

***Mikrocytos mackini* detection by *in situ* hybridization (ISH)**Adapted from Meyer *et al.* (2005)**Content**

1. Scope	2
2. References.....	2
3. Equipment and environmental conditions.....	2
4. Reagents and suppliers.....	3
5. Procedure	3
5.1. Probe preparation	3
5.2. Hybridization.....	3
5.2.1. Reactives	3
5.2.2. Controls.....	4
5.2.3. Deparaffinization	4
5.2.4. Deproteinization.....	4
5.2.5. Hybridization	4
5.2.6. Washing and detection	5
5.3. Counter staining and mounting	5
5.3.1. Reactives	5
5.3.2. Counter staining.....	5
5.3.3. Mounting.....	5
6. Interpretation.....	5

Editions

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1. Scope

This procedure explains a standard diagnostic test used for *Mikrocytos mackini* detection by *In Situ* Hybridization (ISH) to confirm a previous histological diagnosis at the genus level.

Important note: The different steps of the preparation of the histological slides are explained in the procedure “Molluscs processing for diagnosis by histopathology”. The only differences are the use of some aminoalkylsilane coated slides instead of classical histological slides and the thickness of the tissue section (5 µm instead of 2 µm).

Note: it is also possible to perform ISH analysis for the detection of *Mikrocytos* sp. at the genus level using a probe generated by PCR with Msp443F and MSp746R primers as described by Gagné et al. 2008. This SOP does not explain this HIS protocol.

2. References

Meyer, G. R., Bower, S. M., & Carnegie, R. B. (2005). Sensitivity of a digoxigenin-labelled DNA probe in detecting *Mikrocytos mackini*, causative agent of Denman Island disease (mikrocytosis), in oysters. *Journal of invertebrate pathology*, 88(2), 89-94.

Carnegie, R. B., Meyer, G. R., Blackbourn, J., Cochenec-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.

Gagné, N., Cochenec, N., Stephenson, M., McGladdery, S., Meyer, G. R., & Bower, S. M. (2008). First report of a *Mikrocytos*-like parasite in European oysters *Ostrea edulis* from Canada after transport and quarantine in France. *Diseases of aquatic organisms*, 80(1), 27-35.

OIE (2019). Manual of Diagnostic Tests for Aquatic Animals, section 2.4.9, Paris, France. *See the Manual of Diagnostic Tests (online)*: <http://www.oie.int/en/international-standard-setting/aquatic-manual/access-online/>

3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for ISH assays:

- Sets of pipettes (2 µl; 20 µl; 200 µl and 1000 µl) for the different *in situ* hybridization steps.
- Pipette tips (20 µl; 200 µl and 1000 µl) to collect and dispense Proteinase K suspension, probes and buffers on slides
- A humid chamber to maintain slides in humid conditions
- A heated slide moat to denature target DNA present in slides
- An oven to maintain slides during hybridization step at 42°C and under gentle shaking
- Paper towelling
- Racks for histological slides
- Aminoalkylsilane coated slides (SIGMA)
- Fume hood
- Hybridization chamber (ABgene) to be placed on slides during probe and antibody incubation steps
- A timer

Manipulator must wear a lab coat and some gloves during all the different steps. **Hybridization and revelations steps must be performed under a fume hood.**

4. Reagents and suppliers

Product	Supplier	Reference
Anti-Digoxigenin-AP, Fab fragments	Roche	11 093 274 910
Bismark brown Y	Sigma	B2759
Blocking reagent	Roche	11096176001
Denhart 50X	Euromedex	EU0505A
Dextran sulfate	Euromedex	1020A
Dig dUTP	Roche	11 093 088 910
EDTA	Sigma	E5134
Deionised formamide 100%	Euromedex	1117
Maleic acid	Sigma	M0375
MgCl ₂	Sigma	M0250
NaCl	Euromedex	1112A
NBT/BCIP	Roche	11681451001
Proteinase K	Euromedex	EU 0090-B
SSC 20X	Euromedex	EU 0300-A
t ARN	Sigma	R8508
Tris	Sigma	T1503

5. Procedure

5.1. Probe preparation

The *M. mackini*-specific oligonucleotide probe MACKINI-1 5'-AGCCCACAGCCTTCAC-3'; (Carnegie et al., 2003) was purchased with a 3'end digoxigenin label (for example from Eurogentec)

5.2. Hybridization

5.2.1. Reactives

- Xylene
- Ethanol 100 %
- Ethanol 95 %
- TE :

Tris base	1 M	50 ml
EDTA 0.5M, pH 8		20 ml
NaCl 5 M		2 ml
Distilled water for 1 l		
- Proteinase K initial solution at 10 mg/ml in TE is used diluted 100 X
- SSC 20X :

NaCl(0.3 M)	88.2 g
NaCl (3 M)	175.3 g
Distilled water for 1 l	
Ajust pH at 7	
- Denhart 50 X (kept at -20°C)
- Hybridization buffer:

dextran sulfate	1 g
SSC 20X	2 ml
tARN from yeast at 10 mg/ml	150 µl

Denhart 50 X	200 µl
Distilled water for a final volume of 5 ml	
Heat the suspension and finally add:	
Dionized formamid at 100 %	5 ml
- Buffer Dig 1:	
Maleic acid	11.61 g
NaCl 5M	30 ml
Distilled water for 1 l	
Adjust at pH 7.5 (by adding about 15.5 g of NaOH)	
Autoclave	
- Buffer Dig 2:	
Blocking reagent	1 g
Buffer Dig 1 for 100 ml	
Mix at 50°C	
Kept at -20°C	
- Buffer Dig 3:	
Tris 1 M, pH 8	50 ml
NaCl 5 M	10 ml
MgCl ₂ 1 M	25 ml
Distilled water for 500 ml	
Ajust at pH 9.5	
Autoclave	
- Buffer Dig 4:	
Tris 1 M, pH 8	5 ml
EDTA 0.5 M, pH 8	1 ml
Distilled water for 500 ml	
Autoclave	
- Detection solution:	
NBT/BCIP (67 % in DMSO, v/v)	20 µl
Buffer Dig 3	1 ml

5.2.2. Controls

Two types of control are used:

- **Negative** controls consist of sample tissue without probe (for each sample to analyse, prepare one slide with probe and one slide without probe).
- **Positive** controls consist of tissue sections from controlled **infected** oysters

5.2.3. Deparaffinization

1. Sections are immersed twice in xylene for 10 min.
2. Sections are dehydrated by two successive immersions in ethanol 100% for 10 min.
3. Sections are air dried.

5.2.4. Deproteinization

1. 200 µl of proteinase K (100 µg/ml in TE) are displayed on each section.
2. Slides are incubated for 15 min at 37°C in a humid chamber.
3. Slides are dehydrated in ethanol 95 % for 1 min and then in ethanol 100 % for 1 min.

5.2.5. Hybridization

1. Adhesive frames (ABgene) are fixed on each slide
2. 100 µl of a mix consisting of hybridization buffer and digoxigenin labelled probe (10 ng/µl buffer) are spread inside the frame on the tissue.
3. Sections are covered with plastic coverlids.
4. Slides are denaturated for 5 min at 94°C and cooled on ice.

5. Slides are then incubated at 42°C in a humid chamber overnight

5.2.6. Washing and detection

- Adhesive frames are deleted.
- Slides are immersed in SSC 2X at room temperature twice for 5 min.
- Slides are immersed in SSC 0.4X at 42°C for 10 min.
- Slides are immersed in buffer Dig 1 for 1 min.
- 200 µl of buffer Dig 2 are spread on the tissue and slides are incubated in a humid chamber for 30 min at room temperature.
- 200 µl of a mix consisting of buffer Dig 2 and anti-digoxigenin- alkaline phosphatase conjugate (1:500 in buffer Dig 2) are added on the sections
- Slides are incubated in a humid chamber at room temperature for 1 h.
- Slides are washed in buffer Dig 1 twice for 1 min.
- Slides are immersed in buffer Dig 3 for 10 min
- 200 µl of detection solution are added on each slide
- Slides are incubated for 30 minutes in the dark or more if the positive control does not show positive signal
- Reaction is stopped by immersion in buffer Dig 4

5.3. Counter staining and mounting

5.3.1. Reactives

- Bismark brown Y
 - Bismark brown Y 0.5 g
 - Ethanol at 30 % 100 ml
- Filter the solution on a coffee filter
- Ethanol 100
- Ethanol 95
- Xylene
- Mounting medium (e.g. Eukitt® resin)

5.3.2. Counter staining

1. Slides are immersed in Bismark brown Y for 1 min
2. Slides are rinsed under tap water
3. immersed in ethanol 95 and 100 30 sec for each

5.3.3. Mounting

1. Slides are rinsed 10 to 30 sec in Xylene
2. A drop of mounting medium (e.g. Eukitt® resin) is added to mount the cover-slip
3. Slides are observed under a light microscope

6. Interpretation

- Negative controls must appear negative.
- Positive controls must appear positive.
- Slides are checked for positive signal looking like dark purple spots (Figures 1 and 2).
- A positive signal with MACKINI-1 probe in an individual detected positive by histology allows concluding that it is an infection with *Mikrocytos mackini*.

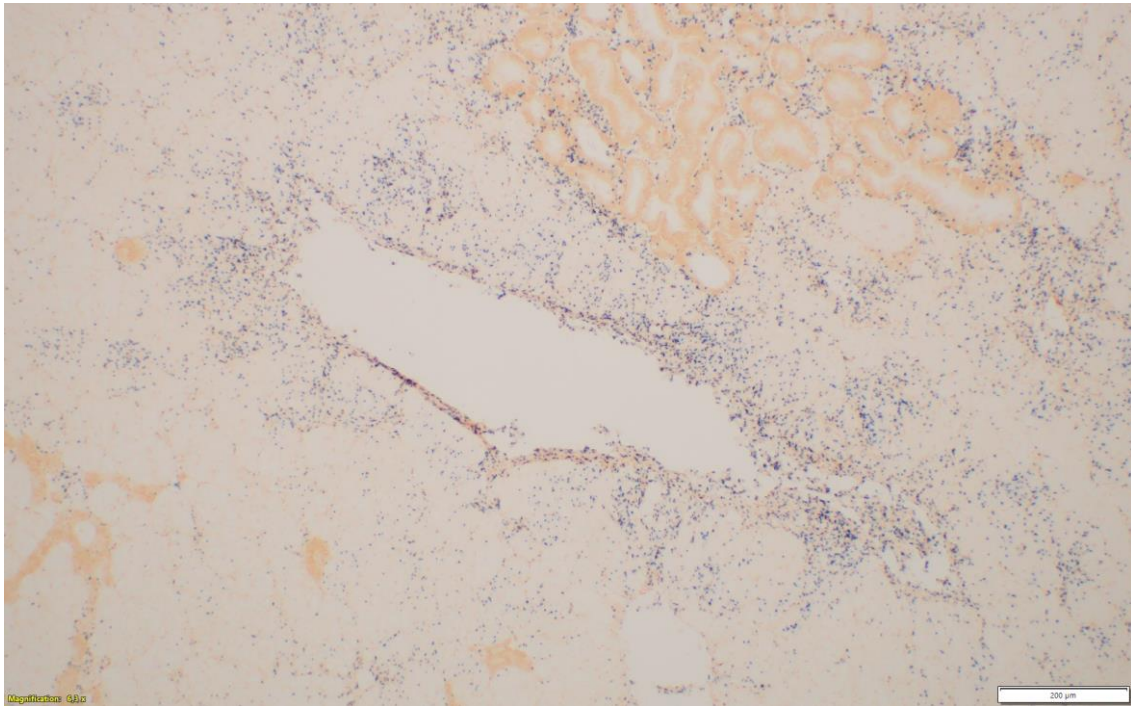


Figure 1. Pacific oyster *Crassostrea gigas* infected with *Mikrocytos mackini* tested by *in situ* hybridization with the MACKINI-1 probe: the parasites appear as dark spots in the connective tissues of the digestive gland and mantle. (Picture B.Chollet)

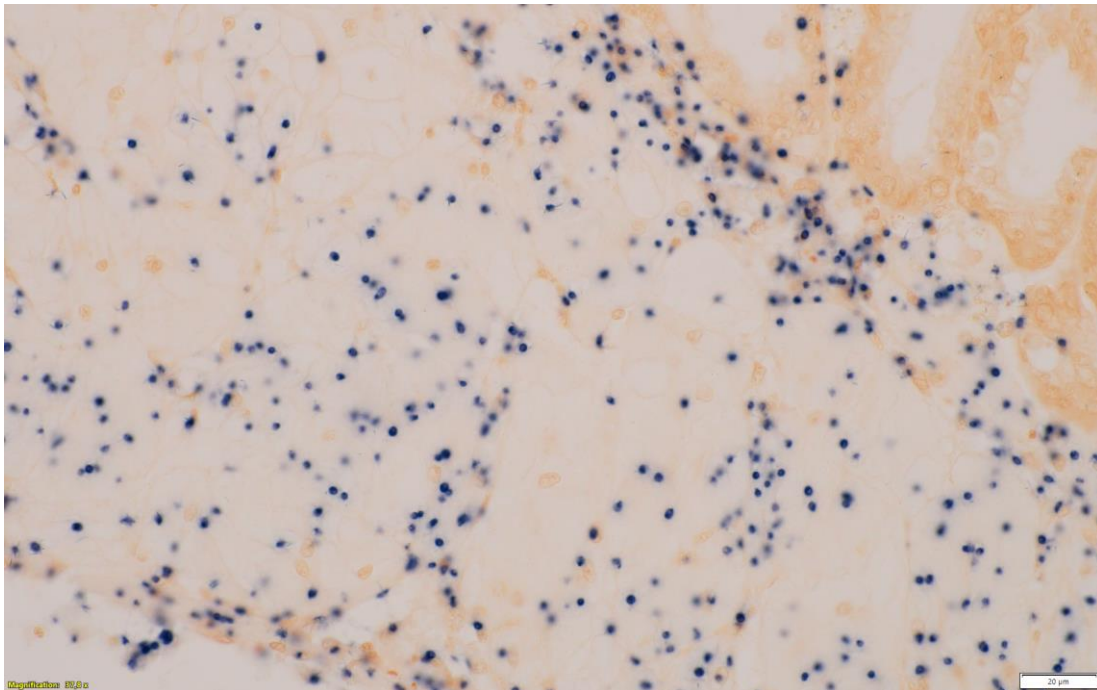


Figure 2. Pacific oyster *Crassostrea gigas* infected with *Mikrocytos mackini* tested by *in situ* hybridization with the MACKINI-1 probe: the parasites appear as dark spots in the connective tissues of the digestive gland. (Picture B.Chollet)