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

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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.

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Guidelines

Protocol

Viral DNA/RNA Extraction

Step 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.

PROTOCOL

. **Isolation of Viral Like Particles (VLP) from Tissues of Molluscs**

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NOTES

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♠ 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

Sampling

Step 1.1.

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

NOTES

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

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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.

Releasing VLP from Tissues

Step 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℃.

TEMPERATURE

4 °C Additional info:

NOTES

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

Releasing VLP from Tissues

Step 1.3.

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

Releasing VLP from Tissues

Step 1.4.

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.

Separation of VLP from Cell Debris

Step 1.5.

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

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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.

Concentrating VLP via Ultracentrifuging

Step 1.6.

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

Separation of VLP from Cell Debris

Step 1.7.

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

NOTES

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

Concentrating VLP via Ultracentrifuging

Step 1.8.

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

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

Releasing VLP from Tissues

Step 1.9.

Put above homogenate into three freezing and thawing cycles

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

Wiping out Free DNA Fragments

Step 1.10.

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.

Concentrating VLP via Ultracentrifuging

Step 1.11.

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

NOTES

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

Wiping out Free DNA Fragments

Step 1.12.

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

Wiping out Free DNA Fragments

Step 1.13.

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

Viral DNA/RNA Extraction

Step 2.

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

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

Viral DNA/RNA Extraction

Step 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.

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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

Step 4.

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

REAGENTS

REPLI-g Cell WGA & WTA 150052 by Qiagen

NOTES

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♠ We havn't fully assessed the performances of three amplification strategies.

♣ However, we found that the production of WTA is usually than WTA&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

Amplification Viral RNA (WTA)

Step 5 - WTA (viral RNA amplification).

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

Amplification Viral RNA (WTA)

Step 6 - WTA (viral RNA amplification).

Incubate at 24°C for 5 min.

TEMPERATURE

24 °C Additional info:

Amplification both DNA and RNA (WGA&WTA)

Step 5 - WGA&WTA (both viral DNA and RNA amplification).

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

Amplification both DNA and RNA (WGA&WTA)

Step 6 - WGA&WTA (both viral DNA and RNA amplification).

Incubate at 24°C for 5 min

TEMPERATURE

24 °C Additional info: Lysising