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

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Editions

Edition	Date	Updated part
N° 1	06/05/2019	Creation
N° 2	02/02/2023	Revision and addition of an Annex “PCR run validation”

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1. Scope

This procedure explains a standard diagnostic test used for the concomitant detection of *Bonamia ostreae* and *Bonamia exitiosa* in flat oysters, using a mutiplex TaqMan® real-time PCR approach. Two sets of primers were designed to specifically amplify a fragment of actin genes of *B. ostreae* and *B. exitiosa.* Signal detection relies on the use of two specific labelled probes (HEX label for *B. ostreae,* FAM label for *B. exitiosa*.).

2. Reference

López-Flores I., Suárez-Santiago V. N., Longet D., Saulnier D., Chollet B., Arzul I., 2007. Characterization of actin genes in *Bonamia ostreae* and their application to phylogeny of the Haplosporidia. Parasitology 134, 1941–1948.

Prado-Alvarez M., Couraleau Y., Chollet Y., Tourbiez D., Arzul I., 2015. Whole-genome amplification: a useful approach to characterize new genes in unculturable protozoan parasites such as *Bonamia exitiosa*. Parasitology 142, 1523–1534.

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

As a general rule, we recommend performing DNA extraction on pool of tissues (about 20 mg) from **digestive gland, gills and mantle** for each animal (this combination of organs allows to look for the presence of the main mollusc pathogens). If analyses only concern *Bonamia* parasites, DNA extraction should be performed **at least on gills and mantle**.

DNA is extracted from live or freshly dead (not decaying) animals.
These animals can be stored frozen or fixed in 96-100% ethanol before being processed for DNA extraction.

DNA extraction can be performed using 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 for the presence of PCR inhibitor in analysed DNA samples using an internal control. Optionally, the quality and efficacy of the extraction can also be checked 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 assays, DNA are diluted 1/10 with molecular grade water.
Note: alternatively, pure DNA can tested by PCR and only DNA sample showing the presence of inhibitors are diluted.

4.2. Real Time Polymerase Chain Reaction

4.2.1. Reactives

The PCR mix can be prepared using the following Master Mix: Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies® ref: # 600881), SsoAdvanced Universal Probes Supermix (BioRad®,ref: #1725281) or TaqPath™ qPCR Master Mix (AppliedBiosystem, ref:# A16245). 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.

H_20 (molecular grade, free of DNA and RNA)

Internal control kit, for example: Universal exogenous pPCR Positive Control for TaqMan assays (Eurogentec, ref: # RT-IPCY-B02).

4.2.2. Primers and Probes

BO2_F	5’ AAATGGCCTCTTCCCAATCT 3’
BO2_R	5’ CCGATCAAACTAGGCTGGAA 3’
BO2_probe	5’ TGACGATCGGGAATGAACGC 3’ (HEX-BHQ-1)

Note: PCR product size = 127 bp

BEa_F	5’ GACTTTGACCATCGGAAACG 3’
BEa_R	5’ ATCGAGTCGTACGCGAGTCT 3’
BEa_probe	5’ GGCAGCGAATCGATGGGAAT 3’ (FAM-BHQ-1)

Note: PCR product size = 108 bp

4.2.3. Controls

Negative controls aim at detecting potential cross contamination of working environment during the DNA extraction and the PCR. **Positive controls** allow verifying that DNA extraction and PCR assays have performed correctly. **Internal controls** allow to check for the presence of PCR inhibitors in DNA samples, and avoid false negative result reporting.

Controls for the DNA extraction step:
At least one negative control and one positive control per extraction. Extraction negative controls could be tissues from an oyster known to be uninfected or an empty tube extracted at the same time as the samples. Extraction positive controls could be tissues from an oyster known to be infected with *B. ostreae* or *B. exitiosa* ,or plasmidic DNA containing the PCR target added in a tube of negative oyster tissues before the extraction step (plasmidic DNA may be added after the lysis step to avoid its deterioration).

The parasite concentration of this positive control should preferentially be close to the detection limit of the method. Note: Alternatively, extraction can be monitored by amplifying a host gene in each DNA samples using specific primers and probe at the PCR step.

Controls for the PCR step:
-At least two negative controls per PCR run. PCR negative controls could be water added in the PCR mix instead of the DNA samples (for example: water used to dilute DNA and water used to prepare the PCR mix).
- At least one positive control per target per PCR run (one control for *B. ostreae* and one for *B. exitiosa*). Positive controls consist of DNA extracted from oyster known to be infected with *B. ostreae* or *B. exitiosa*, or plasmidic DNA containing the PCR target. The parasite concentration of these positive controls should always be the same to be able monitor PCR performances over time (for example, 10^4 copies/ µl for plasmids).

-Internal control (IC): ideally, each DNA sample should be checked for the presence of PCR inhibitors using an internal control kit. IC consists in the addition of a synthetic DNA in the DNA samples that will be amplified using specific primers and probes. Late or no amplification of the IC DNA reveals the presence of PCR inhibitors.

4.2.4. PCR Mix

PCR mix for each tube is:

	Initial concentration	**Volume per tube**	**Final concentration**
qPCR Master Mix*	2 X	10 µl	**1X**
Primer BO2_F	10 µM	0.6 µl	**0.3 µM**
Primer BO2_R	10 µM	0.6 µl	**0.3 µM**
Probe BO2 (HEX)	10 µM	0.4 µl	**0.2 µM**
Primer BEa_F	10 µM	0.6 µl	**0.3 µM**
Primer BEa_R	10 µM	0.6 µl	**0.3 µM**
Probe BEa (FAM)	10 µM	0.4 µl	**0.2 µM**
H_2O		1.8 µl	

**It is recommended to shake thoroughly the Mix before adding it to the PCR mix.*

15 µL of this **PCR mix** is dispensed in each Real Time PCR plate well
5 µL of **extracted DNA** (diluted 1/10) is added to each tube

Note 1: Real Time PCR mix should be prepared in excess (for example, prepare mix for 10% more wells)
Note 2: DNA samples should be tested in parallel for the presence of PCR inhibitors

4.2.5. Amplification

Amplification cycles are performed using a thermocycler for real-time PCR (for example CFX96 from Biorad®)

-(optional: UNG incubation : **2 min* at 50°C** – for PCR master mix containing dUTP and Uracil-N-glycosylase)
-Initial denaturation: **3 min* at 95°C**
-Amplification: **40 cycles (15 sec at 95°C and 20 sec at 60°C)**

* UNG incubation and initial denaturation 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. Cts are calculated automatically by the thermocycler software.

PCR run validation

Before concluding about the status of the tested samples, results obtained with control samples should be checked. A PCR run is valid if negative controls produce a negative result ("not detected") and if positive controls present the expected amplification (HEX amplification for the *B. ostreae* positive control, FAM amplification for the *B. exitiosa* positive control). Note: a range of expected Ct values can be defined for positive controls.

If one or several controls produce noncompliant results, analysis may be repeated from the DNA extraction or the PCR step (See example in Annex).

Interpretation of samples results

DNA of *B. ostreae* is considered as detected if a PCR amplification is observed in the HEX channel with a Ct value below 38 (Ct <38).

DNA of *B. exitiosa* is considered as detected if a PCR amplification is observed in the FAM channel with a Ct value below 38 (Ct <38).

DNA of *B. ostreae* or *B. exitiosa* is considered as not detected if no amplification is observed, or if an amplification is observed with a Ct value above 38 (Ct ≥ 38) AND if no PCR inhibitors was detected in the corresponding DNA sample.

If PCR inhibitors are detected in a "negative "sample, this sample should be considered as uninterpretable. Such sample can be repeated from the PCR step after being diluted (for example: dilution 1/10 if the sample was tested pure, or dilution 1/100 was tested at 1/10).

Important note: Recommendations for result interpretation presented in this document have been established under the EURL 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.

In case of an unusual detection of *B. ostreae* or *B. exitiosa* (i.e in a new location / new host) sample should be sent to the EURL for confirmation.

ANNEX PCR run validation

Example for the interpretation of quality controls

Negative controls		Interpretation	Action
T- Ext	**T- PCR**		
-	**-**	Valid	Process with sample results interpretation
+	**-**	Not valid, possible contamination at the extraction step.	Repeat analyses from DNA extraction step, at least for samples showing positive results
-	**+**	Not valid, possible contamination at the PCR step.	Repeat PCR analyses, at least for samples showing positive results
+	**+**	Not valid, possible contamination at the PCR step or/and the DNA extraction step	Repeat PCR analyses. If still not valid, repeat analyses from the extraction step.

Positive controls		Interpretation	Action
T+ Ext	**T +PCR**		
+	**+**	Valid	Process with sample results interpretation
-	**+**	Not valid, possible technical problem during the Extraction step	Repeat analyses from DNA extraction step, at least for samples showing negative results
+	**-**	Not valid, possible technical problem during the PCR step.	Repeat PCR analyses, at least for samples showing negative results
-	**-**	Not valid, possible technical problem at the PCR step or/and the DNA extraction step	Repeat PCR analyses. If still not valid, repeat analyses from the extraction step.

Result obtained with controls: "-" = not detected, "+" = detected , compliant results are in green, non-compliant results are in red

T – Ext = extraction negative control

T+ Ext = extraction positive control

T- PCR = PCR negative control

T+ PCR = PCR positive control