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RNA extraction and purification from cultured diatom viruses

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Abstract

This protocol describes a workflow for processing viral lysates to obtain genome sequences of marine RNA viruses. The procedure includes lysate clarification, virus concentration by tangential flow filtration, chloroform-based purification, RNA extraction and conversion to cDNA, and final nucleic acid quantification. cDNA produced using this protocol is suitable for sequencing genomes of diatom-infecting RNA viruses. This protocol is adapted from the method described in Arsenieff et al. (2019).

Image Attribution

Transmission electron micrograph of *M.comicus* (RCC4660) infection with an Bacillarnavirus (RCC7291).

Guidelines

Guides and Manuals:
MasterPure Complete guide
SuperScript III Reverse Transcriptase guide

Materials

Equipments :
weighing machin & pH meter
Centrifuge Avanti J-25 XP & Rotor JA-10 (Bekman Coulter)
Centrifuge **5804 R** (Eppendorf)
Centrifuge **5427 R** (Eppendorf)
Chemical hood
Micropipets
Hand pump and pump
Eppendorf thermomixer & smartblock 1,5ml
Thermocycler
Microwave
Electrophoresis system & generator **(Sub Cell Model 96 cell)**
Image Quant LAZ4000 (Ge Healthcare)
Qubit 4 fluorimeter (**Invitrogen Q33238**)

Consumables:
Autoclaved 500mL Centrifuge bottles, PPCO/HDPE/PC/FEP, with screw cap, Nalgene® (VWR, cat no : 525-2313)
1L Ventilated Culture flask (Starlab, cat no : CC7682-4822)
0,45µm Filtration unit (VWR, cat no : 2566881-514-0343)
Vivaflow® 200 30Kda, MWCO, PES (Sartorius, cat no : VF20P2)
Vivaspin® 20 30Kda, MWCO, PES (Cytiva, cat no : 28932361)
Syringe filter 0,2µm 25mm, PES (Fisher scientific, cat no : 15392388)
Falcon Tubes 50mL, 15mL
Microtubes 1,5mL ,2mL, 0,2µL (Eppendorf-type)
Filter tips

Reagents:
KH2PO4 (Merck Sigma, cat no : 60218-100G)
Na2HPO4 (Merck Sigma, cat no : 71642)
Chloroform (Merck Sigma, cat no : C2432)
Isopropanol (Merck Sigma, cat no : I9516)
Absolute ethanol (Fisher scientific, cat no : 10342652)
Tris-Base (Merck Sigma, cat no : T6791)
Acetic acid (>99.7%) (Fisher Scientific, cat no : 11463473)
0,5 M EDTA pH 8,0 (Fisher Scientific, cat no : 15575-020)

Master Pure complete DNA and RNA purification kit (Epicentre, cat no : MC85200)
Qubit RNA BR Assay Kit (Invitrogen, cat no : Q10210)
Qubit dsDNA BR Assay Kit (Invitrogen, cat no : Q32853)

SuperScript III Reverse Transcriptase (Invitrogen, cat no : 18080044)

RNAse OUT Recombinant Rnase Inhibitor (Invitrogen, cat no : 10777019)
Random primers, 250ng (Invitrogen, cat no : 48190011)
dNTP mix, 10mM (Invitrogen, cat no : 18427013)
Primers *RdRp* Forward and Reverse (10µM)
DNA polymerase Taq platinium; MgCl2 (50mM); Rxn PCR buffer (10X); dNTP mix (10mM) (Fisher scientific, cat no : 10966018)
Nuclease-Free Water (Invitrogen, cat no : AM9937)

Ice

Loading buffer
Agarose (Interchim, cat no : 31272L)
Ethidium bromide (Merck Sigma, cat no : E1510)
SmartLadder - 200 to 10000 bp (Eurogentec, cat no : MW-1700-10)

Before start

Solution preparation:

cold isopropanol at -20°C
cold 70% ethanol at -20°C

10mM phosphate solution (10 mM KH_2PO_4 + 10 mM Na_2HPO_4), pH 7.2 filtered through a 0,2µm filter

TAE Buffer (Tris Acetate EDTA) 50X :
- Tris-Base (2M final) : 242.5 g (Merck Sigma, cat no : T6791)
- Acetic acid (>99.7%, 2M final) : 7 ml (Fisher Scientific, cat no : 11463473)
- 0,5 M EDTA pH 8,0 (50 mM final) : 100 ml (Fisher Scientific, cat no : 15575-020)
- dH20 qsp 1L

RNA virus culture.

3w

1 One liter of diatom host culture is grown exponentially in K+Si medium (Keller et al., 1987) at 18 °C under a 12:12 h light:dark cycle with an irradiance of 100 µmol photons·m^{-2}·s^{-1}, provided by white fluorescent light (Philips Master TL-D 18W/865). A freshly produced viral lysate, filtered through a 0.45 µm PES membrane, is added to the host culture at a final concentration of 10–20% (v/v). The infected culture is incubated under the same conditions as the host culture until complete lysis is observed.

1 L

RNA virus concentration.

3d 7h 38m

2 **Lysate clarification :**

30m

- Centrifuge the viral lysate (2×500mL) 5353 x g, 4°C, 00:30:00 (Avanti J-25XP, Beckman Coulter-JA-10 Rotor).
- Carefully transfer the supernatant into a 0.45 µm filtration unit (Stericup), avoiding disturbance of the pellet.
- Filter the supernatant completely using a hand pump.

3 **Viral concentration :**

1d 4h

- Concentrate the clarified viral lysate to 50 mL using a Vivaflow® 200 cartridge (30 kDa MWCO, PES, Sartorius).
- Further concentrate the sample to ~1 mL using a Vivaspin® 20 (30 kDa MWCO, PES, Cytiva) by centrifugation 6000 x g, 4°C, 00:10:00
- Keep the Vivaspin at 4°C overnight.
- Rinse the Vivaspin® 20 by adding 1 mL of 10 mM phosphate solution, and transfer the rinse to the Falcon tube containing the sample.
- Repeat the rinsing step until a final volume of 10 mL is reached.

4 **Viral Purification :**

2d 3h 8m

Under a chemical hood :

- Add 1 volume of chloroform (v/v, 10mL) into the Falcon tube containing the sample.
- Vortex thoroughly.
- Centrifuge. 2200 x g, 12°C

- Carefully transfer the aqueous phase (upper phase containing viruses) into a new Falcon tube.
- Add again 1 volume of chloroform
- Repeat previous steps a total of 7 times. 03:00:00
- Leave the tube (containing 5mL of sample) open overnight under the chemical hood to evaporate residual chloroform. Overnight
- Concentrate the sample in 1mL by using Vivaspin®20 6000 x g, 12°C, 00:05:00
- Collect sample and add 1mL of nuclease-free water. Centrifuge in the same Vivaspin®20 6000 x g, 12°C, 00:03:00 . Repeat this whashing step 3 times.
- Incubate the Vivaspin®20 overnight at 4°C and, then, collect the final sample (1mL).

Safety information

Chloroform is a CMR chemical reagent.

RNA extraction using MasterPure Complete DNA & RNA purification kit. 2h

5 Extraction of 500µL viral nucleic acids using MasterPure Complete DNA & RNA Purification Kit (Epicentre). 2h

Cellular lysis:

- Split in two tubes 250µL of viral sample : "tube 1" and "tube 2".

This two tubes will be pooled and the end of extraction for concentration.

- Thoroughly mix the several buffers to homogenize them before dispensing.
- Dilute 4µL of Proteinase K into 600µL of 2X T and C Lysis Solution.
- Vortex 10s.
- Add 250µL of 2X T and C Lysis Solution containing the Proteinase K to each tube of viral sample and mix thoroughly.

- Incubate at 65°C for 15 minutes in a Eppendorf thermomixer at 1000rpm with smartblock 1,5mL.
- Place the samples on ice for 5 minutes to inactivate the proteinase K.

Nucleic acids precipation and extraction :

- Add 250 µL of MPC Protein Precipitation Reagent to 500 µL of lysed sample.
- Vortex vigorously for 10 seconds.
- Centrifuge. 11000 x g, 12°C, 00:10:00
- Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
- Centrifuge. 11000 x g, 12°C, 00:10:00
- Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
- Add 840 µL of cold isopropanol to the recovered supernatant. Invert tubes 40 times.
- Centrifuge tubes to pellet the total nucleic acid. 20800 x g, 12°C, 00:10:00
- Carefully pour off the isopropanol without dislodging the pellet.

Contaminating DNA digestion with DNase :

- Prepare DNAse I solution (1/40) : 10µL Rnase Free Dnase I into 390µL Dnase Buffer.
- Completely resuspend the total nucleic acid pellet in 200 µL of DNase I solution (1/40).
- Incubate at 37°C for 10 minutes.
- Add 200 µL of 2X T and C Lysis Solution.
- Vortex for 5 seconds.
- Add 200 µL of MPC Protein Precipitation Reagent.
- Vortex 10 secondes.
- Place on ice for 4 minutes.
- Centrifuge 11000 x g, 12°C, 00:10:00 to pellet the debris.
- Transfer the supernatant containing the RNA into a clean microcentrifuge tube and discard the pellet.
- Centrifuge 11000 x g, 12°C, 00:03:00 keep and transfer the supernatant into a new tube.
- Add 840µL of cold isopropanol to the supernatant. Invert tubes 40 times.
- Centrifuge 20800 x g, 12°C, 00:10:00 and discard supernatant.

RNA precipitation:

- Rinse twice with cold 70% ethanol, being careful to not dislodge the pellet.
- Centrifuge. 11000 x g, 12°C, 00:00:30
- Remove all the ethanol by pipetting and keep at room temperature the tube open during 10 minutes for the ethanol evaporation .
- Resuspend the total nucleic acids (RNA) of "tube 1" in 35 µL of TE Buffer.

- Re use this solution for resuspend the total nucleic acids of the "tube 2" in the aim of concentrate sample.
- Keep 2µl for Qubit quantification and store RNA extract at -80°C.

RNA Quantification using Qubit 4 fluorimeter.

40m

6
- Quantify the RNA concentration using the Qubit 4 fluorimeter and the RNA BR Assay kit which provides a detection range of 10 and 1200ng of RNA.

Retrotranscription to cDNA.

2h

7 By sample, add in 0,2 ml microtube :

- 1µL Random primers (250ng)
- 11µL RNA extract (Total RNA should range between 10pg - 5µg - complete with RNA-free water if necessary)
- 1µL 10mM dNTP

qsp 13µL : Add Nuclease Free water to complete.

Thermocycler parameter : 65°C 5min.
2min in ice.

In the same microtube, add :

- 4µL 5X First strand buffer
- 1µL 0,1M DTT
- 2µL Superscript III RT (400U/µl)
- 1µL RNAse OUT Recombinant Rnase Inhibitor.

Thermocycler parameter : 25°C 10min > 50°C 60min > 70°C 15min.

Store cDNA at -20°C.

cDNA Quantification using Qubit 4 fluorimeter.

8 Quantify the DNA concentration using the Qubit 4 fluorimeter and the dsDNA BR Assay kit which provides a detection range of 4 and 2000ng of DNA.

Protocol references

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