European Union Reference Laboratory
for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL– *B. xylophilus*-qPCR (BX-qPCR)_Version 01

December/ 2022

Protocol for *Bursaphelenchus xylophilus* detection by real-time PCR from wood and insect vectors

Foreword

These methods are recommended by:

ANSES - Plant Health Laboratory - Nematology Unit

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The consortium between both laboratories is designated the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

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Contents

1. Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934; Nickle, 1970), is the only plant-parasitic nematode (PPN) listed among the 20 priority quarantine organisms in the European Union (Commission Delegated Regulation (EU) 2019/1702), and is also classified as an EU quarantine organism (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2021/2285 amending Implementing Regulation (EU) 2019/2072 as regards the listing of pests). The totality of EU countries must conduct official controls at EU borders and perform annual surveys for this nematode species. If detected, eradication measures should be taken to avoid their spread within the EU territory (Commission Implementing Decision (EU) 2012/535 and Regulation (EU) 2016/2031). This PPN causes the pine wilt disease (PWD) (Figure 1A), which in general only affects *Pinus* spp., known as the most susceptible host; however, it can also be found in other trees species of *Abies*, *Chamaecyparis*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* (Evans *et al*., 1996). After *B. xylophilus* was exported from North America through infected timber to Japan at the beginning of the 20th century, the nematode spread further to other Asian countries (China and Korea). It was detected for the first time in Europe, in Portugal, in 1999, and 2008 in Spain (Mota *et al*. 1999; Abelleira *et al*. 2011; Fonseca *et al*. 2012). Check the EPPO Global Database (EPPO, 2022) for more updated information on geographical distribution.

As common among Aphelenchid species, the *B. xylophilus* feeds on both fungal (mycophagous) and plant cells, and is transmitted to dead or dying trees during oviposition by insect vectors from the genus *Monochamus* (Coleoptera: Cerambycidae) or to healthy trees during maturation feeding by the vector beetle (Mota & Vieira. 2008). The life cycle of *B. xylophilus* involves two forms: the propagative form (J1 to J4 and adult stages), which is present under suitable conditions, and the dispersal form (dauer juveniles J_{III} and J_{IV}) that is induced under unsuitable conditions, such as desiccation, food shortage, or environmental deterioration due to overpopulation. The dispersal form is intimately related to the vector beetle (Futai 2013). Thus, *B. xylophilus* can be found and detected in adult insects of *Monochamus* spp. (Figure 1B), including *M. alternatus* in Japan, China, and Korea, *M. carolinensis* in the United States, and *M. galloprovincialis* in Portugal. When *B. xylophilus* is transmitted during oviposition, the nematodes remain relatively close to the introduction site. However, when transmission occurs through the young shoots and when the tree succumbs to PWD, the nematodes are distributed throughout the whole tree, destroying wood tissues such as epithelial cells, parenchyma cells of axial and radial resin canals, cambium and phloem. *Bursaphelenchus xylophilus* can also be found in roots, even when the above-ground part of the tree is already dead, dried out or felled. Symptoms may first appear on one or a few branches but often develop quickly throughout the crown, and trees may die only 1 or 2 months after symptoms appear. Symptoms start with needle discolouration, progressing rapidly from a greyish green to yellow and brown. *Bursaphelenchus xylophilus* feeds on fungi in the wood, including the bluestain fungi that are transmitted by engravers and other bark beetles.

Next to their occurrence in vector insects (*Monochamus* spp.), *B. xylophilus* can be present in several coniferous products: wood, wood products and packaging, wood chips/shavings, plants for planting, branches, isolated bark, but not in needles, cones or seeds. Therefore, samples of imported wood and standing conifer trees should be taken and verified for the presence of the nematode.

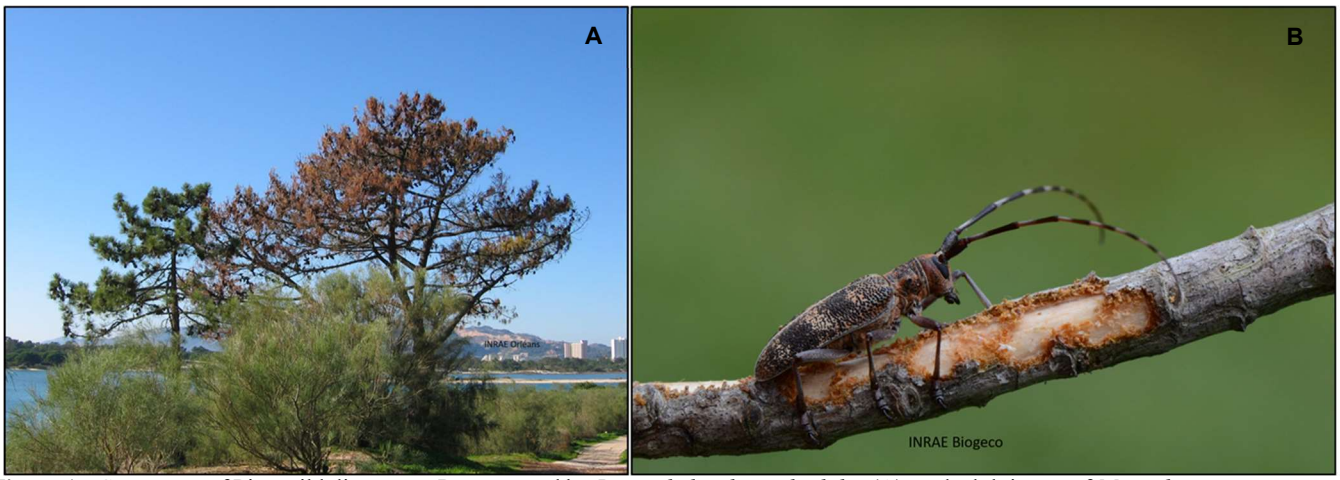


Figure 1 – Symptoms of Pine wild disease on *Pinus* caused by *Bursaphelenchus xylophilus* (A), and adult insect of *Monochamus* spp., vector of *B. xylophilus* (B). Pictures from INRAE-Biogeco and INRAE Orléans, France.

The EURL recommended protocols are based on literature reviews and IPPC and EPPO standards[1], when available. These recommended methods, including operational procedures, were performed with the scientific experience and technical expertise of the EURL team. These methods have been adapted, optimised and further validated by the EURL laboratory.

The purpose of this EURL recommended protocol[2] is to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostic analyses by providing them with details on analytical operating procedures for the detection and identification of *B. xylophilus*.

The material linked to this diagnostic protocol, such as slide presentations, technical videos & media, can be found on the EURL Plant Parasitic Nematodes website.

Warning and safety precautions: The user of these methods should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations. All actions taken in accordance with this method must be performed by employees who have attended relevant training.

[1] For more information on *B. xylophilus* biology, detection, and identification, view the standards EPPO PM 7/4 (4) and IPPC ISPM27 (DP10).

[2] The use of chemical brands or equipment in this recommended protocol does not imply the exclusion of others, which may also be appropriate.

2. Terms, abbreviations and definitions

- bp: base pairs
- Cycle threshold (Ct): the number of cycles required for the fluorescent signal to cross the threshold
- D: Detected
- ND: Not Detected
- EU: European Union
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- J1: first second-stage juveniles
- J2: second-stage juveniles
- J3: third-stage juveniles
- J4: fourth-stage juveniles
- J_{III}: Juvenile dispersal third-stage
- J_{IV}: Dauer juvenile (dispersal fourth-stage)
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematodes
- PWN: pine wood nematode
- PWD: pine wood disease

3. Purpose and scope

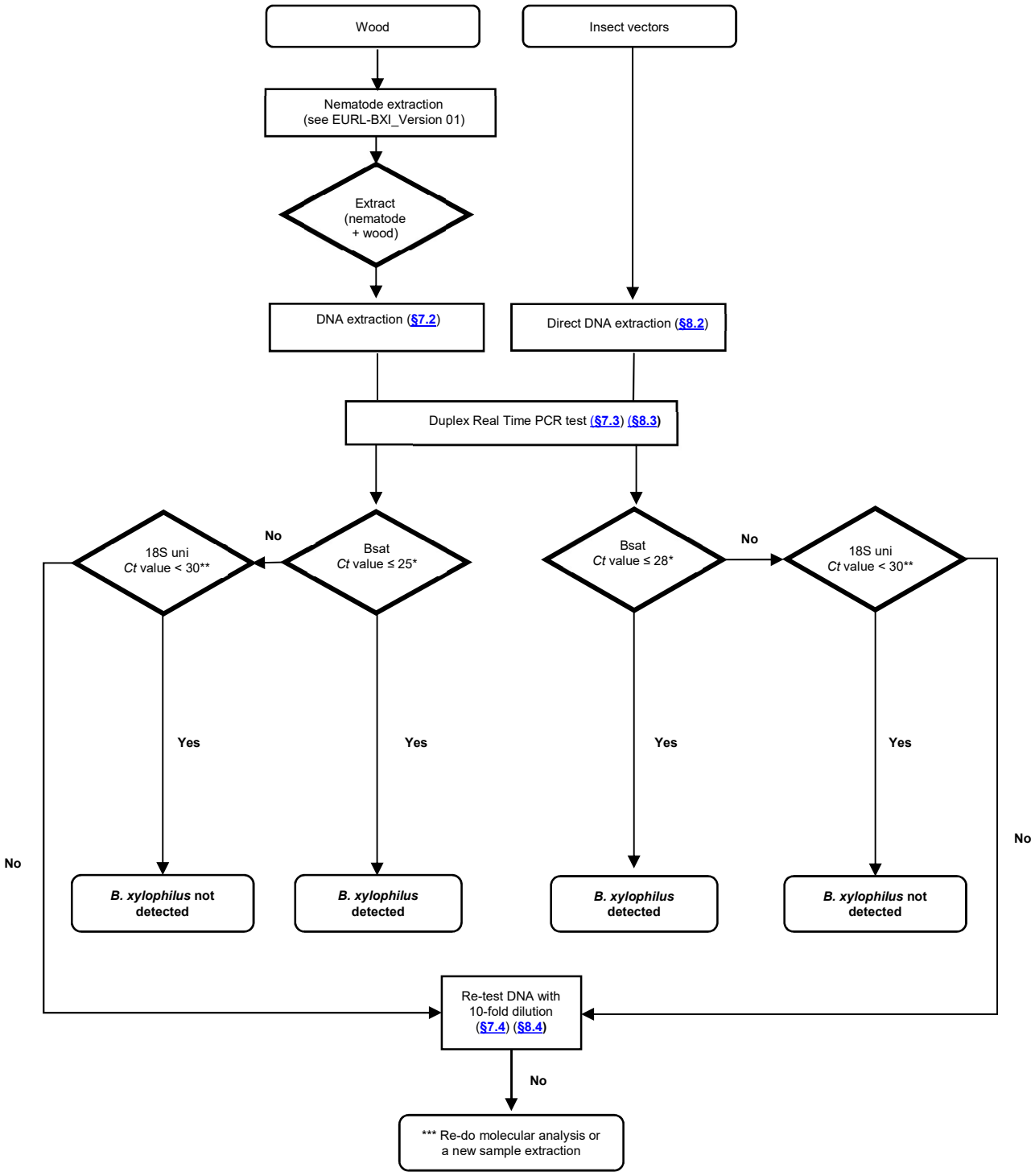
The purpose of this document is to describe a EURL diagnostic protocol for the detection of *Bursaphelenchus xylophilus* from coniferous wood and insect vectors using real-time PCR. The methods allow the detection of this nematode species from wood and insect vectors by performing a total DNA extraction from the nematodes extracted from wood (using the protocol EURL-BXE_Version 01) or directly from insect vectors, followed by real-time PCR detection. This approach can be used as a first screening in routine analysis by diagnostic laboratories in the EU. For all positive test results and in the case of inconclusive results, additional morphological analysis (see **§5** from EURL- BXI_Version 01, internal document, 2022) should be carried out, in particular, to ensure that the positive result is not linked to the presence of dead nematodes in treated wood packaging.

The real-time PCR methods based on the test from François et al. (2007) presented in this protocol were developed, optimised and validated by Anses-LSV Nematology Unit (FR), and can be used to detect *B. xylophilus* directly from wood extract or insect vectors.

The test is published as an official test for wood extracts (Anses, 2017) and vectors (Anses, 2018) and is also described in the updated EPPO PM 7/4.

The performance characteristics of these methods are available in appendix **§9**.

4. Schematic procedure of the methods



* The test is positive if a C_t value ≤ 25 (for specific test "Bsat primers/probes" on wood extract); ≤ 28 (for specific test "Bsat primers/probes" on insect vector), and an exponential amplification curve for each primer are observed.
**The test is negative if no C_t value ≤ 25 (for specific test test "Bsat primers/probes" on wood extract); ≤ 28 (for specific test test "Bsat primers/probes" on insect vector), and no exponential curve is observed; but a C_t value < 30 (for 18Suni primers/probes) and its exponential amplification curve is observed.
*** If a final conclusion is not possible, redo the molecular analysis, and/or analyse the samples according to another specific test or method and/or perform a new analysis on the remaining material or after a new extraction from the matrix.

Note: *The set C_t values were obtained under our conditions (i.e. equipment/materials and reagents); therefore, each laboratory needs to define/verify the C_t values using their conditions before implementing the test.*

5. Material and consumables

The equipment' small material and the consumables described below are the same for both molecular methods presented in this protocol.

5.1 Equipment and small materials

In addition to the standard equipment for molecular biology (pipettes, centrifuges, shaker, water bath, etc.), the following equipment is considered necessary for certain steps of the analysis:

5.1.1 For wood samples

- Glass beads (e.g. 3 mm and 1 mm)
- Shaking tissue grinder for approximately 2 mL microtubes (e.g., Tissulyser, Qiagen®) or equivalent equipment
- Conical bottom tubes (30 mL minimum)

5.1.2 For insects vectors

- Steel beads (e.g. 1 cm)
- Rotating shaker
- Conical bottom tubes (50ml)
- Real-time PCR thermal cycler
- Use pipette tips with filter plug during manipulation for PCR reactions.

5.2 Consumables

In general, the manipulator must ensure (either by the use of consumables known as molecular biology quality or by cleaning, sterilisation or other appropriate treatment) that the water and products or consumables used are free of contamination with DNA/RNA, nucleases, inhibitors or any other element that may interfere with the molecular analysis and the result.

Any commercial DNA extraction kit and/or PCR master mix suitable for real-time PCR application can be used as long it has been found to meet the requirements in an in-lab validation study.

Suppliers' recommendations regarding storage conditions before use should be followed, as well as conservation during usage. If impossible, the laboratory should define the most optimal conditions.

The reagent or brands[3] and solution preparations are the following:

- Molecular biology grade water
- **DNA extraction kit:** QIAamp® DNA mini kit (Qiagen) (ref. 51306).
- **Complete for insects with Buffer AL:** (Qiagen) (ref. 19075), Protease (30AU) (Qiagen) (ref. 19157) and PBS Buffer
- **Master mix:** LightCycler® 480 Probes Master mix (Roche Diagnostics) (ref. 04707494001).

[3] Brand names or suppliers may be mentioned in the description of the products necessary to implement this method. This information does not mean that the EURL recommends the exclusive use of these products. Any other DNA extraction kit or protocol may be used if their performance is validated and leading to the same results (see § 9).

- **Primers:**

Target PPN	Test reference	Primer/probe Real-time PCR	Sequence 5'- 3'
Bursaphelenchus xylophilus	François *et al*. (2007)*	**BSatF**	TGA-CGG-AGT-GAA-TTG-ACA-AGACA
		BSatR	AAG-CTG-AAA-CTT-GCC-ATG-CTA-AA
		Probe: BsatS	**FAM**-ACA-CCA-TTC-GAA-AGC-TAA-TCGCCT-GAG-A **BHQ1**
Eukaryote 18S rDNA	Ioos *et al.* (2009)**	**18S uni-F**	GCA-AGG-CTG-AAA-CTT-AAA-GGA-A
		18S uni-R	CCA-CCA-CCC-ATA-GAA-TCA-AGA
		Probe: 18S uni-P	**JOE**-ACG-GAA-GGG-CAC-CAC-CAG-GAG-T-**BHQ1**

* The primer set targets a 77 bp amplicon of the target sequence from *B. xylophilus* MspI satellite DNA monomeric unit (accession number L09652)
** The universal primer set targets a 150 bp amplicon for universal control, which is used as an inhibition control.

6. Controls and their purpose

The following controls are mandatory to check the correct performance of DNA extraction and PCR steps.

Controls	Purpose	Expected result *
Negative process control (NPC)	DNA extracted from the matrix (wood extract or insect vector) without the target nematode. The DNA is conditioned, extracted and tested similarly to the tested sample. Verify the absence of any flaws during the analytical process.	Negative
Positive process control (PPC)	DNA extracted from the matrix (wood extract or insect vector) containing the target nematode. The DNA is conditioned, extracted and tested similarly to the tested sample. Verify the absence of any flaws during the analytical process.	Positive
Positive PCR control (PC)	It contains all the elements of the PCR reaction mix, including a DNA extract from *B. xylophilus* (viable juveniles); this control verifies that the PCR reaction has proceeded correctly, allowing the amplification of the samples containing the target.	Positive
No Template Control (NTC)	It contains all the elements of the PCR reaction mix, but no DNA is added; this control checks the absence of contamination during the PCR preparation and reaction.	Negative
Negative specificity control (NSC)	It contains all the elements of the PCR reaction, including non-target DNA; this allows for checking the absence of cross-reaction during the PCR. This type of control is not required for the universal test (Ioos *et al.,* 2009)**.**	Negative
Quality control (QC)	Quality control of the DNA extraction process and check the presence of inhibitors in negative results (where no amplification is observed). This control is carried out using the 18S uni rDNA primers/probe combination (Ioos et al. 2009). This test control will be performed in the same reaction as the *B. xylophilus* test (duplex). However, the analysis of the 18S uni primers/probe fluorescence curves will be limited to the data acquired during the first 30 cycles only. A DNA will be said to be positive for the 18S uni test if the C_t or the average C_t generated is within an acceptable C_t range, previously determined experimentally by the laboratory, on this type of matrix (wood or insect) and under its own conditions.	Positive**

* The results are only valid if the expected results are met.
If available, the target species may be supplied by the EURL.

**Positive result, however, different target, i.e. positive result in wells with no amplification for Bsat.

7. Direct detection from wood samples

7.1 Conditioning nematode suspension from wood

After extracting the nematodes from wood samples (see EURL- BXE_Version 01, 2022), the resulting suspension (nematode + wood in water) is transferred into a conical bottom tube (of at least 30 mL) for at least 3 h, to let the nematodes settle it to the bottom of the tube.

Note: *The extract can be refrigerated for longer storage, but it should not be stored for more than 7 days at this stage*

7.2 DNA extraction

Each extraction series should include the following controls: NPC and PPC.

1) Take approximately 1.5 mL of the deposit (settled nematodes in water) and transfer it into a 2 mL microtube (e.g. conical bottom tube).
2) Centrifuge for 10 min at 15000 g, and discard the supernatant, keeping the pellet.
   Note: *at the end of this step, the samples can be stored in the freezer until further processing*
3) Add glass or metal beads of different diameters (e.g. 2 beads of 3 mm and a few beads of 1 mm) to the tube.
4) Add the lysis buffer solution and volume supplied and recommended, respectively, with the DNA extraction kit. In the case of the QIAamp DNA mini kit (Qiagen), this is 180 µL of lysis buffer (ATL) + 20 µL of proteinase K).
5) Suspend the pellet.
6) Grinding: place the tube on a tissue grinder rack ([e.g. using a Tissulyser II (Qiagen®) shake at a frequency 30 Hz for 40 sec.
7) Place the tube in a water bath at approximately 56°C for at least one hour.
8) Briefly centrifuge. Add 200 µL of AL buffer and vortex well.
9) Incubate for 10 min at 70 °C.
10) Briefly centrifuge. Add 200 µL of ethanol (96 -100%) and homogenise well.
11) Transfer the contents of the tube to the column supplied with the QIAamp kit.
12) Centrifuge for 1 min at 6000 g.
13) Place the column on a clean collector and add 500 µl of AW1 buffer.
14) Centrifuge for 1 min at 6000 g.
15) Place the column on a clean collector and add 500 µl of AW2 buffer.
16) Centrifuge for 3 min at 20 000 g.
17) Place the column on a clean, labelled 1.5 mL microtube and add 100 µL of buffer AE or molecular-grade water.
18) Incubate for a few minutes at room temperature and then centrifuge for 1 min at 6000 g.
19) The resulting DNA solutions are directly analysed by real-time PCR or frozen (<10 °C) until analysis.

7.3 Real-time PCR test (based on François *et al*. 2007)

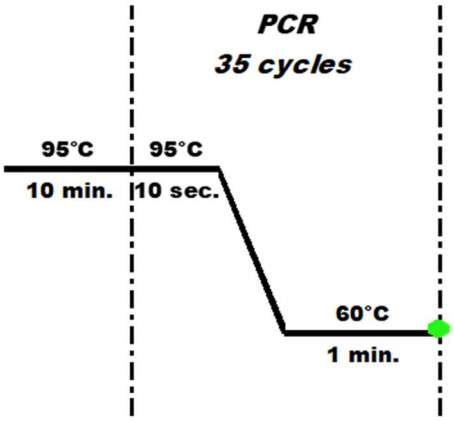
Each DNA sample is tested at least in duplicate.

- **Duplex PCR reaction**

	Real-time PCR test
Reagents – Master mix	**Final concentration per reaction tube**
Total volume	20 µL
LightCycler® 480 Probe Master (Roche)	1 X
BSatF	0.3 µM
BSatR	0.3 µM
Probe: BsatS	0.1 µM
18S uni-F	0.3 µM
18S uni-R	0.3 µM
Probe: 18S uni-P	0.1 µM
Molecular-grade water water	Adjust to 15 µL
Add DNA to 15 µL of reaction mix	5 µL

DNA amplification is carried out under the following conditions:

- **PCR program:**



Monitoring PCR: the fluorescent signal of the reporter dye is measured at the end of the hybridization phase (hydrolysis probe).

7.4 Evaluation and reporting the results

The results obtained by real-time PCR are preferably processed using software for automatic analysis or, failing that, by defining and applying the same threshold line. A C_t value must be accompanied by an exponential curve to be considered valid. The result interpretation of the molecular analysis is carried out according to the above flowchart (see **§4)**. The molecular analysis result is a synthesis of the results obtained from each of the microtubes analysed. The correct interpretation of the results is carried out by observing the exponential fluorescence curves measured by the real-time PCR and generated from the various controls. The analysis is valid if, and only if, all of the following conditions are met:

Checking the controls for specific primers (Bsat):

- The NPC, NTC and NSC: no amplification is observed in any of the replicates (C_t>25)
- All replicates of PC, PPC: amplification of the amplicon in accordance with the requirements.

If the results of one or more controls do not comply with those expected (as defined above), the analysis is not valid and, depending on the non-compliance observed, all or part of the analysis must be repeated.

After validation of the controls and for each of the fluorescence signals, observe the *Ct* value and the appearance of the amplification curve: sample results should be interpreted as follows for the target species under investigation (*B. xylophilus*):

Checking the samples:

Analysis		**Sample Results**
Well 1	Well 2	
+	+	Positive test for the target species
+	-	The PCR is repeated. If 1 out of 2 is still positive, the result is interpreted as positive.
-	-	* For the universal 18S uni test (see **§6** Quality Control) and for the relevant test run: If QC amplification is observed, i.e. the DNA of the test sample is positive for the18S uni (C_t value < 30) in duplex reaction, the **test result is negative for *B. xylophilus*** If no amplification of QC is observed, i.e. the DNA of the test sample is negative for the18S uni in duplex reaction, a new PCR reaction is performed **with 10-fold dilution** from the DNA. If unable to conclude, **perform a new analysis on the remaining sample according to another method and/or new extraction from the matrix**

+: observation of an exponential fluorescence curve (for Bsat) with a C_t value ≤ 25 (for wood extract) in duplex reaction.

- : the absence of an exponential fluorescence curve or observation of an exponential amplification curve with a C_t value ≥ 25 (for wood extract

**** Note:***

The universal 18S uni test (QC control) is only analysed in the case of Bsat-negative samples to verify the DNA extraction process and the absence of inhibition in those samples. For information: Bsat amplification overcomes 18S uni amplification. This means that in the case of a positive sample, it is very likely that an amplification will not be observed with the universal primers despite the absence of inhibitors.

If you have any question about this protocol, please, send an e-mail to eurl.nematode@anses.fr

Acknowledgements

These recommendations were prepared by the EURL consortium composed of ANSES - Plant Health Laboratory - Nematology Unit and ILVO - Plant Unit - Nematology in the frame of EURL activities.

8. Direct detection from insect vectors

8.1 Conditioning insect vector samples

1) The DNA is extracted from the metathorax and abdomen of one to several *Monochamus* specimens. The posterior part of the insect is removed with a single-use scalpel, as shown in figure 2.

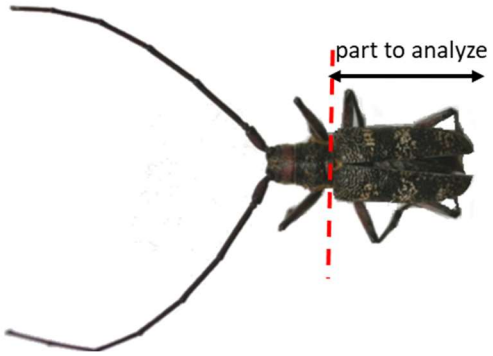


Figure 2. *Monochamus* preparation for DNA extraction.

2) Pack the collected posterior parts in one or more tubes (at least 50 mL) with a conical bottom with a maximum of 20 insects per tube. Prepare as many tubes as necessary
   - ***Note:*** *at the end of this step, the samples can be stored in the freezer until further processing*

8.2 DNA extraction

3) Add to each tube, 1 steel bead of approximately 1 cm , and add approximately 5 mL of PBS buffer[4] 1X
4) Place the racks containing the 50 mL tubes in a rotating shaker (e.g. Reax rotating shaker, HEIDOLPH) and stir at low speed (30 to 40 rpm) for approximately one hour
5) Briefly centrifuge
6) Add 500 µL of protease solution (Qiagen)
7) Add 12 mL of AL buffer (Qiagen)
8) Vortex to homogenise
9) Incubate the tubes for 10 min at 70°C in a pre-heated thermostatic bath.
10) Briefly centrifuge the tubes
11) Add 10 mL of 96-99% ethanol.
12) Homogenise well and incubate for a few minutes at room temperature
13) Transfer 700µL of the tube's content (be careful not to pick up any debris) to the column provided in the QIAamp DNA mini kit (Qiagen)
14) Centrifuge for 1 min at 6000 g
15) Add 500 µL of buffer AW1
16) Centrifuge for 1 min at 6000 g
17) Add 500 µL of buffer AW2
18) Centrifuge for 3 min at 20000 g
19) Place the column on a clean, labelled 1.5 mL microtube and add 100 µL of buffer AE or molecular-grade water
20) Incubate for a few minutes at room temperature and then centrifuge for 1 min at 6000 g
21) The resulting DNA solutions are directly analysed by real-time PCR or frozen (<10 °C) until analysis.

[4] 10× Phosphate buffered saline (PBS), pH 7.2 (dilute 1:10 before use) NaCl 80.0 g, $NaH_2PO_4.2H_2O$ 4.0 g, $Na_2HPO_4.12H_2O$ 27.0 g, Distilled water 1000 mL.

8.3 Real-time PCR test (based on François et al. 2007)

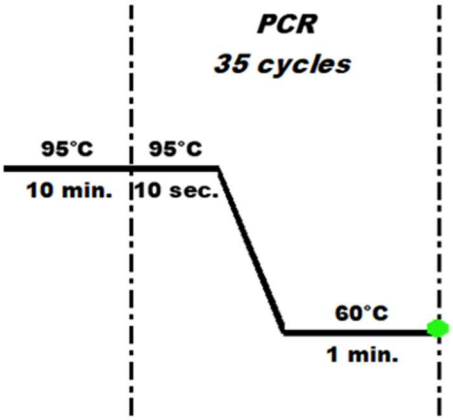
Each DNA sample is tested at least in duplicate.

- **Duplex PCR reaction**

	Real-time PCR test
Reagents – Master mix	**Final concentration per reaction tube**
Total volume	20 µL
LightCycler® 480 Probe Master (Roche)	1 X
BSatF	0.3 µM
BSatR	0.3 µM
Probe: BsatS	0.1 µM
18S uni-F	0.3 µM
18S uni-R	0.3 µM
Probe: 18S uni-P	0.1 µM
Molecular grade water water	Adjust to 15 µL
Add DNA to 15 µL of reaction mix	5 µL

DNA amplification is carried out under the following conditions:

- **PCR program:**



Monitoring PCR: the fluorescent signal of the reporter dye is measured at the end of the hybridization phase (hydrolysis probe).

8.4 Evaluating and reporting the results

The results obtained by real-time PCR are preferably processed using software for automatic analysis or, failing that, by defining and applying the same threshold line. A C_t value must be accompanied by an exponential curve to be considered valid. The result interpretation of the molecular analysis is carried out according to the above flowchart (see **§4)**. The molecular analysis result is a synthesis of the results obtained from each of the microtubes analysed. The correct interpretation of the results is carried out by observing the exponential fluorescence curves measured by the real-time PCR and generated from the various controls. The analysis is valid if, and only if, all of the following conditions are met:

Checking the controls for specific primers (Bsat):

- The NPC, NTC and NSC: no amplification is observed in any of the replicates, C_t >28. Notice that the threshold is higher than for wood samples.
- All replicates of PC, PPC: amplification of the amplicon are in accordance with the requirements.

If the results of one or more controls do not comply with those expected (as defined above), the analysis is not valid and, depending on the non-compliance observed, all or part of the analysis must be repeated.

After validation of the controls and for each of the fluorescence signals, observe the *Ct* value and the appearance of the amplification curve: sample results should be interpreted as follows for the target species under investigation (*B. xylophilus*):

Checking the samples:

Analysis		**Sample Results**
Well 1	Well 2	
+	+	Positive test for the target species
+	-	The PCR is repeated. If 1 out of 2 is still positive, the result is interpreted as positive.
-	-	* For the universal 18S uni test (see **§6** Quality Control) and for the relevant test run: If QC amplification is observed, i.e. the DNA of the test sample is positive for the18S uni (C_t value < 30) in duplex reaction, the **test result is negative for *B. xylophilus*** If no amplification of QC is observed, i.e. the DNA of the test sample is negative for the18S uni in duplex reaction, a new PCR reaction is performed **with 10-fold dilution** from the DNA. If unable to conclude, **perform a new analysis on the remaining sample according to another method and/or new extraction from the matrix**

+: observation of an exponential fluorescence curve (for Bsat) with a C_t value ≤ 28 (for insect vector) in duplex reaction.
- : the absence of an exponential fluorescence curve or observation of an exponential amplification curve with a C_t value ≥ 28 (for insect vector)

**** Note:***

- The universal 18S uni test (QC control) is only analysed in the case of Bsat negative samples to verify the DNA extraction process and the absence of inhibition in those samples.

- For information: Bsat amplification overcomes 18S uni amplification. This means that in the case of a positive sample, it is very likely that an amplification will not be observed with the universal primers despite the absence of inhibitors.

9. Performance characteristics and validation of the real-time PCR test

The performance characteristics below were obtained within the framework of the molecular test validation by ANSES laboratory. The molecular test in this protocol was evaluated and validated according to the following criteria:

- Specificity (= analytical specificity-exclusivity): Ability of the method to not detect the target from a range of non-target populations (absence of false positives);
- Sensitivity (= analytical specificity-inclusivity): Ability of the method to detect the target from a range of target populations (absence of false negatives);
- Repeatability: Ability of the method to reproduce identical results under identical analytical conditions from samples at low concentrations.
- Accuracy: Ability of the method to detect the target species from a range of populations from the target species and not to detect the target species from a range of populations from the non-target species.
- Reproducibility: the ability of the method to reproduce identical results under different conditions (equipment, operators, etc.) from samples at low concentrations.
- Limit of detection (= analytical sensitivity): the smallest amount of target species that gives a positive result in all replicates.

Concerning the specificity, the molecular test was applied to the following species/populations:

- 7 populations of *Bursaphelenchus xylophilus*
- 4 populations of *B. mucronatus* ;
- 2 populations of *B. sexdentati ;*
- 1 population of *B. doui;*
- 1 population of *B. fraudulentus;*
- 1 population of *B. singaporensis ;*
- 1 population of *B. macromucronatus;*
- 1 population of *B. hoffmani*;
- 1 population of *B. vallesianus*;
- 1 population of *B. willibaldi*
- 3 populations of *B. sp* from China, France and Vietnam.

Summary of the validation results for the identification of *B. xylophilus* from wood extract and insect vector matrices by real-time PCR.

Characteristics	**Results**	
	Wood samples	**Insects vector**
Analytical sensitivity (LOD)	1 nematode	5 nematodes /pool of 20 insects
Repeatability of the method *	100%	100%
Reproducibility of the method *	100%	100%
Analytical specificity-exclusivity):	100%	100%
Analytical specificity-inclusivity	100%	100%
Accuracy	100%	100%

*: At the LOD

10. References

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