

**Vibrio aestuarianus detection by Real Time Polymerase Chain Reaction**

**CONTENTS**

**1. SCOPE .....2**

**2. REFERENCES.....2**

**3. EQUIPMENT AND ENVIRONMENTAL CONDITIONS .....2**

**4. PROCEDURE .....2**

**4.1. SAMPLE PREPARATION.....2**

        4.1.1. DNA extraction from a representative colony.....3

        4.1.2. DNA extraction from bivalve tissues .....3

**4.2. REAL TIME POLYMERASE CHAIN REACTION .....4**

        4.2.1. Reactives .....4

        4.2.2. Primers and Probes.....4

        4.2.3. PCR Mix.....4

        4.2.4. Negative Controls.....5

        4.2.5. Standard curves .....5

        4.2.6. Amplification .....5

        4.2.7. Interpretation .....5

**Editions**

| <b>Edition</b> | <b>Date</b> | <b>Updated part</b> |
|----------------|-------------|---------------------|
| N° 1           | 07/08/2013  | Creation            |
| N°2            | 13/03/2015  | Revision            |
| N°3            | 06/10/2015  | Revision            |

## *Vibrio aestuarianus* detection by Real Time Polymerase Chain Reaction

### 1. Scope

This procedure explains a standard diagnostic test used for the detection of the bacteria *Vibrio aestuarianus* in bivalves. It allows detecting all the strains of *V. aestuarianus* known until now based on the amplification of a fragment of the *dnaJ* gene encoding heat shock protein 40 (GenBank # AB263018).

### 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

**Garnier M, Labreuche Y, Nicolas JL, 2008.** Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp *francensis* subsp nov., a pathogen of the oyster *Crassostrea gigas*. *Systematic and Applied Microbiology* 31(5), 358-365.

**Nhung PH, Shah MM, Ohkusu K, Noda M, Hata H, Sun XS, Iihara H, Goto K, Masaki T, Miyasaka J, Ezaki T, 2007.** The *dnaJ* gene as a novel phylogenetic marker for identification of *Vibrio* species. *Systematic and Applied Microbiology*, 30(4), 309-315.

**Tison DL, Seidler RJ, 1983.** *Vibrio aestuarianus* - a new species from estuarine waters and shellfish. *International Journal of Systematic Bacteriology* 33(4), 699-702.

### 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 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 or PCR tubes
- A thermocycler for real-time PCR

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, sample dispensing and amplification.

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

DNA can be extracted from a representative colony selected after bacteria isolation or from bivalve tissues.

#### 4.1.1. DNA extraction from a representative colony

Predominant bacteria are isolated from marine mollusc tissues according to the SOP “Isolation of predominant bacteria in marine molluscs” available on the EURL website : <https://www.eurl-mollusc.eu/SOPs>.

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 for ten minutes at 98°C. After a quick cooling at 4°C, five µl are immediately used for the assay.

#### 4.1.2. DNA extraction from bivalve tissues

Live or freshly dead (not decaying) bivalves, 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 the soft tissues from each animal are grinded **individually**.
- d) For animals bigger than 15 mm, pieces of **gills** 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 1.5 mL 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 (overnight). Vortex occasionally during incubation to disperse sample. Briefly centrifuge the 1.5 mL 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 1.5 mL 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 1.5 mL microcentrifuge tube (not provided in the kit) and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 100 µL of distilled 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. Prepare dilution of your samples in order to have a final DNA concentration of 2-5 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 Polymerase Chain Reaction

### 4.2.1. Reactives

Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies® ref: # 600881),  
H<sub>2</sub>O (free of DNA and RNA)

### 4.2.2. Primers and Probes

*dnaJ*-F                    5' GTATGAAATTTTAACTGACCCACAA 3'  
*dnaJ*-R                    5' TCAATTTCTTTTGAACAACCAC 3'  
*dnaJ* -probe            5' Texas Red TGGTAGCGCAGACTTCGGCGAC BHQ-2 3'

Note: Primers amplify a PCR product of 267 bp.

### 4.2.3. PCR Mix

PCR mix for each tube is:

|   | Volume per tube | Final concentration |
|---|-----------------|---------------------|
| 2X Brilliant III Ultra-Fast QPCR Master Mix | 10µL            | 1X                  |
| <i>dnaJ</i> -F                              | 0.3µL           | 0.3µM               |
| <i>dnaJ</i> -R                              | 0.3µL           | 0.3µM               |
| <i>dnaJ</i> -probe                          | 0.2µL           | 0.2µM               |
| H <sub>2</sub> O                            | 4.2µL           |                     |

15 µL of this 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<sub>2</sub>O (5 µL for 15 µL of Real Time PCR Mix). They aim at detecting potential reactive contamination of working environment during the DNA extraction and the PCR. Two negative controls should be included for each test: one extraction negative control and one PCR negative control.

#### 4.2.5. Standard curves

**Standard curves** are prepared using dilutions of bacterial or plasmidic DNA suspension. This suspension corresponds to a known amount of bacterial DNA copies (extracted from bacteria culture) or to a known amount of plasmidic DNA including the target region *dnaJ* - F- *dnaJ* - R.

This suspension is 1:10 serially diluted. The standard curve should include at least 6 dilutions:

| Copies of <i>Vibrio aestuarianus</i> DNA/µL in the initial DNA suspension |
|---|
| 1 <sup>E8</sup>   |
| 1 <sup>E7</sup>   |
| 1 <sup>E6</sup>   |
| 1 <sup>E5</sup>   |
| 1 <sup>E4</sup>   |
| 1 <sup>E3</sup>   |

Standard curves aim at checking the efficacy of the PCR reaction and at estimating the amount of bacteria 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 a thermocycler for real-time PCR (for example Mx3000 Thermocycler sequence detector from Stratagene®)

-Initial denaturation: 3 min at 95°C

-Amplification: 40 cycles (10 sec at 95°C and 20 sec at 60°C)

The fluorescence was recorded at the end of each cycle with ROX or Texas Red filter

#### 4.2.7. Interpretation

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

## Controls

Before concluding about the status of the tested samples regarding the presence of *Vibrio aestuarianus*, negative control should not present any amplification and the standard curve should fulfil the following requirements:

- difference between duplicated values should not exceed 1 Ct
- the efficiency of the standard curve should be contained between 90 and 110%
- the slope of the standard curve should be close to -3.3 and regression coefficient should be at least 0.98

## Samples

A tested sample is considered positive if its Ct value is below 37 ( $\leq 37$ )

A tested sample is considered negative if there is no amplification or if its Ct value is above 39 ( $\geq 39$ )

A tested sample is considered doubtful if its Ct value is between 37 and 39 ( $37 < x < 39$ ) and should be tested again