

Note that this version of the protocl was adopted to V14 chemistry of ONT.

This RCA-NGS were optimized for an NGS machine, MinION. These methods do not require nucleic acid amplification with virus-specific PCR primers, physical viral particle enrichment, and RACE.

These methods enable whole RNA viral genome sequencing by combining the following techniques:

- 1) removal of unwanted DNA and RNA other than the RNA viral genome by nuclease treatment
- 2) the terminal of viral genome sequence determination by barcoded linkers ligation
- 3) Amplification of the viral genomic cDNA using an isothermal DNA amplification technique, such as rolling circle amplification (RCA).

This method can be exploited to determine any whole RNA viral genomes (i.e., single-stranded, double-stranded, positive-stranded, negative-stranded, non-segmented or multi-segmented genomes).

#### **MATERIALS**

Micrococcal Nuclease - 320,000 gel units New England Biolabs Catalog
#M0247S

**Keywords:** Oxford Nanopore Technology, RNA virus, Sequence method, MinION, Nanopore sequencing, RCA-NGS

- X High Pure Viral RNA Kit Roche Catalog #11858882001
- Turbo DNA-free Kit Invitrogen Thermo Fisher Catalog
  #AM1907
- NucleoSpin RNA Clean-up XS Takara, Catalog #740903.10
- T4 RNA Ligase 2, truncated KQ 2,000 units **New England Biolabs Catalog** #M0373S
- The barcode-polyA linker DNA (e.g., The cSP6-polyA linker DNA)
- Superscript IV Thermo Fisher Scientific Catalog #18090050
- SP6 primer (e.g., 5' phosphorylated SP6 primer)
- Deoxynucleotide (dNTP) Solution Mix New England Biolabs Catalog #N0447S
- Superase-In RNase Inhibitor Thermofisher Catalog #AM2694
- Dr.GenTLE Precipitation Carrier Takara Catalog #9094
- RNase H 250 units **New England Biolabs Catalog** #M0297S
- Agencourt AMPure XP Beckman Coulter Catalog
  #A63880
- CircLigase II ssDNA Ligase Biosearch Technologies Catalog #CL9021K
- GenomiPhi V3 Ready-To-Go DNA Amplification Kit Cytiva Catalog #25-6601-24
- T7 Endonuclease I 250 units New England Biolabs Catalog #M0302S
- NEBNext FFPE DNA Repair Mix 24 rxns New England Biolabs Catalog #M6630S
- NEBNext Ultra II End Repair/dA-Tailing Module 24 rxns New England Biolabs Catalog #E7546S
- Blunt/TA Ligase Master Mix 50 rxns New England Biolabs Catalog #M0367S
- NEBNext Quick Ligation Module 20 rxns New England Biolabs Catalog #E6056S
- Native Barcoding Kit 24 V14 Oxford Nanopore Technologies Catalog #SQK-NBD114.24
- Qubit 4 Fluorometer **Thermo Fisher Scientific Catalog** #Q33238
- Qubit 1X dsDNA HS Assay Kit Thermo Fisher Scientific Catalog #033230
- DNA LoBind Tube 1.5ml Eppendorf Catalog #022431021

- 0.2 ml PCR Tube strips **Eppendorf Catalog** #0030124359
- 100 % ethanol
- 70 % ethanol
- TE(pH8.0)
- nuclease-free H<sub>2</sub>O

### SAFETY WARNINGS

•

Follow your facility's regulations and biosafety practices.

### **BEFORE START INSTRUCTIONS**

This method was only confirmed to work with the working stocks that contain isolated RNA viruses at least  $3.0 \times 10^5$  TCID<sub>50</sub> per ml.

It is recommended to check no bacterial contamination(e.g., Mycoplasma spp.).

# **Preparation for virus supernatant**

1 Centrifuge the working stock virus to remove debris.

10m

6000 x g, Room temperature, 00:10:00

- Transfer  $\boxed{180 \, \mu L}$  virus supernatant to a 1.5ml screw cap tube.
- Unwanted DNA and RNA mainly originating from the virus-infected cells are digested using

  Micrococcal Nuclease 320,000 gel units New England Biolabs Catalog

  #M0247S
- 3.1 Total 201 µl reaction

1h

- 🗸 180 µL virus supernatant
- I 20 uL 10X Micrococcal Nuclease Reaction Buffer
- 🗓 1 µL Micrococcal nuclease

Mix by pipetting and spin down.



## The viral genomic RNA extraction

- 4 The viral genomic RNA extraction is performed using
  - ₩ High Pure Viral RNA Kit Roche Catalog #11858882001
- 4.1 Add  $\underline{A}$  400  $\mu$ L of binding buffer (with  $\underline{A}$  4  $\mu$ L PolyA carrier RNA).

10m

Mix gently by ~5 times pipetting and flicking thoroughly the tube, and spin down.

Room temperature 00:10:00

**4.2** Transfer the sample to a High Pure Filter Tube.

8000 x g, Room temperature, 00:01:00

Discard the flow-through liquid and Collection Tube, and insert the Filter Tube into a new Collection Tube.

4.3 Add A 500 ul of inhibitor removal bo transfer the sample to a High Pure Filter Tube.

8000 x g, Room temperature, 00:01:00

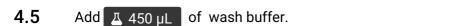
Discard the flow-through liquid and Collection Tube, and insert the Filter Tube into a new Collection Tube.

4.4 Add  $\pm$  450  $\mu$ L of wash buffer.

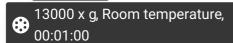
8000 x g, Room temperature, 00:01:00

Discard the flow-through liquid and Collection Tube, and insert the Filter Tube into a new Collection Tube.

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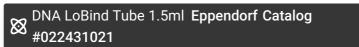


1m



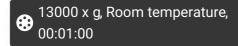
and discard the flow-through liquid.

Discard the Collection Tube and insert the Filter Tube into a 1.5 ml tube -



4.6 Add Δ 50 μL Elution Buffer.



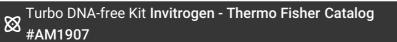


Note

The eluted RNA can be stored at -80°C.

## **Remove unwanted DNA**

5 Unwanted DNA mainly from the virus-infected cells in the RNA sample is digested using a



**5.1** Total 56 μl reaction



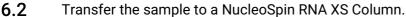
- Δ 50 μL the eluted RNA
- <u>Δ 5 μL</u> 10X reaction buffer
- 🗸 1 µL DNase I

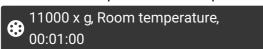
Mix gently by pipetting and spin down.



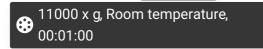
The viral RNA is purified using **NucleoSpin RNA Clean-up XS - Takara, Catalog #740903.10.** 





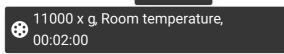


6.3 Wash the column by A 400 µL Buffer RA3.



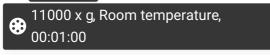
Discard the flow-through liquid and Collection Tube, and insert the NucleoSpin RNA XS Column into a new Collection Tube.

6.4 Wash the column by A 200 µL Buffer RA3.

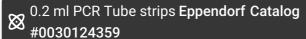


Discard the flow-through liquid and Collection Tube, and insert the NucleoSpin RNA XS Column into a Nuclease-free Collection Tube(1.5 ml).

6.5 Add  $\blacksquare$  10  $\mu$ L RNase-free H<sub>2</sub>O.



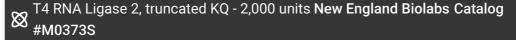
Transfer the sample to a 0.2 ml PCR tube -



# cSP6-polyA Linker DNA ligation

7

The viral RNA is ligated with cSP6-polyA Linker DNA using



The RNA is ligated to the 3' end with the barcoded(complementary sequence of SP6 (cSP6)) polyA linker DNA. It is able to identify the 3' terminal viral genome sequence. The PolyA sequence is required for reverse transcription for ONT kit (SQK-PBK004/ PCS109).

....

1m

#### Note

### 7.1 Total 20 µl reaction

- I 10 µL Purified RNA
- Δ 1 μL
   10 μM the cSP6-polyA linker DNA
- Δ 2 μL 10X T4 RNA Ligase Reaction Buffer
- Δ 6 μL 50% PEG8000 solution
- Δ 1 μL
   T4 RNA Ligase 2, truncated KQ

Mix gently by pipetting and spin down.

The linker-ligated viral RNA is purified using **NucleoSpin RNA Clean-up XS - Takara, Catalog** #740903.10



Fill the sample to 100  $\mu$ l with 80  $\mu$ l TE (pH 8.0) and add 100  $\mu$ l (equal volume) of Buffer RCU.

Eluted the RNA in  $\boxed{\text{\em L}}$  10  $\mu\text{\em L}$  RNase-free H<sub>2</sub>O and transfer the sample to a 0.2 ml PCR tube.

# **Reverse transcription**

**9** The viral RNA is reverse transcribed using

Superscript IV Thermo Fisher Scientific Catalog #18090050

5' phosphorylated SP6 primer is used for reverse transcription.

#### Note

**SP6 primer (**5' phosphorylated SP6 primer**)**; 5' [Phos]GATTTAGGTGACACTATAG 3' 5' phosphorylation is due to circularization.

### **9.1** Set up pre-mixture

6m

- Д 10 µL RNA (~ 50ng)
- Δ 1 μL 50 μM SP6 primer
- 🔼 1 µL nuclease-free H<sub>2</sub>O
- <u>Δ 1 μL</u> 10mM dNTP -

Deoxynucleotide (dNTP) Solution Mix New England Biolabs Catalog #N0447S

Mix gently by flicking the tube, and spin down.

### 9.2 Total 20 µl reaction

20m

- 🗸 13 µL pre-mixture sample
- A µL 5X SSIV Buffer
- <u>Δ 1 μL</u> 100mM DTT
- Z 1 μL RNase OUT -

**⋈** Superase-In RNase Inhibitor **Thermofisher Catalog** #AM2694

Δ 1 μL SuperScript IV Reverse Transcriptase

Mix gently by flicking the tube, and spin down.

\$\cdot \ 55 \cdot \ \cdot \ 00:10:00 \\ \cdot \ 80 \cdot \ \cdot \ \cdot \ 00:10:00 \\ \cdot \ \cdot \ 00:10:00 \\ \cdot \ \cdot \cdot \cdot \cdot \cdot \cdot \ \cdot \

## **RNase H treatment**

# cDNA purification using AMPure XP

11 Agencourt AMPure XP Beckman Coulter Catalog cDNA is purified using #A63880

Prepare AMpure XP beads for use; resuspend by vortexing.

Transfer amplified DNA sample to 1.5ml low binding tube.

11.1 AMPure XP reagent and mix by pipetting. volume)

Incubate on rotor mixer.

- **(?)** 00:05:00 ! Room temperature
- 11.2 Spin down and pellet on a magnet.

Wait for 00:01:00 and pipette off the supernatant.

- 11.3 Wash twice by A 100 µL 70 % ethanol and remove the ethanol using a pipette and discard.
- 11.4 Spin down and pipette off any residual ethanol.
- 11.5 Resuspend pellet in A 12 µL TE(pH 8.0).

Incubate on a rotor mixer.

**(:)** 00:05:00 Room temperature

11.6 Spin down and pellet the beads on the magnet until the elute is clear and colourless. 

# (Optional step) Short cDNA fragment removal

12 Short cDNA fragment is removed from the viral RNA sample using

Agencourt AMPure XP Beckman Coulter Catalog #A63880

Prepare AMpure XP beads for use; resuspend by vortexing.

Transfer amplified DNA sample to 1.5ml low binding tube.

#### Note

If a significant proportion of the reads obtained from an NGS run fail to match with the NCBInr database (i.e., no hits), it could indicate a large number of short cDNA fragments in the sample. In such instances, re-performing the optional step following step 11 by adding x1.8 volume of AMPure XP could significantly enhance the outcomes.

- 12.1 Add  $\blacksquare$  8  $\mu$ L of TE to the  $\blacksquare$  12  $\mu$ L of elute to adjust  $\blacksquare$  20  $\mu$ L
- 13 Size selection of the cDNA sample is performed using

Agencourt AMPure XP Beckman Coulter Catalog #A63880

X0.8 volume of AMPure beads recovers more than 200 bp of nucleic acids.

13.1 Add  $\mu$  16  $\mu$ L (X0.8 AMPure beads and mix by pipetting.

Incubate on rotor mixer.

© 00:05:00 S Room temperature

13.2 Spin down and pellet on a magnet.

Wait for 00:01:00 and pipette off the supernatant.

13.3 Wash twice by Δ 100 μL 70 % ethanol and remove the ethanol using a pipette and discard.
13.4 Spin down and pipette off any residual ethanol.
13.5 Resuspend pellet in Δ 12 μL nuclease-free water. Incubate on a rotor mixer.
② 00:05:00 Room temperature
13.6 Spin down and pellet the beads on the magnet until the elute is clear and colourless.
13.7 Remove retain Δ 12 μL elute into a new tube.

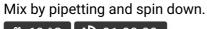
# **Circularization of cDNA**

1h 10m

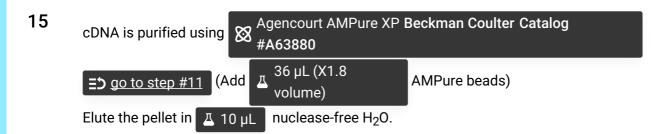
- 14 The cDNA is circularized using CircLigase II ssDNA Ligase Biosearch Technologies Catalog #CL9021K.
- **14.1** Total 20 μl reaction

1h 10m

- <u>I</u> 12 µL cDNA
- I 2 µL 10X reaction buffer
- <u>Δ 1 μL</u> 50 mM MnCl<sub>2</sub>
- A μL 5M Betaine
- I μL CircLigase II







# Amplification of cDNA by rolling circle amplification (RCA)

- 16 cDNA is amplified by Rolling circle amplification (RCA) using **GenomiPhi V3 Ready-To-Go DNA**Amplification Kit Cytiva Catalog #25-6601-24.
- **16.1** Total 20 μl reaction

3m

- <u>A</u> 10 µL cDNA
- 🔼 10 µL 2X denaturation buffer

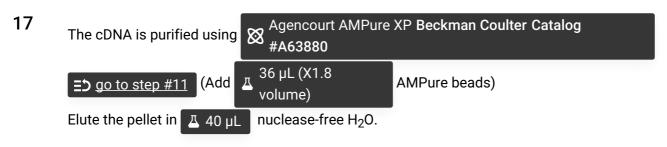
Mix by pipetting and spin down.



16.2 Add 20 μl denatured sample to Ready to go GenomiPhi cake.







18 DNA concentration is measured using a Qubit 4 Fluorometer with

# $\bowtie$ Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog** #Q33230

- 🗸 199 µL 1X working solution
- A 1 µL DNA

Mix by vortexing.

Incubate 00:02:00 Room temperature and measure.

#### Note

Confirm the total amplified cDNA to be over 1500 ng, as confirmed using, for instance, a Qubit 4 Fluorometer and Qubit 1X dsDNA HS Assay Kit.

### T7 endonuclease treatment

19 The amplified cDNA by RCA is digested using

T7 Endonuclease I - 250 units **New England Biolabs Catalog** #M0302S

to remove

branching.

The following protocol is modified based on the Native barcoding amplicons (with EXP-NBD104, EXPNBD114, and SQK-LSK109) protocol (NBA\_9093\_v109\_revA\_12Nov2019) provided by Oxford Nanopore Technologies website.

**19.1** Total 30 μl reaction

30m

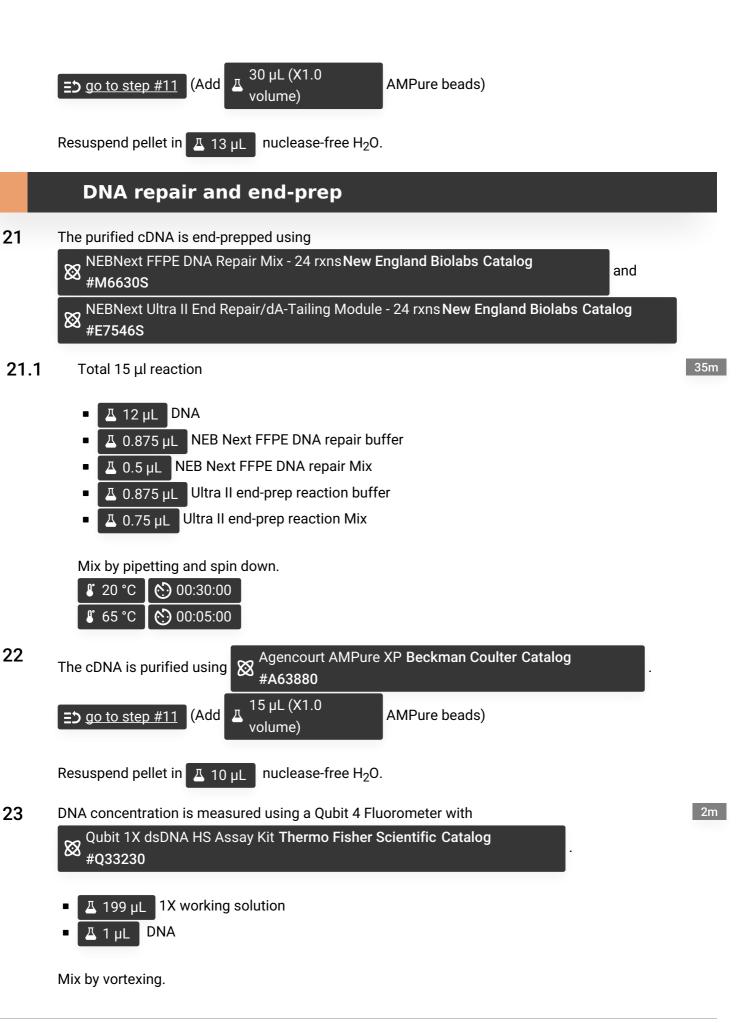
- Δ<sub>X</sub> μL (1.0 μg) DNA
- 🗓 3 µL NEBuffer 2
- 🗸 1.5 µL T7 endonuclease I
- $\triangle$  25-x  $\mu$ L nuclease-free H<sub>2</sub>O

Mix by pipetting and spin down.

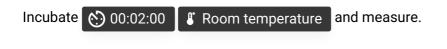
**37 °C ♦** 00:30:00

The cDNA is purified using

Agencourt AMPure XP **Beckman Coulter Catalog** #**A63880** 



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### Note

Confirm the purified cDNA to be approximately 700 ng or more using, for instance, Qubit 4 Fluorometer with a Qubit 1X dsDNA HS Assay Kit.

#### Note

The cDNA can be stored at 4°C overningt.

# **Native barcode ligation**

The end-prepped cDNA is ligated with native barcode using Native Barcoding Kit V14 - Oxford Nanopore Technologies Catalog #SQK-NBD114.24 and

Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs Catalog** #**M0367S** 

**24.1** Total 20 μl reaction

20m

- 🚨 x µL DNA(about 400ng)
- I 1.5 µL native barcode
- 🗓 10 µL Blunt/TA ligase master mix
- $\blacksquare$  8.5-x µL nuclease-free H<sub>2</sub>O

Mix by pipetting and spin down.



25 Add Δ 20 μL TE(pH8.0).



The cDNA is purified using Agencourt AMPure XP Beckman Coulter Catalog #A63880

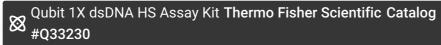




AMPure beads)

Resuspend pellet in  $\boxed{\text{\em L}}$  12  $\mu\text{L}$  nuclease-free H<sub>2</sub>O.

27 DNA concentration is measured using a Qubit 4 Fluorometer with



- Д 199 µL 1X working solution
- 🗸 1 µL DNA

Mix by vortexing.

Incubate 00:02:00 Room temperature and measure.

Convert nanogram(ng) into femtomole(fmol) by a calculator.

#### Note

The molar concentration of the cDNA sample can be converted based on the length of the major band confirmed by electrophoresis after T7 endonuclease treatment. Typically, the fragment lengths are around 2000 bases pairs.

# **Adaptor ligation**

20m

Pool each barcoded sample into a 0.2ml PCR tube (Total 100–200 fmol).

#### Note

Even when the V14 kit was used, we have good results with using total 100 to 200 fmol of samples.

29 Adaptor Ligation with pooled samples is performed using
Ligation Sequencing Kit - Oxford Nanopore Technologies Catalog #SQK-NBD114.24 and

# NEBNext Quick Ligation Module - 20 rxns New England Biolabs Catalog #E6056S

**29.1** Total 20 μl reaction

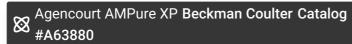
20m

- Д x µL DNA (100-200 fmol)
- Z 2 μL Native Adapter (NA)
- Д 4 μL NEB Next Quick Ligation Reaction Buffer(5X)
- 🗓 2 µL Quick T4 DNA ligase
- 🔼 12-x µL nuclease-free H<sub>2</sub>O

mix gently and incubate.



**30** The adaptor-ligated cDNA is purified using



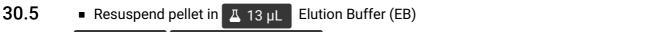
Prepare AMpure XP beads for use; resuspend by vortexing. Transfer amplified DNA sample to 1.5ml low binding tube.

30.1 Add  $\frac{10 \,\mu\text{L} (\text{X}0.5 \,\text{Volume})}{\text{Volume}}$  AMPure XP reagent and mix by pipetting.

Incubate on a rotor mixer.



- 30.2 Spin down and pellet on a magnet. Wait for 00:01:00 and pipette off the supernatant.
- 30.3 Wash twice by  $\Delta$  100  $\mu$ L Short Fragment Buffer(SFB) and remove the SFB using a pipette and discard.
- **30.4** Spin down and pipette off any residual SFB.



© 00:05:00 Room temperature and tapping occasionally.

- 30.6 Spin down and pellet the beads on the magnet until the elute is clear and colourless.
- 30.7 Remove retain  $\boxed{\text{L}}$  13  $\mu\text{L}$  elute into a new tube.
- 31 DNA concentration is measured using a Qubit 4 Fluorometer with

Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog** #Q33230

- 🔼 199 µL 1X working solution
- **Z** 1 µL DNA

Mix by vortexing.

Incubate 00:02:00 Room temperature and measure.

# **Sequencing by MinION**

32 Sequencing according to the manufacturer's instructions.