Mikrocytos mackini detection by Real Time Polymerase Chain Reaction
According to Polinski et al. (2015)

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Editions

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

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Mikrocytos mackini 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 *Mikrocytos mackini* in bivalves based on the amplification of a fragment of the ITS2 region (rRNA gene) using a TaqMan® real-time PCR approach.

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

2. References

Polinski, M., Lowe, G., Meyer, G., Corbeil, S., Colling, A., Caraguel, C., & Abbott, C. L. (2015). **Molecular detection of Mikrocytos mackini in Pacific oysters using quantitative PCR**. Molecular and biochemical parasitology, 200(1-2), 19-24. Saulnier D., De Decker S, Haffner P., 2009.

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 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 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 real time PCR assay, DNA suspensions are diluted 1/10 or adjusted at 5ng/µl with distilled water.

4.2. Real Time Polymerase Chain Reaction

4.2.1. Reactives

MasterMix for Taqman® PCR, for example : BioRad SsoAdvanced Universal Probes Supermix (#1725280), or Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies® ref: # 600881),

H_20 (free of DNA and RNA)

4.2.2. Primers and Probes

MikMack primer F (5.8S) 5’ GCCTATGACAGCACGAAGCA 3’

MikMack Primer R (ITS-2) 5’ TGGCCGAATGACGTAGTTG 3’

MikMack Probe (ITS-2) 5’ -CTGCACGCCGAAGC- 3’ (FAM - MGB)

Note: Primers amplify a 59 bp PCR product.

4.2.3. PCR Mix

PCR mix for each tube is:

	Initial concentration	**Volume per tube**	**Final concentration**
2X Master Mix	2X	12,5 µL	1X
MikMack primer F	20 µM	0.75 µL	0.6µM
MikMack primer F	20 µM	0.75 µL	0.6µM
MikMack probe (FAM)	10 µM	0.5 µL	0.2µM
H_2O		8,5 µL	

23 µL of this PCR mix is dispensed in each Real Time PCR plate well
2 µL of extracted DNA (diluted 1/10 or adjusted 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 *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 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: **40 cycles (30 sec at 95°C and 60 sec at 60°C)**

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

The fluorescence is recorded at the end of each cycle with FAM 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.

Controls

Before concluding about the status of the tested samples regarding the presence of *M. mackini*, 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.

Samples

A sample is considered positive when a characteristic amplification curve is observed.

A sample is considered negative when there is no amplification curve.

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