Detection of mollusc pathogens by *in situ* hybridization (ISH)

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Editions

Edition	Date	Updated part
N° 1	December 2024	Creation of a general SOP for *in situ* hybridization, connected to diseases specific SOPs This SOP is based on previously published species-specific SOPs and includes additional technical recommendations

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1. Scope

In Situ Hybridization (ISH) assays are useful to confirm a previous histological diagnosis at the genus level or at the species level, depending on the probes used in the assay.
This procedure describes the general steps to perform *In Situ* Hybridization (ISH) assays for the detection of mollusc pathogens using digoxigenin (DIG) labelled probes and an alkaline phosphatase revelation system.
Preparation of probes and results interpretation are presented pathogen-specific SOP.

2. Principle

ISH allows localising a pathogen on an histological slide using a labelled DNA probe.

Histological slides are deparaffinised and deproteinized, then DNA is denatured.

The tissue sections are incubated with the **DIG-labeled probe**. The probe binds specifically to its complementary target DNA sequence in the tissue. Excess unbound probe is washed away.

The tissue sections are incubated with an anti-DIG antibody that recognizes the DIG label on the probe. This antibody is conjugated to the **alkaline phosphatase (AP)** enzyme.

AP enzyme allows for the conversion of a colorless substrate (**BCIP/NBT**) into a colored precipitate at the site of hybridization. The precipitate forms where the probe has bound, thus revealing the location of the target nucleic acid.

Note : other revelation systems can be used.

3. References

WOAH (2024). Manual of Diagnostic Tests for Aquatic Animals, section 2.4. Diseases of molluscs, Chapter 2.4.0 General information and disease-specific chapters*)*: https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-manual-online-access/

Connected SOPs :
Bonamia sp.detection and characterization by *in situ* hybridization (ISH)
Marteilia sp. detection and characterization by *in situ* hybridization (ISH)
Perkinsus marinus detection by *in situ* hybridization (ISH)
Mikrocytos mackini detection by *in situ* hybridization (ISH)

4. 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.**

5. 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

6. Probe Preparation

Two types of probes can be used:

Labelled PCR amplicons
The probe consists in a PCR amplicon in which digoxigenin (DIG) has been incorporated.
The PCR is performed as usual PCR except that DIG dUTP 25 mM is added to the reaction mixture.
DIG labelling should be checked by agarose gel electrophoresis.

Labelled nucleonucleotids
Pathogen-specific oligonucleotide probes are to be purchased with a 5' end digoxigenin label (for example from Eurogentec).

Probe preparation is detailed in pathogen-specific SOPs

7. Reagent preparation

- 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	

- 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:	
Deionised 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

8. Procedure

8.1. 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
- **Genus-specific probes** can be used to control the integrity of DNA on analysed slides

Additionally, a universal probe targeting Eukaryotes (For example the CS1-CaS1 probes produced by PCR with primers CS1 GTACGGGGAATCAGGGTTCG -CaS1 GGTGCCCTTCCGTCAATTCC, Le Roux et al. 1999), can be used to check if histological blocks are suitable for ISH analysis. This control is particularly useful in case of negative results with both the genus and species-specific probes. As the CS1 probe produce a strong signal, only 1µl of PCR product /100µl mix is used to avoid false positive signal.

8.2. ISH protocol

8.2.1. Slide preparation

The different steps of the preparation of 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).

8.2.2. 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.

8.2.3. 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.
4. Adhesive frames (ABgene) are fixed on each slide

Note: digestion time depends on the quality of histological blocks and the thickness of tissue slices, and can be optimised to improve the accessibility of the parasite DNA (generally between 10 and 30 minutes).

8.2.4. Prehybridization (optional)

1. 100 µl of a mix consisting of hybridization buffer
2. Incubated slide at 42°C during 30 min
3. Eliminated hybridization buffer

Note: A pre-hybridization step (hybridization buffer without the probe) may be added before the hybridization to decrease the background noise if needed.

8.2.5. Hybridization

1. 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.
   Note: The following concentrations of probes are generally used:
   - *for labelled oligonucleotides, 0.5 to 10µl of probe at 100µM / 100µl of hybridization mix;*
   - *for probes prepared by PCR: 1 to 5µl of labelled PCR product.*

   Lower quantity of probes is usually required for probes targeting the 18S compared to probes targeting the ITS or IGS.
2. Sections are covered with plastic coverlids.
3. Slides are denaturated for 5 min at 94°C and cooled on ice.
4. Slides are then incubated at 42°C in a humid chamber overnight

8.2.6. 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

8.3. Counter staining and mounting

8.3.1. 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)

8.3.2. Counter staining

1. Slides are immersed in Bismark brown Y for 1 min
2. Slides are rinsed under tap water and air-dried to remove alcool (alcool reduce the signal)
3. immersed in ethanol 95 and 100 30 sec for each

8.3.3. 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

9. Interpretation

- Negative controls must appear negative.
- Positive controls must appear positive.
- Slides are checked for positive signal looking like dark purple spots (See pathogen-specific SOPs for more details)