

Marteilia refringens detection and typing by
by Taqman® Real Time Polymerase Chain Reaction

CONTENTS

1. SCOPE		2
2. REFERENCES		2
3. EQUIPMENT AND ENVIRONMENTAL CONDITIONS		2
4. PROCEDURE		2
4.1. <i>SAMPLE PREPARATION</i>		2
4.2. <i>REAL TIME POLYMERASE CHAIN REACTION</i>		3
4.2.1. <i>Reactives</i>		3
4.2.2. <i>Primers and Probes</i>		3
4.2.3. <i>PCR Mix</i>		3
4.2.4. <i>Controls</i>		3
4.2.5. <i>Amplification</i>		4
4.2.6. <i>Interpretation</i>		4

Editions

Edition	Date	Updated part
N° 1	2016	Creation
N° 2	2020	Minor protocol changes, change of probe dyes.

Bonamia ostreae and *Bonamia exitiosa* detection by Taqman® Real Time Polymerase Chain Reaction

1. Scope

This procedure explains a standard diagnostic test used for the detection and characterization of the parasite *Marteilia refringens* in bivalves including flat oysters *Ostrea edulis* and mussels, *Mytilus edulis* and *M. galloprovincialis*.

This multiplex Real-Time PCR assay amplifies a fragment of the ITS-1 (Internal Transcribed Spacer) of *Marteilia refringens* genome. It allows a specific diagnosis between *M. refringens* type O and type M as defined by Le Roux et al. (2001) by using type-specific probes.

2. References

Le Roux F., Lorenzo G., Peyret P., Audemard C., Figueras A., Vivarès C., Gouy M. & Berthe F., 2001. Molecular evidence for the existence of two species of *Marteilia* in Europe. *J. Eukaryot. Microbiol.* 48, 4: 449-454.

OIE (2019). Manual of Diagnostic Tests for Aquatic Animals, section 2.4.4, Paris, France, (http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.4.04_M_REF.pdf)

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 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.

4. Procedure

4.1. Sample preparation

DNA is extracted from the digestive gland of 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 DNA extraction 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 4°C +/- 3°C until PCR analyses are performed.

Just before performing the real time PCR assay, final DNA concentration is adjusted at 5ng/µl with distilled water.

4.2. Real Time Polymerase Chain Reaction

4.2.1. Reactives

The PCR mix can be prepared using the Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies® ref: # 600881). Other commercial Master Mix for Multiplex Taqman® real-time PCR may be used as long as it has been demonstrated that it gives similar results.

H₂O (free of DNA and RNA)

4.2.2. Primers and Probes

- TaqMar F 5' GTGTTTCGGCACGGGTAGT 3'
- TaqMar R 5' TGATCTGATATTATTCAGCTGTTCG 3'
- TaqProb M HEX 5' GCGCTTGCCCTACGGCCGTGC 3' BHQ-1
- TaqProb O FAM 5' GCCCTTTCCCGACGGCCG 3' BHQ-1

4.2.3. 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
TaqMar F	20 µM	0.13 µl	0.1 µM
TaqMar R	20 µM	0.38 µl	0.3 µM
TaqProbe M (HEX)	20 µM	0.31 µl	0.25 µM
TaqProbe O (FAM)	20 µM	0.31 µl	0.25 µM
H ₂ O		6.37 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)

4.2.4. 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 allow verifying that PCR assays have performed correctly, and that no PCR inhibitors are present. Positive controls consist of DNA extracted from oyster known to be infected with *M. refringens* type M and type O., or plasmidic DNA containing the PCR target. Two positive controls (one for each *M. refringens* type) should be included for each PCR test.

4.2.5. 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 (30 sec at 95°C and 60 sec at 60°C)**

Note: PCR cycle times may vary depending on the Master mix used

The fluorescence is recorded at the end of each cycle with **HEX** and **FAM** filters.

4.2.6. Interpretation

Threshold cycle (C_t) is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected. C_ts are calculated automatically by the 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 *M. refringens*, results obtained with control samples should be checked. A PCR run is valid if negative controls do not present any amplification and positive controls present amplification (HEX amplification for the *M. refringens* type M positive control, FAM amplification for the *M. refringens* type O positive control).

Samples

A tested sample is considered positive if both C_t values are below 37 (≤ 37)

A tested sample is considered negative if there is no amplification or if its C_t value is above 37 (≥ 37)

A tested sample with a C_t value below 37 and a C_t above 37 is questionable and should be re-tested.

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