# *Bonamia* spp. detection by Polymerase Chain Reaction and species characterization by Restriction Fragment Length Polymorphism

According to Cochennec et al. (2000)

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## Editions

| Edition | Date | Updated part |
|---|---|---|
| N° 1 | October 2008 | Creation |
| N° 2 | May 2019 | Revision<br>Format changes, minor changes in the protocol and additional information on interpretation |

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# 1. Scope

This procedure explains a standard diagnostic test used for Haplosporidian *Bonamia* spp. detection and species characterization in flat oysters (e.g. *Ostrea edulis*) to confirm a previous histological or cytological diagnosis at the genus level. It allows distinguishing between *Bonamia ostreae* and *B. exitiosa* but **needs to be completed by sequencing to confirm the species**.

# 2. References

Cochennec N., Le Roux F., Berthe F. & Gérard A., 2000. Detection of *Bonamia ostreae* based on small subunit ribosomal probe. *J. Invetebr. Pathol.* 76:26-32.
OIE (2006). Manual of Diagnostic Tests for Aquatic Animals, section 2.2, Paris, France, (web format of Manual of Diagnostic Tests : http://www.oie.int/eng/normes/fmanual/A_summry.htm?e1d11)
Abollo E., Ramilo A., Casas S.M., Comesaña P., Cao A., Carballal M.J. & Villalba A. (2008). First detection of the protozoan parasite *Bonamia exitiosa* (Haplosporidia) infecting flat oyster *Ostrea edulis* grown in European waters. Aquaculture, 274 : 201-207.

# 3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for 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.
- Two different pipettes: one pipette (2 µl) to dispense samples in PCR mix, one pipette (20µl) for BET sampling and to load PCR products in agarose gels
- Filter pipette tips (2 µl; 20 µl; 200 µl and 1000 µl) for DNA extraction, PCR mix preparation and sample dispensing
- Pipette tips (20 µl) to collect BET and to load amplification products in agarose gel
- A thermal cycler to perform amplifications
- A microwave to melt agarose
- A horizontal electrophoresis system for PCR products electrophoresis
- An UV equipment to observe PCR products after agarose gel electrophoresis and to acquire pictures of the gels

Manipulator must wear a lab coat and some gloves during all the different steps described bellow. Lab coat and gloves must be changed preferably after each main step: DNA extraction, preparation of PCR mix, sample dispensing, amplification and gel loading.
It is recommended to perform these different steps in different rooms. More particularly, amplification and gel loading/electrophoresis 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 pieces of gills ( or heart) using commercial DNA extraction kits such as Wizard Genomic DNA Purification Kit (PROMEGA), or QIAamp® DNA Mini Kit (QIAGEN) and following instructions for Tissue Protocol.

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 PCR assay, final DNA concentration is adjusted at 50ng/µl with distilled water.

### 4.2. Polymerase Chain Reaction (PCR)

#### 4.2.1. Reactives

- 10 X Buffer (furnished with the Taq DNA polymerase)
- $MgCl_2$ (furnished with the DNA polymerase) (25 mM)
- Taq DNA Polymerase (Taq Diamond, Eurogentec) 5 U/µl
- dNTP Master Mix (20mM)
- dATP 5 mM
- dCTP 5 mM
- dGTP 5 mM
- dTTT 5 mM
- $H_20$ (free of DNA and RNA)

Other commercial Taq polymerase may be used as long as it has been demonstrated that it yields similar results.

#### 4.2.2. Primers

BO 5' CAT TTA ATT GGT CGG GCC GC 3'
BOAS 5' CTG ATC GTC TTC GAT CCC CC 3'

#### 4.2.3. PCR Mix

PCR mix for each tube is:

| | Initial concentration | Volume per tube | Final concentration |
|---|---|---|---|
| Buffer | 10X | 5 µl | **1X** |
| $MgCl_2$ | 25 mM | 2.5 µl | **1,25 mM** |
| dNTPs | 20 mM | 0.5 µl | **200µM (50 µM for each)** |
| BO primer | 20 µM | 2,5 µl | **1 µM** |
| BOAS primer | 20 µM | 2,5 µl | **1 µM** |
| Taq polymérase | 5U/µl | 0,5 µl | **2,5 U** |
| $dH_2O$ | | 34,5 µl | |

- 48 µl of PCR mix is dispensed in each PCR tube
- 2 µl of extracted DNA (50 ng/µl) is added to each tube

#### 4.2.4. Controls

**Negative controls** aim at detecting potential cross contamination of working environment during the DNA extraction and the PCR. Two types of negative controls should be included: extraction negative control (could be a known negative sample or water extracted at the same time as the samples) and PCR negative control (could be water added in the PCR mix instead of the DNA sample). One negative control should be included every 10 samples or before and after each batch of samples.

**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, or plasmidic DNA containing the PCR target. At least one positive control should be included (*Bonamia* sp. positive control). In

case the RFLP analysis is intended, two positive controls (for *B. ostreae* and *B. exitiosa*) should be included in the PCR test.

### 4.2.5. Amplification

Amplification cycles are performed in a thermal cycle apparatus (PTC-100 MJ Research, Inc.Perkin).

- Initial denaturation: 5 min at 94°C
- Amplification: 30 cycles (1 min at 94°C, 1 min at 55°C and 1 min at 72°C)
- Final elongation: 10 min at 72°C

PCR products are then analysed by agarose gel electrophoresis, see section 4.4.

### 4.2.6. Interpretation

A positive result is an amplicon of the appropriate size (300 bp) with all negative controls negative and all positive controls positive.

Note: this PCR assay has been shown to amplify several haplosporidian species and not only parasites of the genus *Bonamia*.

Multi-band profiles can sometime be observed for some samples (see Figure 1). Such profiles should be considered as non-specific amplifications and thus negative regarding the presence of parasites of the genus Bonamia. Multi-bands can be avoided by changing the Taq polymerase, or by re-extracting DNA from the samples.

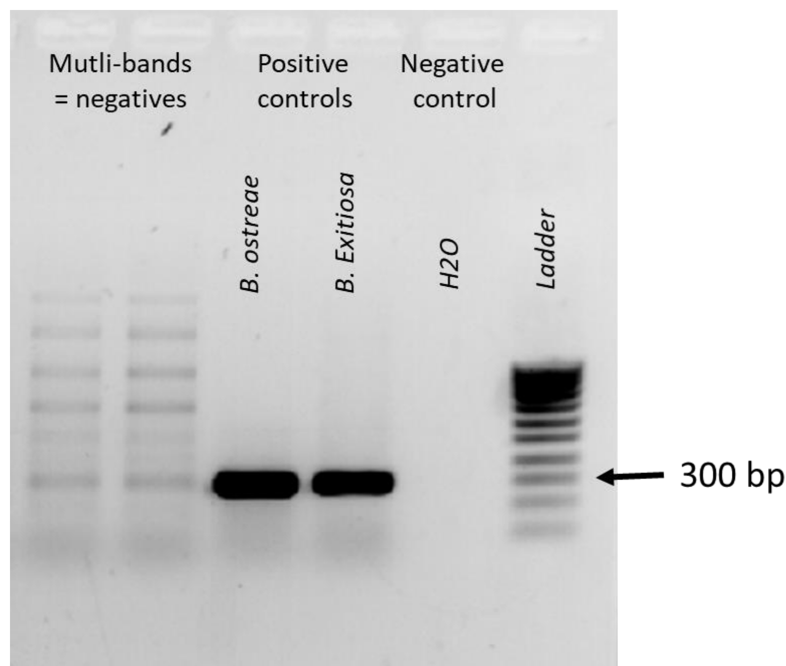


Figure 1: example of multi-band profiles

## *4.3. Restriction Fragment Length Polymorphism (RFLP)*

### 4.3.1. Reactives

- 10 X Buffer (furnished with the restriction enzyme)
- $H_20$ (free of DNA and RNA)
- *Bgl*I **or** *Hae*II (10U/µl)

### 4.3.2. Digestion mix

-Digestion mix for each tube is:
- 2 µl of the appropriate buffer
- 0.5 µl of **one** enzyme (final concentration: 0.25U/µl)
- 7.5 µl of $dH_2O$

-10 µl of this digestion mix is dispensed in each tube
-10 µl of PCR products are added to each tube

### 4.3.3. Digestion

Digestion is then performed by incubating samples for 2 hours at the temperature indicated by the manufacturer.

PCR products are then analysed by agarose gel electrophoresis, see section 4.4.

### 4.3.4. Interpretation

Bo-Boas PCR products will be digested differently according to the parasite species (Figure 2).
Table below indicates expected restriction profiles (however species confirmation requires sequencing):

| | ***Bgl*I** | ***Hae*II** |
|---|---|---|
| *Bonamia ostreae* | 120 bp + 180 bp | 115 bp + 185 bp |
| *Bonamia exitiosa* | Not digested | 117 bp + 187 bp |
| *Bonamia rouyghleyi* | Not digested | Not digested |
| *Bonamia perspora* | Not digested | 117 bp + 187 bp |

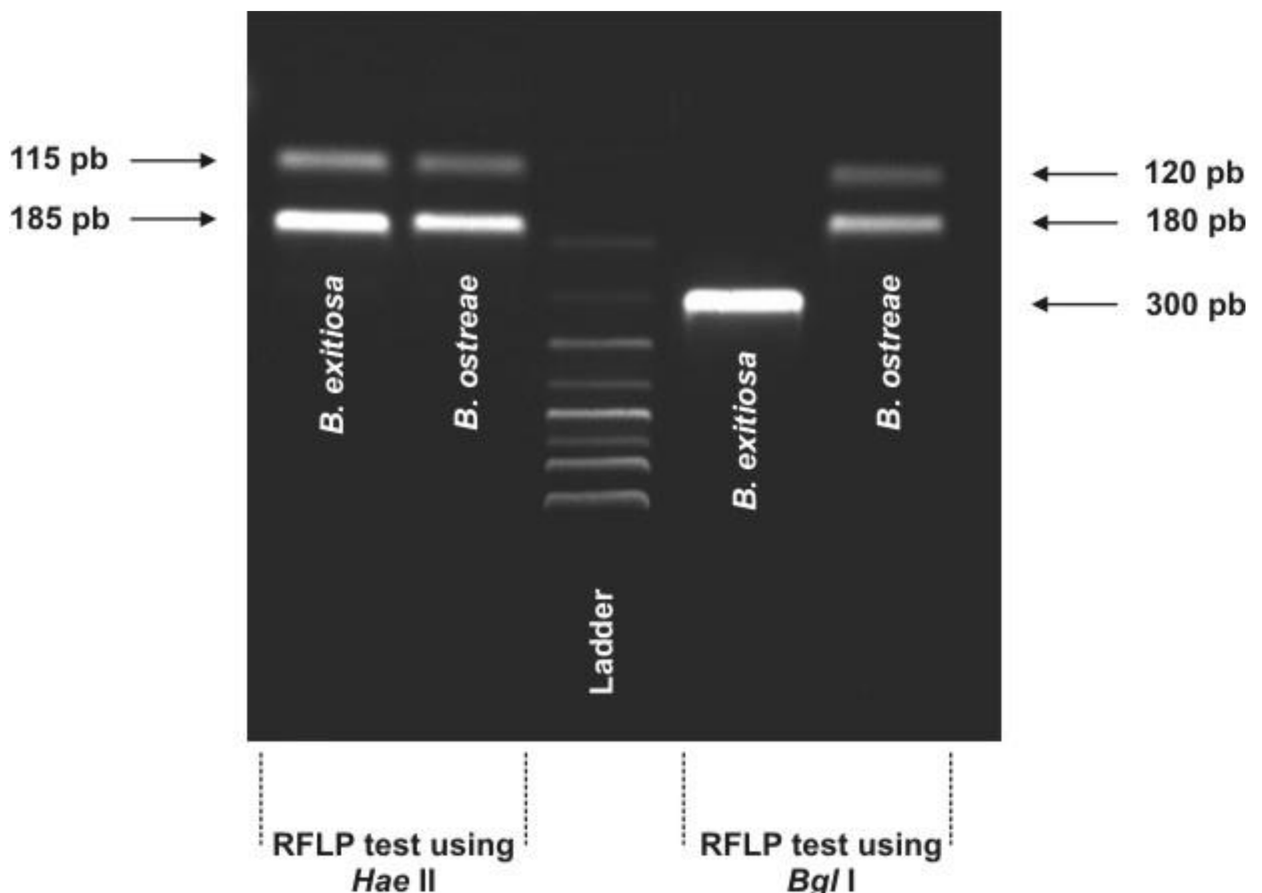


**Figure 2**: Restriction profiles obtained after digestion of Bo-Boas PCR products using *Hae* II and *Bgl* I.

### 4.4. Electrophoresis

#### 4.4.1. Reactives

- 50 X TAE (can be bought directly ready for use):

| | |
|---|---|
| Tris base (40 mM) | 242 g |
| Acetic glacial acid (40 mM) | 57,1 ml |
| $Na_2EDTA.2H_2O$ (1 mM) | 18,61 g |
| $dH_2O$ | for 1 liter |

Ajust at pH 8

- Agarose gel:
  1 % for PCR products or 2% for RFLP products of agarose in 1X TAE
  Ethidium bromide (0,5 µg/ml) added after cooling the gel.

- Loading blue dye:

| | |
|---|---|
| Bromophenol blue | 0,25 % |
| Cyanol xylene FF | 0,25 % |
| Sucrose | 40 % |

Keep at 4°C.
Use diluted 6 times (2 µl of loading blue buffer for 10µl of PCR products).

- Molecular weight marker:
  SmartLadder SF (Eurogentec): a ready-to-use molecular weight marker including 9 regularly spaced bands from 100 to 1000 bp.

#### 4.4.2. Agarose gel preparation

1. Weight X g of agarose, add 100 x X ml of 1X TAE and heat until the mix is melted.
2. After cooling the solution, ethidium bromide (10 mg/ml) is added (5 µl for 100 ml of agarose gel) and the solution is disposed in a specific mould equipped with combs (to form wholes).
3. When gel is polymerised, combs are removed and placed in a horizontal electrophoresis system containing enough 1X TAE to the cover agarose gel.
4. 5 µl of PCR products or 20 µl of RFLP products are mixed with 2 µl (for PCR products) or 4 µl (for RFLP products) of blue dye (6X) and disposed in the wholes
5. Two wells is dedicated to the molecular weight marker (3-5 µl)
6. A voltage of 50 to 150 volts is applied during 30 min to 1 hour depending on the gel size and thickness
7. Gel is observed under UV (*See picture below*).