# *Perkinsus marinus* detection by *in situ* hybridization (ISH)

Adapted from Moss *et al*. (2006)

## Content



## Editions

| Edition | Date | Updated part |
|---|---|---|
| N° 1 | April 2021 | Creation |

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## 1. Scope

This procedure explains a standard diagnostic test used for *Perkinsus marinus* 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 *Perkinsus* sp. at the genus level using the probe Perksp700DIG (18S) described by Elston et al. 2004. This SOP does not explain this HIS

## 2. References

**Moss, J. A., Burreson, E. M., & Reece, K. S. (2006).** Advanced *Perkinsus marinus* infections in *Crassostrea ariakensis* maintained under laboratory conditions. Journal of Shellfish Research, 25(1), 65-72.

**Elston, R. A., Dungan, C. F., Meyers, T. R., & Reece, K. S. (2004).** *Perkinsus* sp infection risk for manila clams, *Venerupis philippinarum* (A. Adams and Reeve, 1850) on the Pacific coast of North and Central America. Journal of Shellfish Research, 23(1), 101.

**OIE (2019).** Manual of Diagnostic Tests for Aquatic Animals, section 2.4.6, 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*

The *P. marinus*-specific oligonucleotide probe PmarLSU-181DIG 5′-GACAACGGCGAACGACTC-3′ is to be purchased with a 3′end digoxigenin label (for example from Eurogentec).

## *5.2. Hybridization*

### 5.2.1. Reactives

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

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|---|---|
| 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 :

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|---|---|
| NaCl (0.3 M) | 88.2 g |
| NaCl (3 M) | 175.3 g |
| Distilled water for 1 l | |
| Ajust pH at 7 | |

- Hybridization buffer:

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|---|---|
| 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: | |

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|---|---|
| Deionised formamid at 100 % | 5 ml |

- Buffer Dig 1:

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|---|---|
| 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:

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|---|---|
| Blocking reagent | 1 g |
| Buffer Dig 1 for 100 ml | |
| Mix at 50°C | |
| Kept at –20°C | |

- Buffer Dig 3:

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|---|---|
| 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:

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|---|---|
| Tris 1 M, pH 8 | 5 ml |
| EDTA 0.5 M, pH 8 | 1 ml |
| Distilled water for 500 ml | |
| Autoclave | |

- Detection solution:

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| 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 slides without probe).
- **Positive** controls consist of tissue sections from controlled **infected** oysters

### 5.2.3. Deparaffinization

1. Sections are immersed twice in xylen 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 (between 5 and 10 ng/µl of 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

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| 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 PmarLSU-181DIG in an individual detected positive by histology allows concluding that it is an infection with *Perkinsus marinus.*

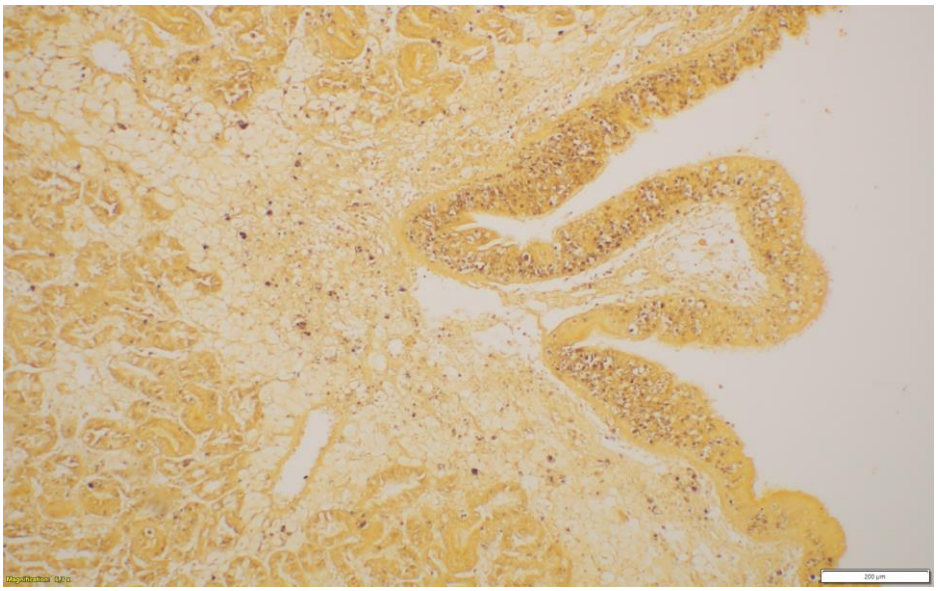

Figure 1. Eastern oyster *Crassostrea virginica* infected with *Perkinus marinus* tested by *in situ* hybridization with the PmarLSU probe: the parasites appear as dark spots in the epithelia of the digestive tract and in the connective tissues.

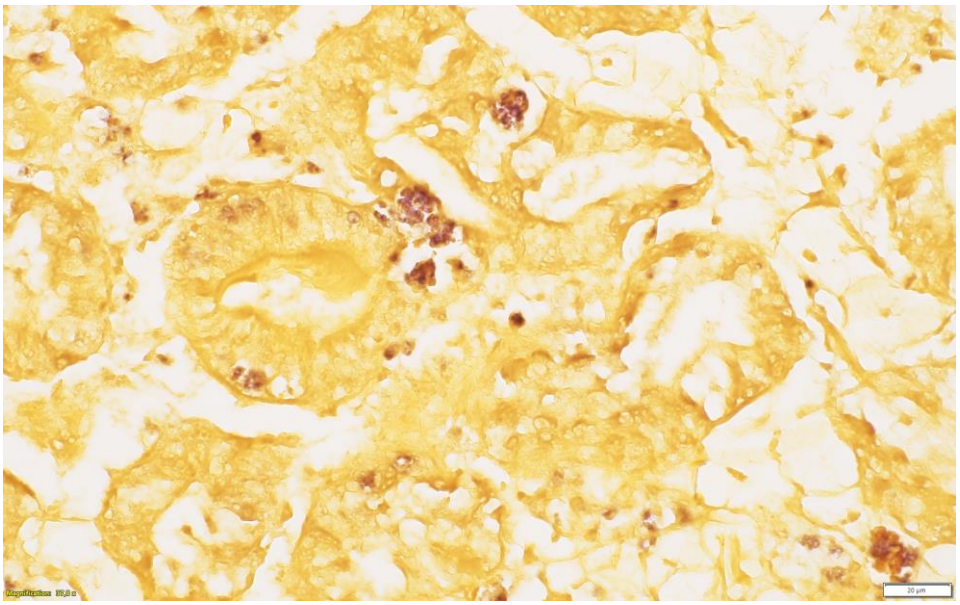

Figure 2. Eastern oyster *Crassostrea virginica* infected with *Perkinus marinus* tested by *in situ* hybridization with the PmarLSU probe: the parasites appear as dark spots in the epithelia of the digestive tubules.