OsHV-1 detection and quantification by Real Time Polymerase Chain Reaction

CONTENTS

Editions

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1. Scope

This procedure explains a standard diagnostic test used for the detection and the quantification of the Ostreid Herpesvirus type 1 (OsHV-1) in the Pacific Cupped Oyster *Crassostrea gigas*. It allows detecting all the variants of OsHV-1 described until now but it does not allow distinguishing between OsHV-1 (GenBank # AY509253) and OsHV-1 µvar (GenBank # HQ842610) for example.

2. References

Pépin J.F., Riou A., Renault T. 2008. Rapid and sensitive detection of ostreid herpesvirus1 in oyster samples by real-time PCR. Journal of Virological Methods 149, 269–276

Davison AJ, Trus BL, Cheng NQ, Steven AC, Watson MS, Cunningham C, Le Deuff RM and Renault T, 2005. A novel class of herpesvirus with bivalve hosts. J. gen. Virol., 86, 41-53.

Segarra A, Pépin JF, Arzul I, Morga B, Faury N, Renault T, 2010. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France. Virus Research 153: 92–99

Webb SC, Fidler A and Renault T, 2007. Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): Application in a survey of New Zealand molluscs. Aquaculture, 272, 126-139.

3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for Real Time PCR assays:

- A closed hood equipped with an UV producing system to eliminate potential contaminations when preparing PCR mix
- Two complete sets of pipettes (2 µl; 20 µl; 200 µl and 1000 µl), the first one for DNA extraction, and the second one for Real Time PCR mix preparation.
- An additional pipette (20 µl) to dispense samples in Real Time PCR mix
- Filter pipette tips (2 µl; 20 µl; 200 µl and 1000 µl) for DNA extraction, Real Time PCR mix preparation and sample dispensing
- Real Time PCR plates and caps
- A thermocycler for real-time PCR (for example Mx3000 Thermocycler sequence detector from Stratagene®)

Manipulator must wear a lab coat and gloves during all the different steps described below. Lab coat and gloves must be changed preferably after each main step: DNA extraction, preparation of PCR mix and sample dispensing.
It is recommended to perform these different steps in different rooms. Amplification particularly should take place in a room separate from DNA extraction, PCR mix preparation and DNA dispensing.

4. Procedure

4.1. Sample preparation

Live or freshly dead (not decaying) oysters, which can be previously frozen, are processed for DNA extraction. Samples are processed differently according to their size:

a) For larva, **pools** of 50 mg of whole animals (including the shell) completed with 200 µl of distilled water are crushed and centrifuged at 1000 g for 1 minute.
b) For spat smaller than or of 6 mm, **pools** of 300 mg of whole animals (including the shell) completed with 1200µl of distilled water are crushed and centrifuged at 1000 g for 1 minute.
c) For spat between 6 and 15 mm in size, all or part of the soft tissues from each animal are grinded **individually**.

d) For animals bigger than 15 mm, pieces of gills and mantle are sampled and processed **individually**.

DNA extraction is performed by using the QIAamp® DNA Mini Kit (QIAGEN) and following the instructions for Tissue Test Protocol.

The further sample preparation is performed in the following order:

1. Place 100 µl of supernatant for samples **a** and **b** or 10 to 50 mg of tissues for samples **c** and **d** in a microcentrifuge tube and add 180 µl of Buffer ATL.

2. Add 20 µl Proteinase K, mix by vortexing and incubate at 56°C until the tissue is completely lysed (1-3 h at least or overnight). Vortex occasionally during incubation to disperse sample. Briefly centrifuge the microcentrifuge tube to remove drops from the lid.

3. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 sec. and incubate at 70°C for 10 minutes. Briefly centrifuge the microcentrifuge tube to remove drops from the lid.

4. Add 200 µl ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 sec. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the lid.

5. Carefully apply the mixture from step 4 to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. Close the cap and centrifuge at 10 000 rpm for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided in the kit) and discard the tube containing the filtrate.

6. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 10 000 rpm for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided in the kit) and discard the collection tube containing the filtrate.

7. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (14 000 rpm) for 3 min.

8. (Optional) Place the QIAamp Spin Column in a new 2ml collection tube (not provided in the kit) and discard the collection tube containing the filtrate. Centrifuge at full speed (14 000 rpm) for 1 min.

9. Place the QIAamp Spin Column in a clean microcentrifuge tube (not provided in the kit) and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 50 µl of molecular biology grade water. Incubate 5 minutes at room temperature and centrifuge at 10 000 rpm for 1 min.

10. Control the quality and efficacy of the extraction (for example by measuring DO (260 nm) under spectrophotometer or after electrophoresis in agarose gel).

11. Just before performing the real time PCR assay, prepare dilution of your samples in order to have a final DNA concentration of 5 ng/µl.

12. DNA solutions are kept at 4°C until PCR analyses are performed.

Other commercial kits may be used for the DNA extractions provided they have been demonstrated to give similar results.

4.2. Real Time Quantitative Polymerase Chain Reaction

4.2.1. Reactives

2X Brilliant SYBR® Green Q PCR Master Mix (Stratagene® ref: # 600548),

H_20 (free of DNA and RNA)
Other commercial Q PCR Master Mix may be used provided they have been demonstrated to give similar results.

4.2.2. Primers

HVDP-F 5' ATTGATGATGTGGATAATCTGTG 3' (5 µM)
HVDP-R 5' GGTAAATACCATTGGTCTTGTTCC 3' (5 µM)

Note: These primers amplify a PCR product of 197 bp

4.2.3. Real Time PCR Mix

Real Time PCR mix for each tube is:

	Volume per tube	**Final concentration**
2X Brilliant SYBR® Green Q PCR Master Mix	12.5µl	1X
HVDP-F (5µM)	2.5µl	0.5µM
HVDP-R (5µM)	2.5µl	0.5µM
H_2O	2.5µl	

- 20 µl of this Real Time PCR mix is dispensed in each Real Time PCR plate well
- 5 µl of extracted DNA (5 ng/µl) is added to each tube
- each sample should be tested in duplicate
- Real Time PCR mix should be prepared in excess (for example for two additional samples)

4.2.4. Negative Controls

Negative controls or NCT consist of dH_2O (5 µl for 20 µl of Real Time PCR Mix). They aim at detecting potential reactive contamination of working environment. One negative control should be included in each Real Time PCR plate.

4.2.5. Standard curves

Standard curves are prepared using dilutions of viral or plasmidic DNA suspension.
This suspension corresponds to a known amount of viral DNA copies (extracted from purified virus particles) or to a known amount of plasmidic DNA including the target region HVDP-F-HVDP-R.
This suspension is 1:10 serially diluted. The standard curve should include at least 5 dilutions:

Copies of OsHV-1 DNA/µl
100 000
10 000
1 000
100
10

Standard curves aim at checking the efficacy of the PCR reaction and at estimating the amount of viral copies present in tested samples.
One standard curve should be included for each Real Time quantitative PCR analysis.

4.2.6. Amplification

Amplification cycles are performed using an Mx3000 Thermocycler sequence detector (Stratagene®).

- Initial denaturation: 10 min at 95°C

- Amplification: 40 cycles (30 sec at 95°C, 1 min at 60°C and 45 sec at 72°C)
- Melting temperature curve analysis: 1 min at 95°C, 30s at 60°C and 30s at 95°C

4.2.7. Interpretation

Threshold cycles (C_t), defined as the cycle at which a statistically significant increase in fluorescence output above background is detected. Ct are calculated automatically by the Stratagene thermocycler software. Reported Ct values are calculated as averages of the duplicates within each reaction.

4.2.1.7. Controls

Before concluding about the status of the tested samples regarding the presence and the quantification of OsHV-1, NTC should not present any amplification and the standard curve should fulfil the following requirements:

- difference between duplicated values should not exceed 0.5 Ct
- each dilution should present a unique melting temperature peak 77.2 ±0.4 °C
- the efficiency of the standard curve should be contained between 90 and 110%
- the slope of the standard curve should be closed to -3.3 and regression coefficient should be at least 0.98
- the Ct values of the standard curves should be contained in the following frame (+/- 0.5 Ct):

Copies OsHV-1 ADN/µl	**Ct value**		
	Minimum	**Medium**	**Maximum**
100 000	19.5	20.02	21
10 000	22.5	23.33	24
1 000	26	26.79	27.45
100	29.5	30.27	31
10	32.5	33.18	34

4.2.2.7. Samples

A tested sample is considered positive if:

- its melting temperature peak is 77.2 ±0.4 °C
- its mean Ct value is below 37

Quantification of OsHV-1 can be estimated in a positive sample if:

- the difference between duplicated values is less than 0.5 Ct
- Ct value is included in the frame of the standard curve

Note: “Doubtful samples” (shifting in Tm value; difference in replicates) or samples with Ct values out of standard curve, should be tested again.