Perkinsus marinus detection by Real Time Polymerase Chain Reaction

(According to Audemard et al. 2004)

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Editions

Edition	Date	Updated part
N° 1	15/04/2021	Creation

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Perkinsus marinus detection by Real Time Polymerase Chain Reaction

1. Scope

This procedure explains a standard diagnostic test used for the detection of DNA of the protozoan parasite *Perkinsus marinus* in bivalves based on the amplification of a fragment of the ITS region (rRNA gene) using a SYBR Green® real-time PCR approach.

Perkinsus marinus is an EU regulated pathogen and causative agent of "Dermo" disease in oysters. This pathogen is considered to be exotic in Europe.

2. References

Audemard, C., Reece, K. S. and Burreson, E. M. (2004). **Real-time PCR for detection and quantification of the protistan parasite Perkinsus marinus in environmental waters** .Applied and Environmental Microbiology 70, 6611–6618.OIE - Manual of Diagnostic Tests for Aquatic Animals

OIE - Manual of Diagnostic Tests for Aquatic Animals, section 2.4 diseases of molluscs, chapter 2.4.6 (2019).

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 according to the go-forward principle. 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 **gills, mantle and digestive gland** tissues 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 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, DNA suspensions are adjusted at 5ng/µl with distilled water.

4.2. Real Time Polymerase Chain Reaction

4.2.1. Reactives

MasterMix for SYBR Green® PCR, for example: BioRad SsoAdvanced Universal SYBR Green Supermix (#1725270), or 2X Brilliant SYBR® Green Q PCR Master Mix (Stratagene® ref: # 600548),),

H_20 (free of DNA and RNA)

4.2.2. Primers and Probes

PmarITS-70F 5' CTTTTGYTWGAGWGTTGCGAGATG 3'
PmarITS-600R 5' CGAGTTTGCGAGTACCTCKAGAG 3'

Note: Primers amplify a 509 bp PCR product.

4.2.3. PCR Mix

PCR mix for each tube is:

	Initial concentration	**Volume per tube**	**Final concentration**
2X SYBR Green Master Mix	2X	10 µL	1X
Primer PmarITS-70F	20 µM	0.5 µL	0.5 µM
Primer PmarITS-600R	20 µM	0.5 µL	0.5 µM
H_2O		7 µL	

18 µL of this PCR mix is dispensed in each Real Time PCR plate well
2 µL of extracted DNA (diluted at 5 ng/µL) is added to each tube
Each sample can be tested in simplicate or duplicate
Real-time PCR mix should be prepared in excess (for example for two additional samples)

4.2.4. Controls

Negative controls aim at detecting potential cross contamination of working environment during the DNA extraction and the PCR. At least 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. Positive controls consist of DNA extracted from oyster known to be infected with *P. marinus*, or plasmidic DNA containing the PCR target. At least one positive control should be included for each PCR run.

4.2.5. Amplification

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

-Initial denaturation: **3 min* at 95°C**
-Amplification: **50 cycles (10 sec at 95°C, 5 sec at 69°C, 25 sec at 72°C)**
- Melting curve analysis: **1 min at 95°C, increment 1°C from 60°C to 95 °C for 30 sec with plate read at each step**

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

The fluorescence is recorded at the end of each cycle with SYBR Green filter.

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. Cts are calculated automatically by the thermocycler software. If samples were tested in duplicates, reported Ct values are calculated as averages of both replicates for each reaction.

Melt curve analysis allows checking the specificity of PCR amplification, by looking at the Melting point or Tm. The Tm is the temperature where 50% of the DNA amplicon is single stranded, the Tm depends on the length and content of the DNA fragment. The Tm should be checked for each sample showing an amplification.

A sample is considered positive when an amplification curve is observed and associated with the expected Tm value.

Controls

Before concluding about the status of the tested samples regarding the presence of *P. marinus*, 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 with expected Tm value.

Samples

A sample is considered positive when a characteristic amplification curve is observed and when the melting temperature peak (or Tm) is approximately at 84°C*.

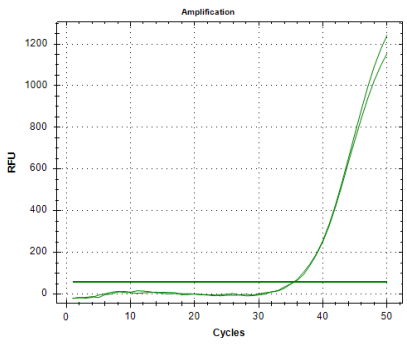
A sample is considered negative when there is no amplification curve, or when an amplification curve is associated with a non-specific Tm.

*Tm may vary depending on reagent and equipment used for the PCR test.

Depending on the context / objective of the analysis, a cut-off Ct value can be used to define if samples are positives or negatives. In such a case, cut-off values need to be established considering laboratory conditions.

Example of a PCR curve and melt peak profiles obtained with a *P.marinus* positive sample:

Amplification curve



Melt peak