Nucleic Acid Extraction, Amplification and Library Construction for Viral Metagenomic Sequencing. V.2

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ABSTRACT

This protocol is a continuation of the previous protocol (dx.doi.org/10.17504/protocols.io.m4yc8xw) for viral metagenomic researches with the tissues of Molluscs, such as abalone, oyster, clam, scallop and so on. This maybe also applicable for other animals, such as shrimp and fish, based on the principle of the protocol. But we havn't verified this idea yet.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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Wei, HY., Huang, S., Wang, JY. et al. Genes Genom (2017). https://doi.org/10.1007/s13258-017-0629-1

ATTACHMENTS

10.1007%2Fs13258-017-0629-1.pdf

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MATERIALS TEXT

STEP MATERIALS

REPLI-g Cell WGA & WTA **Qiagen Catalog #150052** Step 4

NEBNext® Ultra™ II FS DNA Library Prep Kit **New England Biolabs Catalog #E7805, E6177** Step 28

TruePrep DNA Library Prep Kit V2 for Illumina **Contributed by users Catalog #TD501,502,503** Step 28

TruePrep Index Kit V# for Illumina **Vazyme Catalog #TD202-TD207** Step 28

NEBNext Singleplex or Multiplex Oligos for Illumina **New England Biolabs Catalog #E7350, #E7335, #E7500, #E6609, #** Step 28

NEBNext® Ultra™ II FS DNA Library Prep Kit **New England Biolabs Catalog #E7805, E6177** Step 30

NEBNext Singleplex or Multiplex Oligos for Illumina **New England Biolabs Catalog #E7350, E7335, E7500, E6609** Step 30

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Viral DNA/RNA Extraction

1 Now, the supernatant from step 13 of below protocol is ready, We will use HP Viral DNA/RNA Kit (Omega Bio-Tek, Norcross, GA, USA) to extract both the viral DNA and RNA sitimutaniously.

Isolation of Viral Like Particles (VLP) from Tissues of Molluscs
by Jing-Zhe (Ginger) Jiang

PREVIEW RUN

♠ The kit HP Viral DNA/RNA Kit is not indispensable. You can replace it with any brand of kits, such as QIAamp cador Pathogen Mini Kit (Qiagen), as long as it suitable for extraction both viral DNA and RNA from liquid samples.
♣ The entire procedure MUST BE RNase free

1.1 Tissue samples are sliced off and freezed in liquid Nitrogen instantly. Then transfer to -80°C freezer after return to laboratory.

Visceral mass and gills are the best target tissues for isolation, as viruses are easily concentrated in those organs.

We have tested with from minimum 20 mg of oyster spat samples to maximum 20 g of abalone samples for VLP isolation. And we all acquired certain amount of Viral reads after seqenced by Illunima platform.

1.2 Add about 1 to 1.5 times volume of SB (Stabilizing Buffer, 0.2 M NaCl, 50 mM Tris-HCl, 5 mM $CaCl_2$, and 5 mM $MgCl_2$, pH 7.5) and grind with grinding machine at 4°C.

For example, adding 1-1.5 mL SB buffer to homogenize 1g tissues

4 °C

1.3 Add extra SB to fix a final volume at 3-5 times of weightened tissue, and make it fully homogenized.

1.4 Put above homogenate into three freezing and thawing cycles

Use liquid nitrogen and 50°C water bath to proceed the cycle. But it needs to be removed **immediately** after

melting in the water bath.

1.5 Add extra SB to fix a final volume at 5-10 times of weightened tissue, if you want to maximize the dissolution of VLP from cell debris.

1.6 Conduct differential centrifugation at 4 °C, sequentially from 1,000 3,000 5,000 8,000 10,000 g, each time for 5 min.

Cell debris will precipitate to the bottom of tubes, and VLP will be keeped in supernatant.

00:25:00 Centrifuging

1.7 Supernatants then filtrated with Millex-HV 0.45 µm and 0.22 µm filters (Merck Millipore, Billerica, MA, USA) sequentially

0.22 µm filters will effectively remove most of bacterial cells. Please NOTICE that 0.22 µm not suitable for enriching viruses larger than 200 nm in diameter.

1.8 Sucrose cushion (sucrose-SB solution, 28% w/w) was added to the bottom of the ultracentrifuge tube first.

1.9 Transfer supernatants (step 7) into ultracentrifuge tubes, softly laying onto 28% (w/w) sucrose cushion.

Operation with carefulness, as 28% sucrose solution is easily disturbed

1.10 Centrifuging at 300,000 g for 2 h, then remove supernatant. VLPs are just in the precipitation

You can pause at this step, and put VLP pellet into -80℃ for reservation.

02:00:00 Ultracentrifuging

1.11 Add the appropriate amount of DNase solution (820 µl ddH_2O, 90 µl 10× DNase I Buffer, 90 µl DNase I (1 U/µl)) to the tube, and use pipette tip to fully suspend precipitation.

1.12 Incubating at 37 °C for 60 min in shaker at 150 rpm.

01:00:00 DNase Incubating

1.13 Centrifuging at 10000 g for 5 min and then transferr supernatant to a new tube.

00:05:00 Centrifuging

2 Follow the kit protocol, then you will acquire 15-50 µl of viral DNA/RNA solution.

DO NOT add carrier RNA, as carrier RNA will account a high percentage in the dataset from high throughput seqencing.

3 Use Qubit™ (Thermo Fisher) and Qubit™ dsDNA HS Assay Kit (Thermo Fisher) to specifically quantify the concentration of dsDNA. If the amount is high that means previous extraction is successful.

If you like, you can also use Qubit™ RNA HS Assay Kit to specifically quantify RNA. But we don't recommand Qubit™ ssDNA Assay Kit, as it is not specific.

Choosing Amplification Strategy

4 Now, we are ready to use REPLI-g® Cell WGA & WTA Kit (Qiagen) to amplify both viral DNA RNA, or only viral RNA.

♠ We havn't fully assessed the performances of three amplification strategies.
♣ However, we found that the production of WTA is usually lower than WGA and RT-WGA protocol. Sometime, the barren production of WTA will even not be qualified to the further library construction.
♥ And the differences in terms of viral diversity with three strategies are still under assessment.

♦ We just use *half of the required volume* of kit protocol to save some money.

◊ DO READ THROUGH the whole REPLI-g Cell WGA & WTA Handbook

REPLI-g Cell WGA &

WTA **Qiagen Catalog #150052**

Step 4 includes a Step case.

WTA

RT-WGA

WGA

for viral RNA amplification (WTA)

step case

WTA

for viral RNA amplification

5 Place 3 µl nucleic acid, add 2 µl Lysis Buffer. Mix by vortexing and centrifuge briefly.

6 Incubate at 24°C for 5 min.

00:05:00

24 °C

7 Add 1 µl gDNA Wipeout Buffer the lysed sample. Mix by vortexing and centrifuge briefly.

8 Incubate at 42°C for 10 min.

00:10:00

42 °C Wiping out DNA

9 Prepare Quantiscript RT mix (Table 1).

Table 1. Quantiscript RT mix

Component	Volume/reaction
RT/Polymerase Buffer	2 µl
H_2O sc	0.5 µl
Random Primer	0.5 µl
Oligo dT Primer	0.5 µl
Quantiscript RT Enzyme Mix	0.5 µl
Total volume	4 µl

♠ gDNA Wipeout buffer will be deactivate in this Mix. So the DNA is kept during RT reaction.
♣ The using of both random and oligo dT Primers will improve the proformance of RT reaction.
♥ **NOTE:** The Quantiscript RT mix must be prepared fresh.

10 Add 4 µl RT mix to the aliquot (6 µl) in step 8. Mix by vortexing and centrifuge briefly.

11 Incubate at 42°C for 60 min

01:00:00

42 °C Reverse Transcripting

12 Stop the reaction by incubating at 95ºC for 3 min, then cool on ice.

00:03:00

95 °C Stopping RT

0 °C Cooling

13 Prepare the ligation mix (Table 2).

Table 2 Ligation mix

Component	Volume/reaction
Ligase Buffer	4 µl
Ligase Mix	1 µl
Total volume	5 µl

IMPORTANT: When preparing the ligation mix, add the components in the order shown in Table 2.
NOTE: The ligation mix must be prepared fresh.

14 Add 5 µl ligation mix to the WTA ready reaction (10 µl) from step 12. Mix by vortexing and centrifuge briefly.

15 Incubate at 24°C for 30 min.

00:30:00

24 °C Ligating

16 Stop the reaction by incubating at 95°C for 5 min.

00:05:00

95 °C Stopping ligating

17 Prepare REPLI-g SensiPhi amplification mix (Table 3).

Table 3 Preparing REPLI-g Sensiphi amplification mix

Component	Volume/reaction
REPLI-g sc Reaction Buffer	14.5 µl
REPLI-g Sensiphi DNA Polymerase	0.5 µl
Total volume	15 µl

NOTE: The REPLI-g SensiPhi amplification mix must be prepared fresh.

18 Add 15 µl REPLI-g SensiPhi amplification mix to the ligation reaction (15 µl) from step 16. Mix by vortexing and centrifuge briefly.

19 Incubate at 30°C for 2h.

02:00:00

30 °C Amplifying

20 Stop the reaction by incubating at 65°C for 5 min.

00:05:00

65 °C **Stopping Amplifying**

♠ If not being used directly, store the amplified DNA at –15 ºC to –30ºC until required for downstream applications.
♣ We recommend storage of the amplified DNA at a concentration of at least 100 ng/µl.

Cleaning with AMPure Beads

21 Adjust the volume to 100 µl by adding the appropriate volume of TE buffer. Then equilibrate to room temperature (15–25°C) for no longer than 10 min.

00:10:00 RT equilibrating

22 Add Fully mixed 0.6 × volume of AMPure Beads (Beckman Coulter) to the solution from above step. Mix by gently pipetting entire volume up and down 10 times

♠ Take out AMPure Beads from refrigerator to sit in RT for at least 15 min.
♣ Make sure AMPure Beads is full mixed before use, this will ensure the purification effect.

23 Incubate at RT for 15 min.

00:15:00

24 Place the tube on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant.

Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads).

00:05:00

25 Add 200-600 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

00:00:30

26 Repeat above Step to do the ethanol clean again.

27 Put the tube back in the magnetic stand,completely remove the residual ethanol, and air dry beads for 10 minutes while the tube is on the magnetic stand with lid open.

00:10:00

28 Elute the DNA target from the beads with 15 µl nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic stand until the solution is clear.

29 Transfer the supernatant to a clean PCR tube.

30 We recommend using the transposase base library construction strategy. These kit convert the complex steps of DNA fragment, end repair and adaptor connection into a one-step enzymatic reaction, which significantly reduce the demanded amount of the initial DNA and shorten the time of library construction.

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