

Vibrio splendidus et *V. aestuarianus* detection by Real Time Polymerase Chain Reaction

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Editions

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1. Scope

This procedure explains a standard diagnostic test used for the detection of *Vibrio splendidus* and *V. aestuarianus* in the Pacific Cupped Oyster (*Crassostrea gigas*). This multiplex qPCR assay is based on *V. splendidus* 16S rDNA gene sequence and *V. aestuarianus* *dnaJ* gene sequence.

It allows the specific detection of all bacteria from *V. splendidus* polyphyletic group (*V. lentus*, *V. cyclitrophicus*, *V. pomeroyi*, *V. tasmaniensis*, *V. splendidus*, *V. kanaloae*, *V. gigantis* and *V. crassostreae*) (FAM signal) and the specific detection of *V. aestuarianus* strains (Texas Red signal).

2. References

Saulnier D., De Decker S., Haffner P., 2009. Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: a useful tool for epidemiologic studies. *Journal of Microbiological Methods* 77(2), 191-197

Saulnier D., Travers M.A., De Decker S. (In prep). Development of a duplex real-time PCR assay for rapid identification of *Vibrio splendidus*-related and *V. aestuarianus* strains from bacterial colony

3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for bacteriology:

- Sterile environment
- Artificial sterilized marine water (2.3% NaCl, 20mM KCl, 5mM MgSO₄, 2mM CaCl₂)
- TCBS plates (Difco 265020, boiled but not autoclaved)
- Incubator (20°C)

This test also 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 PCR mix preparation.
- An additional pipette (20 µl) to dispense samples in PCR mix
- Filter pipette tips (2 µl; 20 µl; 200 µl and 1000 µl) for DNA extraction, PCR mix preparation and sample dispensing
- Real Time PCR plates
- A thermocycler for real-time PCR (for example Mx3000 Thermocycler sequence detector from Stratagene/Agilent).

Manipulator must wear a lab coat and some 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, sample dispensing and amplification. It is recommended to perform these different steps in

different rooms. More particularly, amplification 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 decomposed) oysters are processed for bacterial analyses.

Samples are processed differently according to their size:

- a. For larvae, **pools** of 50 mg of the 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 the whole animals (including the shell) completed with 1200 µl of distilled water are crushed and centrifuged at 1000 g for 1 minute.
- c. For spat between 6 and 15 mm in size, all the soft tissues of each animal are crushed **individually**.
- d. For animals bigger than 15 mm, pieces of gills and/or mantle are isolated from the whole tissues and processed **individually**.
- e. For animals bigger than 40 mm, hemolymph is also sampled (if possible) and pieces of gills and/or mantle are isolated from the whole tissues and processed **individually**.

Dilutions to the 1/100 and 1/10,000 are performed in Artificial Sterilized Marine Water (ASMW) and fifty (50) µl of those dilutions are plated on TCBS plates.

4.2. *Classical bacteriology*

Petri dishes are incubated for 48 to 96h at 20°C. Colonies observed (in non-confluent plates) with a similar macroscopical aspect are counted.

One colony representative of each majoritary bacterial type is identified by the multiplex qPCR assay, with a maximum of five (5) different colonies per sample.

Bacteria corresponding to one representative colony are sampled with a wooden sterile toothpick and put in suspension into two hundred (200) µl of molecular biology grade water. Too much material can inhibit the reaction; just take a few of the colony (just a touch).

4.3. *DNA extraction*

Bacteria corresponding to one representative colony are sampled with a wooden sterile toothpick and put in suspension into two hundred (200) µl of molecular biology grade water.

Total DNA is extracted by heating the samples ten (10) minutes at 98°C. After a quick cooling to 4°C, five (5) µl are immediately used for the assay.

4.4. Real Time Polymerase Chain Reaction

4.4.1. Reactives

Sure Taq Polymerase (Agilent ref 600804)
 10X Core PCR buffer
 dNTP 20 mM
 MgCl₂ 50 mM
 H₂O (free of DNA and RNA)

4.4.2. Primers and Probes

	<i>V. splendidus</i>	<i>V. aestuarianus</i>
Forward primer	16S SpF2 ATCATGGCTCAGATTGAACG	DNAj F GTATGAAATTTTAACTGACCCACAA
Reverse primer	16S SpR2 CAATGGTTATCCCCACATC	DNAj R CAATTTCTTTCGAACAACCAC
Probe	16S probe FAM CCCATTAACGCACCCGAAGGATTG BHQ1	DNAj probe Texas Red- TGGTAGCGCAGACTTCGGCGAC- BHQ2

4.4.3. PCR Mix

A PCR premix for 100 reactions is prepared and stored at -20 °C for at least 6 months.

qPCR Premix	Volume for 100 reactions	Final concentration
10X core PCR buffer	250 µl	1X
16S-SpF2 (100 µM)	7.5 µl	300 nM
16S-SpR2 (100 µM)	7.5 µl	300 nM
DNAj F (100 µM)	7.5 µl	300 nM
DNAj R (100 µM)	7.5 µl	300 nM
dNTP (20 mM)	100 µl	0.2 mM
MgCl ₂ (50 mM)	250 µl	5 mM
H ₂ O	1340 µl	

After premix defrosting, a fresh PCR mix is prepared.

PCR Mix	Volume per well	Final concentration
PCR premix	19.65 µl	
Sure Taq DNA Polymerase (5u/µl)	0.25 µl	0.05U/µl (1.25U/reaction)
16s probe (100 µM)	0.05 µl	200 nM
DNAj probe (100 µM)	0.05 µl	200 nM
DNA extracts (4.3)	5 µl	

After positioning qPCR plates on a 4°C plate carrier, twenty (20) µl of qPCR mix are distributed per well, and finally, five (5) µl of bacterial DNA are added. Plates are sealed and briefly centrifuged (100 g).

Each sample should be tested in duplicate

4.4.4. Negative and positive controls

Negative controls or NCT consist of dH₂O (5 µl for 20 µl of Real Time PCR Mix). They aim at detecting potential reactive contamination or working environment. One negative control should be included on each Real Time PCR plate.

Two positive controls are realised in each amplification plate : DNA extract obtained as described in 4.3 by boiling *V. splendidus* LGP32 strain (supplied by LGPMM, IFREMER) and DNA extract obtained as described in 4.3 by boiling *V. aesturianus* 01/032 strain (supplied by LGPMM, IFREMER)

4.4.5. Amplification

Amplification cycles are performed using a thermocycler for real-time PCR (for example Mx3000 Thermocycler sequence detector from Stratagene®)

-Initial denaturation: 10 min at 95°C

-Amplification: 40 cycles (15s at 95°C, 90s at 60°C)

4.4.6. Interpretation

Threshold cycles (Ct), 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.

Before concluding about the status of the tested samples regarding the presence of *Vibrio* DNA, NTC should not present any amplification and the positive controls should fulfil the following requirements:

- Difference between duplicated values should not exceed 0.5 Ct
- A tested sample is considered positive if its Ct value is below 37.