Mikrocytos mackini detection by Polymerase Chain Reaction

According to Carnegie et al. (2003)

CONTENT

1. Scope 2
2. References 2
3. Equipment and environmental conditions..................................2
4. Procedure 2
4.1. Sample preparation...2
4.2. Polymerase Chain Reaction (PCR) 3
4.2.1. Reactives 3
4.2.2. Primers 3
4.2.3. PCR Mix 3
4.2.4. Controls...4
4.2.5. Amplification 4
4.2.6. Interpretation...4
4.3. Electrophoresis..4
4.3.1. Reactives 4
4.3.2. Agarose gel preparation 5

Editions

Edition	Date	Updated part
N° 1	April 2021	Creation

Ifremer, Laboratoire de Génétique et Pathologie des Mollusques Marins, Av. de Mus de Loup,
17390 La Tremblade, France

1. Scope

This procedure explains a standard diagnostic test used for the detection of DNA of the protozoan parasite *Mikrocytos mackini* in bivalves based on the amplification of a fragment of the 18S region (rRNA gene) using a conventional PCR approach.

Mikrocytos mackini is an EU regulated pathogen and causative agent of Denman Island disease in oysters. This pathogen is considered to be exotic in Europe.

2. References

Carnegie, R. B., Meyer, G. R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. C., & Bower, S. M. (2003). **Molecular detection of the oyster parasite Mikrocytos mackini, and a preliminary phylogenetic analysis**. Diseases of aquatic organisms, 54(3), 219-227.

OIE Manual of Diagnostic Tests for Aquatic Animals, section 2.4 diseases of molluscs, chapter 2.4.9 (2019).

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 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 according to the go-forward principle. 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 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.

If animals show some **clinical signs representative of *M. mackini* infections**, those lesions should be used in priority for analysis. According to OIE aquatic manual, clinical signs correspond to small focal lesions (ulcerations, abscesses, pustules usually green in colour but can be yellow–brown or colourless) up to 5 mm in diameter observed in the soft tissues and often with brown scars on the shell, adjacent to abscesses on the mantle surface.

In the absence of lesions, small portions of mantle, digestive gland and gills (and labial palps if possible) should be used for DNA extraction (approx. 25mg total).

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 PCR assay, final DNA concentration is adjusted between 10 and 100 ng/µl with distilled water.

4.2. Polymerase Chain Reaction (PCR)

4.2.1. Reactives

- 5 X Buffer (furnished with the Taq DNA polymerase)
- $MgCl_2$ (furnished with the DNA polymerase) (25 mM)
- Taq DNA Polymerase (for example Go Taq,Promega # M3001) 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

MIKROCYTOS-F: 5'- AGA TGG TTA ATG AGC CTC C-3'
MIKROCYTOS-R: 5'- GCG AGG TGC CAC AAG GC – 3'

Note: Primers amplify a 546 bp PCR product.

4.2.3. PCR Mix

PCR mix for each tube is:

	Initial concentration	**Volume per tube**	**Final concentration**
Buffer	5X	10 µl	**1X**
$MgCl_2$ *	25 mM	2 µl	**2,5 mM**
dNTPs	20 mM	0.5 µl	**200µM (50 µM for each)**
MIKROCYTOS-F	20 µM	0,5 µl	**0,2 µM**
MIKROCYTOS-R	20 µM	0,5 µl	**0,2 µM**
Taq polymérase	5U/µl	0,25 µl	**1,5 U/reaction**
dH_2O		34,25 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
- PCR mix should be prepared in excess (for example for two additional samples)

* the buffer of the GoTaq polymerase already contains 1,5mM of MgCl2

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 *M. mackini,* 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 in a thermal cycle apparatus (for example PTC-100 MJ Research, Inc.Perkin).

- Initial denaturation: 3min at 94°C
- Amplification: 40 cycles (1 min at 94°C, 1 min at 60,5°C and 1 min at 72°C)
- Final elongation: 10 min at 72°C

PCR products are then analysed by 1.5% agarose gel electrophoresis. For detailed protocol on agarose gel electrophoresis, see section 4.3.

4.2.6. Interpretation

A positive result shall be positive PCR amplification at the expected size (546 bp), with all negative controls being negative and all positive controls being positive.

4.3. Electrophoresis

4.3.1. 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
Adjust at pH 8	

- Agarose gel:

1.5 % of agarose in 1X TAE
Ethidium bromide (0,5 µg/ml) added after pouring the gel.

- Loading blue dye: (can be bought directly ready for use):

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.

4.3.2. Agarose gel preparation

1. Weight 1.5 g of agarose per 100 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 are mixed with 2 µl (for PCR products) of blue dye (6X) and disposed in the wholes
5. Two wells are dedicated to the molecular weight marker (3-5 µl) at each side of the gel
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.