Marteilia refringens and *Bonamia sp.* detection by Real Time Polymerase Chain Reaction

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1. Scope

This procedure explains a standard diagnostic test used for the detection of the *Marteilia refringens* and *Bonamia sp*. in bivalves, using a Mutiplex TaqMan real-time PCR approach. Two set of primers were designed to specifically amplify a fragment of the 18S rRNA gene of *Marteilia refringens* and a fragment of the 18S rRNA gene of *Bonamia sp.* Signal detection rely on the use of 2 specific labelled probes (HEX label for *Bonamia sp*., FAM label for *Marteilia refringens*).

2. 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 should 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.

3. Procedure

3.1. Sample preparation

DNA is extracted from gills or heart tissues and digestive gland from live or freshly dead (not decaying) animals.
These animals can be stored frozen or fixed in 100% ethanol before being processed for DNA extraction.

DNA extraction can be performed using the Wizard Genomic DNA Purification Kit from Promega®, or the QIAamp® DNA Mini Kit from QIAGEN ®, and following the instructions for Tissue Test Protocol. Other commercial kits may be used for the DNA extractions as long as they have been demonstrated to give similar results.
It is recommended to check the quality and efficacy of the extraction by measuring the optical density (260 nm) with a spectrophotometer, or by electrophoresis in agarose gel.
DNA solutions are kept at 5°C +/- 3°C until PCR analyses are performed.

Just before performing the real time PCR assay, samples are adjusted at a final DNA concentration of 5ng/µl.

3.2. Real Time Polymerase Chain Reaction

3.2.1. Reactives

The PCR mix can be performed using the Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies® ref: # 600881). Other commercial Master Mixes for Multiplex real-time PCR may be used as long as they have been demonstrated to give similar results.

H_20 (free of DNA and RNA)

3.2.2. Primers and Probes

Mar_18S_F 5' ACGATCAAAGTGAGCTCGTG 3'
Mar_18S_R 5' CAGTTCCCTCACCCCTGAT 3'
Mar_18S_IN (probe) 5' GCATGGAATCGTGGAACGGG 3' (FAM-BHQ-1)
Note: PCR product size = 118 bp

Bosp2_18S_F 5' CAGGATGCCCTTAGATGCTC 3'
Bosp2_18S_R 5' GTACAAAGGGCAGGGACGTA 3'
Bosp2_18S_IN (probe) 5' TTGACCCGGCTTGACAAGGC 3' (HEX-BHQ-1)
Note: PCR product size = 199 bp

3.2.3. PCR Mix

PCR mix for each tube is:

	Initial concentration	**Volume per tube**	**Final concentration**
2X Brilliant III Ultra-Fast QPCR Master Mix	2 X	12.5 µl	**1X**
Primer Mar_18S_F	20 µM	0.5 µl	**0.4 µM**
Primer Mar_18S_R	20 µM	0.5 µl	**0.4 µM**
Probe Mar_18S_IN FAM	10 µM	0.75 µl	**0.3 µM**
Primer Bosp2_18S_F	20 µM	0.38 µl	**0.3 µM**
Primer Bosp2_18S_R	20 µM	0.63 µl	**0.5 µM**
Probe Bosp2_18S_IN HEX	10 µM	0.75 µl	**0.3 µM**
H_2O		4 µl	

20 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
Note: Real Time PCR mix should be prepared in excess (for example, prepare mix for 10% more wells)

3.2.4. Controls

Negative controls aim at detecting potential cross contamination of working environment during the DNA extraction and the PCR. Two negative controls should be included for each test: one extraction negative control (could be a known negative sample or water extracted at the same time as the samples) and one PCR negative control (could be water added in the PCR mix instead of the DNA sample) .

Positive controls verify that PCR assays have performed correctly, and that no PCR inhibitors are present. Positive controls consist of DNA extracted from oyster or mussels known to be infected with *Marteilia Refringens* or *Bonamia sp.*, or plasmidic DNA containing the PCR target. Two positive controls (one for each targeted pathogens) should be included for each PCR analysis.

3.2.5. 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 (15 sec at 95°C and 1 min at 60°C)**

* The initial denaturation time may vary depending on the Master mix used

The fluorescence is recorded at the end of each cycle with **HEX** (*Bonamia sp.)* and **FAM** (*Marteilia refringens*) filters.

3.2.6. Interpretation

Threshold cycle (C_t) is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected. Cts are calculated automatically by the Stratagene thermocycler software. Reported Ct 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 *Marteilia refringens* and *Bonamia sp.*, negative controls should not present any amplification and positive controls (*Bonamia sp.* and *Marteilia refringens*) should present amplification.

Samples

A tested sample is considered positive if its Ct value is below 38 (≤ 38)

A tested sample is considered negative if there is no amplification or if its Ct value is above 38 (≥ 38)

Note: Recommendations for result interpretation presented in this document have been established under the LRUE experimental conditions. The cut-off Ct value used to determine if a sample is positive or negative may vary depending on the equipment, reagents and consumables used for the test.