

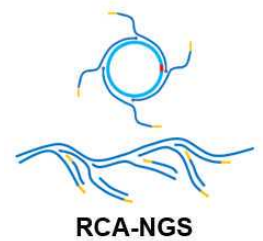
May 23, 2023

# RCA-NGS for RNA viruses with ONT V14 chemistry

 Forked from [RCA-NGS for RNA viruses](#)

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MinION



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**We use this protocol and it's working**

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## Abstract

Note that this version of the protocol 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**
-  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**
-  Superscript-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 #Q33230**
-  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**



## Protocol materials

- ⊠ High Pure Viral RNA Kit **Roche Catalog #11858882001**
- ⊠ Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs Catalog #M0367S**
- ⊠ NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**
- ⊠ T4 RNA Ligase 2, truncated KQ - 2,000 units **New England Biolabs Catalog #M0373S**
- ⊠ Superscript IV **Thermo Fisher Scientific Catalog #18090050**
- ⊠ Micrococcal Nuclease - 320,000 gel units **New England Biolabs Catalog #M0247S**
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- ⊠ Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q33230**
- ⊠ 0.2 ml PCR Tube strips **Eppendorf Catalog #0030124359**
- ⊠ Turbo DNA-free Kit **Invitrogen - Thermo Fisher Catalog #AM1907**
- ⊠ Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog #N0447S**
- ⊠ Superase-In RNase Inhibitor **Thermofisher Catalog #AM2694**
- ⊠ RNase H - 250 units **New England Biolabs Catalog #M0297S**
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✕ Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

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

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

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

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

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

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## Troubleshooting

## Safety warnings

! Follow your facility's regulations and biosafety practices.

## Before start

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.

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

10m

- 2 Transfer 180  $\mu$ L virus supernatant to a 1.5ml screw cap tube.

- 3 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  $\mu$ l reaction

1h

- 180  $\mu$ L virus supernatant
- 20  $\mu$ L 10X Micrococcal Nuclease Reaction Buffer
- 1  $\mu$ L Micrococcal nuclease

Mix by pipetting and spin down.

37 °C water bath 01:00:00

## 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 400  $\mu$ L of binding buffer (with 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.

1m

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  500  $\mu\text{L}$  of inhibitor removal bo transfer the sample to a High Pure Filter Tube.


1m

 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  450  $\mu\text{L}$  of wash buffer.


1m

 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.


- 4.5 Add  450  $\mu\text{L}$  of wash buffer.

1m


 13000 x g, Room temperature, 00:01:00 and discard the flow-through liquid.

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

 DNA LoBind Tube 1.5ml **Eppendorf Catalog #022431021** .

- 4.6 Add  50  $\mu\text{L}$  Elution Buffer.


1m

 13000 x g, Room temperature, 00:01:00

#### Note



The eluted RNA can be stored at  $-80^{\circ}\text{C}$ .

## Remove unwanted DNA


- 5 Unwanted DNA mainly from the virus-infected cells in the RNA sample is digested using a  Turbo DNA-free Kit **Invitrogen - Thermo Fisher Catalog #AM1907** .

- 5.1 Total 56  $\mu\text{L}$  reaction

30m

-  50  $\mu\text{L}$  the eluted RNA
-  5  $\mu\text{L}$  10X reaction buffer



-  1  $\mu\text{L}$  DNase I


Mix gently by pipetting and spin down.

 37 °C  00:30:00

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


- 6.1 Add an equal volume  56  $\mu\text{L}$  of Buffer RCU and mix gently.

- 6.2 Transfer the sample to a NucleoSpin RNA XS Column.

 11000 x g, Room temperature, 00:01:00

1m

- 6.3 Wash the column by  400  $\mu\text{L}$  Buffer RA3.

 11000 x g, Room temperature, 00:01:00

1m

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  200  $\mu\text{L}$  Buffer RA3.

 11000 x g, Room temperature, 00:02:00

2m

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  10  $\mu\text{L}$  RNase-free  $\text{H}_2\text{O}$ .

 11000 x g, Room temperature, 00:01:00

1m


Transfer the sample to a 0.2 ml PCR tube -

 0.2 ml PCR Tube strips **Eppendorf Catalog #0030124359** .

## cSP6-polyA Linker DNA ligation

7

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

 T4 RNA Ligase 2, truncated KQ - 2,000 units **New England Biolabs Catalog #M0373S**










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).

#### Note



The cSP6-polyA linker DNA (5'-5rApp-CTATAGTGTACCTAAATCAAAAAAAAAAAAAAAAAAAAAA-3ddC-3'), which is pre-adenylated at the 5' terminal (5rApp), and consists of the complementary sequence of SP6 (CTATAGTGTACCTAAATC), oligo (dA) 20, and dideoxycytidine (3ddC) at the 3' terminal, was synthesised for 3' linker ligation by Integrated DNA Technologies (Coralville, IA).

### 7.1 Total 20 µl reaction

15m

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


Mix gently by pipetting and spin down.

Incubate  25 °C  00:15:00

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



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

Eluted the RNA in  10 µL 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

- 10  $\mu$ L RNA (~ 50ng)
- 1  $\mu$ L 50  $\mu$ M SP6 primer
- 1  $\mu$ L nuclease-free H<sub>2</sub>O
- 1  $\mu$ L 10mM dNTP -

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

Mix gently by flicking the tube, and spin down.

65 °C 00:05:00 and 4 °C 00:01:00

### 9.2 Total 20 $\mu$ l reaction

20m

- 13  $\mu$ L pre-mixture sample
- 4  $\mu$ L 5X SSIV Buffer
- 1  $\mu$ L 100mM DTT
- 1  $\mu$ L RNase OUT -
- Superscript-III RNase Inhibitor Thermofisher Catalog #AM2694
- 1  $\mu$ L SuperScript IV Reverse Transcriptase

Mix gently by flicking the tube, and spin down.

55 °C 00:10:00



80 °C

00:10:00

## RNase H treatment

20m

10 Add 1  $\mu$ L RNase H - 250 units **New England Biolabs Catalog #M0297S** .

20m

37 °C

00:20:00

## cDNA purification using AMPure XP

11 cDNA is purified 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.

11.1 Add 36  $\mu$ L (X1.8 volume) AMPure XP reagent and mix by pipetting.

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 100  $\mu$ L 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 12  $\mu$ L TE(pH 8.0).

5m

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.

11.7 Remove and retain 12  $\mu$ L elute into a new tube.



## (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 NCBI-nr 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  8  $\mu\text{L}$  of TE to the  12  $\mu\text{L}$  of elute to adjust  20  $\mu\text{L}$  .


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  16  $\mu\text{L}$  (X0.8 volume) AMPure beads and mix by pipetting.


Incubate on rotor mixer.

 00:05:00

 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  100  $\mu\text{L}$  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  12  $\mu\text{L}$  nuclease-free water.

Incubate on a rotor mixer.

 00:05:00

 Room temperature

5m

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



13.7 Remove retain  12  $\mu\text{L}$  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  $\mu\text{L}$  reaction

1h 10m

-  12  $\mu\text{L}$  cDNA
-  2  $\mu\text{L}$  10X reaction buffer
-  1  $\mu\text{L}$  50 mM  $\text{MnCl}_2$
-  4  $\mu\text{L}$  5M Betaine
-  1  $\mu\text{L}$  CircLigase II


Mix by pipetting and spin down.

 60  $^{\circ}\text{C}$   01:00:00

 80  $^{\circ}\text{C}$   00:10:00

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

 [go to step #11](#) (Add  36  $\mu\text{L}$  (X1.8 volume) AMPure beads)



Elute the pellet in  10  $\mu\text{L}$  nuclease-free  $\text{H}_2\text{O}$ .

## 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  $\mu\text{L}$  reaction

3m

-  10  $\mu\text{L}$  cDNA
-  10  $\mu\text{L}$  2X denaturation buffer

Mix by pipetting and spin down.



🌡️ 95 °C ⌚ 00:03:00

🌡️ 4 °C on ice

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

4h 10m

🌡️ 30 °C ⌚ 04:00:00

🌡️ 65 °C ⌚ 00:10:00

17 The cDNA is purified using

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

⇒ go to step #11 (Add 🧪 36 µL (X1.8 volume) AMPure beads)

Elute the pellet in 🧪 40 µL nuclease-free H<sub>2</sub>O.

18 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
- 🧪 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

- x µL (1.0 µg) DNA
- 3 µL NEBuffer 2
- 1.5 µL T7 endonuclease I
- 25-x µL nuclease-free H<sub>2</sub>O

Mix by pipetting and spin down.

37 °C 00:30:00

## 20 The cDNA is purified using

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

(Add 30 µL (X1.0 volume) AMPure beads)

Resuspend pellet in 13 µL nuclease-free H<sub>2</sub>O.

## DNA repair and end-prep

## 21 The purified cDNA is end-prepped using

NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs Catalog #M6630S**

and

NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

## 21.1 Total 15 µl reaction

35m

- 12 µL DNA
- 0.875 µL NEB Next FFPE DNA repair buffer
- 0.5 µL NEB Next FFPE DNA repair Mix
- 0.875 µL Ultra II end-prep reaction buffer
- 0.75 µL Ultra II end-prep reaction Mix

Mix by pipetting and spin down.

20 °C 00:30:00

65 °C 00:05:00



22 The cDNA is purified using

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

(Add 15 µL (X1.0 volume) AMPure beads)

Resuspend pellet in 10 µL nuclease-free H<sub>2</sub>O.

23 DNA concentration is measured using a Qubit 4 Fluorometer with

2m

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

- 199 µL 1X working solution
- 1 µL DNA

Mix by vortexing.

Incubate 00:02:00 Room temperature and measure.

#### 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 overnighgt.

## Native barcode ligation

24 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)
- 1.5 µL native barcode
- 10 µL Blunt/TA ligase master mix





- 8.5-x  $\mu\text{L}$  nuclease-free  $\text{H}_2\text{O}$

Mix by pipetting and spin down.

25 °C 00:20:00

- 25 Add 20  $\mu\text{L}$  TE(pH8.0).

- 26 The cDNA is purified using

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

(Add 20  $\mu\text{L}$  (X1.0 volume) AMPure beads)

Resuspend pellet in 12  $\mu\text{L}$  nuclease-free  $\text{H}_2\text{O}$ .

- 27 DNA concentration is measured using a Qubit 4 Fluorometer with

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

- 199  $\mu\text{L}$  1X working solution
- 1  $\mu\text{L}$  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

- 28 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

-  x µL DNA (100-200 fmol)
-  2 µL Native Adapter (NA)
-  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.

 25 °C  00:20:00

- 30 The adaptor-ligated cDNA is purified 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.

- 30.1 Add  10 µL (X0.5 volume) AMPure XP reagent and mix by pipetting.

Incubate on a rotor mixer.

 00:05:00  Room temperature


- 30.2 Spin down and pellet on a magnet. Wait for  00:01:00 and pipette off the supernatant.

- 30.3 ▪ Wash twice by  100 µL Short Fragment Buffer(SFB) and remove the SFB using a pipette and discard.



30.4 Spin down and pipette off any residual SFB.


30.5 



- Resuspend pellet in  13  $\mu$ L Elution Buffer (EB)  00:05:00  Room temperature and tapping occasionally.

5m



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

30.7 Remove retain  13  $\mu$ 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  $\mu$ L 1X working solution
-  1  $\mu$ L DNA

Mix by vortexing.

Incubate  00:02:00  Room temperature and measure.

## Sequencing by MinION

32 Sequencing according to the manufacturer's instructions.