Test Performance Study for the detection of *Xylella fastidiosa* in Mediterranean dormant plant species Technical sheet

Version N°01 – April 18, 2023

Identification of the test

Code: 23-XfDORM

Title: Test Performance Study "detection of *Xylella fastidiosa* in dormant plant species"

1. Introduction

This Test Performance Study (TPS) aims at evaluating protocols to detect *Xylella fastidiosa* in dormant plant species.

2. Organisation

The TPS is organised by the Plant Health Laboratory of Anses whose address is:

Anses, Plant Health Laboratory, 7 rue Jean Dixméras, 49044 ANGERS Cedex 01, France

The Plant Health Laboratory of Anses is the French National Reference Laboratory for the detection of *Xylella fastidiosa* on host plants.

The coordination of the test is ensured by:

Anne-Laure Boutigny
Email : anne-laure.boutigny@anses.fr
Tel.: +332 41 20 74 54

3. Participation

3.1. *Participation criteria*

The TPS is open to partner laboratories of the Euphresco project "Diagnosis of *Xylella fastidiosa*: detection on dormant plants, important for Mediterranean countries".

3.2. *Codification of laboratories and confidentiality*

Participating laboratories will be identified throughout the TPS by their panel code consisting of the letter L followed by a two-digit number (e.g. "L07"). The results obtained from the participants will be presented anonymously in order to ensure the confidentiality of their results with respect to the other participants. Then each laboratory will be informed of its own panel code for the exploitation of results.
Participating laboratories are informed, upon registration, that the results may be used anonymously for scientific purposes.
Finally, participating laboratories are informed that they may waive confidentiality during the TPS for discussion and mutual assistance or for scientific purposes.

3.3. *Resources required for the TPS*

- **Personnel:**

The working time for preparing the samples, performing the analyses, submitting and sending the results is estimated at 4 to 7 days per participant, according to the number of tested protocols.

- **Consumables:**

All reagents (DNA extraction kits, primers, probes, enzymes and master mix, etc…) are provided by the participant laboratories.
The kits and/or enzymes with supplier/brand are indicated in the different appendixes or protocols in EPPO PM7/24 (4). **It is recommended** to use these reagents. If participants use different reagents, they must report this to the organiser with the results.
The primers and probes are described in the different appendixes or protocols in EPPO PM7/24 (4). Deviations from the recommended sequences are not allowed.

- **Equipment and materials**:

Devices and equipment commonly used in molecular biology and especially for real time PCR are necessary.
It is highly recommended to use a robot for the QuickPick™ DNA extraction, as for example KingFisher™ mL or KingFisher™ Flex (Thermo Fisher Scientific), or equivalent for a better homogeneity of the results. For manual method, follow strictly indication in Appendix 2.
The metrological requirements are those of the participating laboratory.

- **Specific environmental conditions:**

No additional requirements other than those usually implemented in the participating laboratory.

3.4. *Special precautions to be taken by the participants*

Because the testing samples are naturally infected with *Xylella fastidiosa*, a risk of dissemination of the pest organism cannot be excluded. Each operator/laboratory must prevent contamination by applying good laboratory practices and national regulation rules.

4. Framework of the Test Performance Study

Steps	Deadlines
Call for applicants	07 April 2023
Sending of panels	22 May 2023
Performing analyses and submitting results	28 July 2023
Final report	31 October 2023

Table 1: Framework for the Test Performance Study.

5. Communication with participants

5.1. *Information provided to the participants*

TPS steps	Documents provided to participants	Actions to be carried out by the participants
Call for applicants	- the technical sheet (including description of some protocols) - the participant's contract	- Registration by sending back the participant's contract. - Accomplishment of the regulatory formalities allowing the sending of the panel in accordance with the phytosanitary regulation (e.g. : request for LOA and submission of the signed LOA to the organiser for European countries)
Sending of panels	- the letter relating to the panel - the instruction sheet - the acknowledgement of receipt form - the result form - the regulatory documents provided by the participant allowing the sending of the samples in accordance with the phytosanitary regulation	On receipt of the package : - checking the content of the package - sending back the acknowledgement of receipt form Preparing the samples, performing analyses and recording the results according to the technical sheet and instruction sheet Sending back the result form, once completed before the deadline
Sending of the TPS report	- the TPS report - the individual summary sheet	

Table 2: Details of the information provided to the participants and of the actions to be carried out by the participants at the different steps of the TPS.

5.2. *Communication*

If the organiser has to communicate with the participants, the email will be used. For this reason, two email addresses are required upon registration.
Requests for information from the participants will be taken into account; the participants are invited to send their emails to:

anne-laure.boutigny@anses.fr

Moreover, in order to avoid bias in the TPS, participating laboratories are not allowed to communicate with each other about their results until the deadline for submission of results. This is a commitment of the participant's contract.

6. Studied methods

The TPS involves the testing of different DNA extraction methods and molecular methods proposed in the EPPO standard PM7/24 (4) for the detection of *Xylella fastidiosa* and/or the identification of subspecies.
The following protocols can be implemented:

- DNA extraction methods :

- CTAB (EPPO PM7/24 (4))

- QuickPick™ SML Plant DNA kit (Bio-Nobile) (EPPO PM7/24 (4)) : Automate or Manual
- DNeasy Plant Mini Kit (Qiagen) (EPPO PM7/24 (4))
- Other DNA extraction method (choice of participant)

- Molecular methods for Xf detection:

- Real-time PCR Harper *et al*. (2010) (EPPO PM7/24 (4)): Simplex and/or Duplex
- Other PCR for Xf detection (choice of participant, including digital PCR for quantification)

- Molecular methods for subspecies identification:

- Tetraplex real-time PCR Dupas *et al*. (2019) (EPPO PM7/24 (4))
- Simplex real-time PCR Dupas *et al*. (2019)
- MLST Yuan *et al*. (2010) recommended on 7 genes to determine the ST (EPPO PM7/24 (4))
- Real-time PCR Hodgetts *et al.* (2021) (EPPO PM7/24 (4))
- Other PCR for subspecies determination (choice of participant)

It should be noted that in order to evaluate a common protocol, it is recommended that each laboratory use at least the following DNA extraction and PCR technique:

- **DNA extraction CTAB (EPPO PM7/24 (4))**
- **Real-time PCR Harper *et al*. (2010) (EPPO PM7/24 (4))**

The procedure for some methods is given to the participants in the appendixes 1 to 5.

The plant material provided in the panel is not in limited quantity and participating laboratories are welcome to perform additional analyses such as testing other DNA extraction and/or PCR methods. Participants will have to indicate in the participant's contract which DNA extraction methods and real-time PCR methods they will implement in the TPS.

The evaluation does not concern the sampling since this have already been carried out by the organiser in order to ensure the homogeneity and the stability of the samples submitted to the test.

For each sample analysed and for each method, the participants should give a qualitative result under the form "positive", "negative". In case of duplex real-time PCR using an internal control (IC), the final result for Xf target can also be "undetermined" if the result for Xf target and IC are both negative.
For real-time PCR methods, the participants should also give the Ct values obtained for each sample with the threshold applied, and provide curves from thermocycler.

7. Samples to be analysed, sending of panels to participants

7.1. *Composition of the panel*

One panel will be constituted of maximum 10 samples of naturally contaminated and not contaminated dormant twigs. Each sample will contain 10 pieces of twigs of about 2 cm in bags.
The organiser could have the constraint to supply less than 10 samples per panel to some laboratories if the material is not sufficient.

Material should be stored at -20°C upon reception.

7.2. *Validation of the samples*

The assigned value of samples (status assigned to the samples) will result from the experimental work of Anses and confirmed during the homogeneity and stability study by repeated analyses on the samples packaged in their final form.

In addition, the organiser will ensure that the samples used for the TPS are sufficiently homogeneous and stable to make sure that the differences in the results among laboratories can be connected to the performances of methods and not to the sample characteristics.

The homogeneity and the stability of the samples will be studied with the real-time PCR Harper *et al.* (2010) for sample prepared according to QuickPick™ SML Plant DNA kit extraction procedure. The study of homogeneity will cover all samples (contaminated and healthy). The study of stability will be carried out the same way from the deadline for performing the analyses by the participants. To simulate the conditions of transport, the package containing the stability samples will be left at room temperature during the longest period announced by the carrier to ensure the delivery of the packages to the participants at the different destinations (3 working days). This delay may, at the discretion of the coordinator, be extended by a few days if the delivery of a parcel is delayed but is imminent.

Qualitative results, and when available quantitative ones (Ct), will be exploited for the analysis of the results of homogeneity and of stability.

7.3. *Codification of the samples*

The samples will be coded from 01 to 10 according to the following principle: TPS code - panel code - sample code.
e.g. 23-XfDORM-*L01-05-: sample N°5 of panel 1*
The coding of samples will be randomly chosen and will be different for each panel (and consequently for each participant).

7.4. *Transport*

The samples will be sent to the laboratories by a carrier and will be packed with freezing blocks. In case of problems with the samples, the laboratories must contact the organiser within 24 hours of receiving the samples and maximum effort will be made to find a solution.

7.5. *Sample storage, analyses and result recording*

An instruction sheet will be sent to each participant with the package. It will explain the instructions to be followed:

- once the package is received,
- for the storage of the samples,
- for performing analyses,
- for recording and submission of the results.

8. Submission of results

Participants must respect deadlines for performing analyses and for submission of results (Table 1).
To submit their results, participants must use the result form provided by the organiser. Once completed, the result form must be returned by email at the electronic address mentioned in paragraph 5.2.

9. Processing of results

Each protocol will be evaluated according to the qualitative results submitted. The total number of positive accord (PA, if a positive result is obtained when a positive result is expected), negative accord (NA, if a negative result is obtained when a negative result is expected), positive deviation (PD, if a positive result is

obtained when a negative result is expected) and negative deviation (ND, if a negative result is obtained when a positive result is expected) will be determined for each laboratory and each protocol. The ISO 16140 standard (ISO, 2003) stipulates that collaborative studies should be based on data from laboratories with high competence on the techniques that are being compared. Consequently, the results of a laboratory could be excluded for a given method (considered as outliers *i.e.* far removed from the rest of the laboratories), as example, when the expected result for at least one control will not be obtained or when the number of PD or ND results obtained by this laboratory will represent more than 40% of PD or ND results obtained for the method and when 50% of PD or ND results or more will be recorded from the panel of samples.

The performance criteria proposed by the standard PM7/98(2) (EPPO, 2014) and PM 7/76(2) (EPPO, 2010), defined below will be evaluated for each method.

The **analytical specificity** is defined as the degree of correspondence between the responses obtained by the evaluated method and the expected theoretical results (samples' real status), and will be assayed using two criteria:

- the **diagnostic sensitivity** *i.e.*, the ability of the method to detect the target when it is present in the sample,
- the **diagnostic specificity** *i.e.*, the ability of the method to not detect the target when it is not present in the sample.

The **repeatability** is the level of agreement between replicates of a sample tested under the same conditions.
The **reproducibility** is the ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (time, persons, equipment, location etc.).

Details of the statistical analysis of the results will be provided in final report.

10. **TPS report**

The final report will present anonymously the results of all participants. It will be transmitted by electronic format (protected pdf format) to the two electronic addresses communicated in the participant's contract. Concerning the dematerialisation of the TPS report, the convention of proof that applies is specified in the contract. The signature of the contract means acceptance of the convention of proof.
The code of each laboratory will be transmitted individually at the same time and according to the same modalities as the report

Validated the 18th April, 2023
Anne-Laure Boutigny

The coordinator

References

- Dupas E, Briand M, Jacques MA, Cesbron S. Novel Tetraplex Quantitative PCR Assays for Simultaneous Detection and Identification of *Xylella fastidiosa* Subspecies in Plant Tissues. Front Plant Sci. 2019 Dec 27;10:1732. doi: 10.3389/fpls.2019.01732. PMID: 31956326; PMCID: PMC6951419.
- European Plant Protection Organization. PM7/76(2)-Use of EPPO diagnostic protocols. EPPO Bulletin/Bulletin OEPP. 2010; 40:350-2.
- European Plant Protection Organization. PM7/98(2)-Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bulletin/Bulletin OEPP. 2014; 44:117-47.
- European Plant Protection Organization. PM7/24(4)-Diagnostic, *Xylella fastidiosa*, Bulletin OEPP/EPPO. 2019; 49: 175-227.
- European Plant Protection Organization. PM7/122 - Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories. Bulletin OEPP/EPPO Bulletin 2014; 44 (3), 390–399.
- Harper S.J., Ward LI and Clover GRG.. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications, Phytopathology, 2010; erratum 2013; 12: 1282-1288.
- Hodgetts, J., Glover, R., Cole, J., Hall, J. and N. Boonham, N. 2021. Genomics informed design of a suite of real-time PCR assays for the specific detection of each Xylella fastidiosa subspecies, Journal of Applied Microbiology, 131 (2): 855-872.
- International Organization for Standardization. ISO 16140: 2003. Microbiology of foods and animal feeding stuffs-Protocol for the validation of alternative methods.
- Ioos R., Fourrier C., Iancu G., Gordon T.R. 2009. Sensitive Detection of *Fusarium circinatum* in Pine Seed by Combining an Enrichment Procedure with a Real-Time Polymerase Chain Reaction Using Dual-Labeled Probe Chemistry. Phytopathology 99-5-0582
- Yuan X., Morano L., Bromley R., Spring-Pearson S., Stouthamer R., Nunney L., 2010. Multilocus sequence typing of *Xylella fastidiosa* causing Pierce's disease and oleander leaf scorch in the United States. Phytopathology 100, 601-11.

Appendix 1: Sample preparation

One macerate is prepared per DNA extraction method per sample.

1. General Information

One panel contains maximum 10 samples. Each of the 10 samples contains 10 pieces of about 2 cm of dormant twigs which have to be processed. The debarking and cutting protocol is proposed by Anses (LSV Angers).

2. Methods

In order to prepare the plant macerate from which the DNA extraction will be made, it is necessary to remove the bark from the 10 pieces, cut them all in thin slices and weigh the necessary mass according to this protocol :

- The dead bark present on the 10 pieces is removed with a scalpel (no bark to remove on the young green pieces)
- The large pieces can be cut in 2 or 4 lengthwise
- For grapevine samples, the pith (dead central part), if present in large amount, is removed with a scalpel, after cutting the pieces in 2 or 4 lengthwise
- The pieces debarked or not (if green) are cut in thin slices (≈ 1mm) in the direction of the width in order to cut the vessels of the xylem (the totality of the 10 pieces must be cut) and homogenized

- **According to DNA extraction method, the necessary mass is weighed and placed in a bag (e.g.: Bioreba) for preparation of macerate:**
  - **1.5 g for CTAB extraction;**
  - **1 g for Quickpick,**
  - **1 g for DNeasy (we recommend to prepare macerate on 1 g of material for DNeasy extraction rather than 0.2 g as recommended in EPPO PM/7/24 (4) and to adjust the volume of lysis buffer)**
  - **other according to protocol**

Appendix 2: QuickPick™ (Bio-Nobile)-based extraction (EPPO PM7/24 (4))

Two DNA extractions are performed per macerate.
1 g of material is homogenized in 5 mL of water; 2 test samples of 250 µL for DNA extraction

1. Methods

- Weight 1 g of material in a bag (e.g.: Bioreba bag) with 5 mL of sterile water and homogenize using appropriate equipment (pneumatic press, hammer …)
- Perform an ultrasonication of bags (1min at 35-40 kHz)
- Leave bags to soak for at least 15 min under gentle shaking
- Transfer 2 x 250 µL of extract into 2 x 2 mL micro-centrifuge tube and centrifuged for 20 min at 20 000 g.
- The pellet is suspended in 75 µL of lysis buffer with 5 µL of proteinase K, and then the tube is vortexed.
- Tubes are heated 20 min at 65°C under gentle shaking.
- The tubes are centrifuged 5 min at 18 000 g.
- At this stage, three options are possible: option 1: automated extraction (recommended), option 2: by using magnetic rack extraction; option 3: by using magnetic pipet extraction. **Note use of the option 1 is recommended.**

Option 1: automated extraction (e.g. KingFisher mL (Thermo Fisher Scientific))

KingFisher™ tube strip (5 wells)

Hand Strip	A		B	C	D	E
Buffers	Binding	Beads suspension	Wash	Wash	Wash	Elution
Volumes	125 µL	5 µL	250 µL	250 µL	250 µL	50 µL

- Buffers and beads are added into the tube strip as described on the schema above.
- Supernatants are transferred into well A
- The tube strip are inserted on the automate tray.
- Tip combs (Thermo Fisher Scientific) are inserted on the magnetic rod bloc.
- The robot is turned on and the specific program (see below) is started (duration: about 31 min).
- **At the end of the program, eluates are transferred into new tubes for storage.**

Robot extraction program (e.g. KingFisher mL (Thermo Fisher Scientific))

[PLATE LAYOUTS]

_____
Default

Plate type = 5-tube strip (1 ml)
Plate change message = Change Default

A: - Volume = 80, name = Lysat
- Volume = 5, name = magnetic particules
- Volume = 125, name = binding buffer

B: volume = 250, name = Wash buffer
C: volume = 250, name = Wash buffer
D: Volume = 250, name = Wash Buffer
E: Volume = 50, name = Elution Buffer

[STEPS]

<table>
<tr>
<td><u>STEP 1 : BIND</u>
Step parameters
• Name = Binding
• Well = A, Default
Beginning of step:
• No Action = Yes
Bind parameters:
• Bind time = 10min 0s, speed = Medium
End of step:
• Collect beads = Yes, count = 10</td>
<td><u>STEP 2 : WASH 1</u>
Step parameters
• Name = Wash 1
• Well = B, Default
Beginning of step:
• Release = Yes, time = 10s, speed = Medium
Wash parameters:
• Wash time = 20s, speed = Medium
End of step:
• Collect beads = Yes, count = 10</td>
</tr>
<tr>
<td><u>STEP 3 : WASH 2</u>
Step parameters
• Name = Wash 2
• Well = C, Default
Beginning, Wash and End of step: same as before</td>
<td><u>STEP 4 : WASH 3</u>
Step parameters
• Name = Wash 3
• Well = D, Default
Beginning, Wash and End of step: same as before</td>
</tr>
<tr>
<td colspan="2"><u>STEP 5 : ELUTION</u>
Step parameters
• Name = Elution
• Well = E, Default
Beginning of step:
• Release = Yes, time = 10s, speed = Medium
Elution parameters:
• Elution time = 10min 0s, speed = Slow
Pause parameters:
• Pause for manual handling = No
Remove beads:
• Remove beads = Yes, collect count = 10, disposal well = D</td>
</tr>
</table>

Note: Software version Bindtl 3.3.1 or 3.2. or more recent version

<u>Option 2</u>: <u>by hand extraction with use of a magnetic rack</u>
<u>Respect of time incubation is critical.</u>
For this by hand extraction, requisite magnetic racks as for example “DynaMag™-2 Magnet” adapted for 1.5 - 2 mL microtubes.
<u>Binding</u>

- Supernatants are transferred into 2 mL microtubes containing 125 µL of Binding buffer and 5 µl of beads suspension.
- Tubes are incubated at room temperature during about 10 minutes under gentle shaking.
- Tubes are centrifuged 5 s at 250 g.
- Tubes are put down on the magnetic rack during **at least** 5 min in order to pellet beads along the tube wall.
- Discard the supernatant with a pipette avoiding removing beads

<u>Washing</u>

- Tubes are removed from the magnetic rack.
- 250 µL of washing buffer are added in each tube.
- Tubes are shaken by hand during 1 min.
- Tubes are put down on the magnetic rack during **at least** 5 min in order to pellet beads along the tube wall.
- Discard the supernatant with a pipette avoiding removing beads
- The previous washing steps are repeated twice.

Elution

- Tubes are removed from the magnetic rack.
- 50 µL of Elution buffer are added and tubes are incubated at room temperature during about 10 min under gentle shaking.
- Tubes are centrifuged 5 s at 250 g.
- Tubes are put down on the magnetic rack during at least 5 min in order to pellet beads along the tube wall.
- Eluates are transferred into new tubes for storage.

Option 3: by hand extraction with magnet pipette

For this by hand extraction, requisite magnetic pipette as for example QuicPick™ 1-magnet tool (Bio-Nobile)

During the lysis step pipette QuickPick™ SML Plant DNA reagents into tubes as follows:

- Tube 2: 5µL Plant DNA Magnetic Particles and 125µL Plant DNA Binding Buffer

*Important: never vortex the magnetic particles, but gently suspend and homogenize the particles before pipetting.

- Tube 3: Plant DNA Wash Buffer
- Tube 4: Plant DNA Wash Buffer
- Tube 5: Plant DNA Wash Buffer
- Tube 6: Plant DNA Elution Buffer

Binding

- Gently transfer the supernatant into tube 2.
- Mix tube 2 gently and incubate at room temperature for 10 min. Mix the suspension continuously during this step.

Washing

- Pick up the QuickPick tip with the QuickPick 1. Collect the magnetic particles from tube 2 and release them into tube 3 (washing buffer).
- Wash the magnetic particles by mixing the suspension gently for 20 seconds using the QuicPick tip.
- Repeat the washing steps in tubes 4 and 5 (Wash Buffer).

Elution

- Collect the Magnetic Particles from tube 5 with the QuicPick 1 and release them into tube 6 (Elution Buffer).
- Mix tube 6 continuously and incubate at room temperature for 10 minutes (use a tube rotator or mix manually). During elution Magnetic Particles should disperse.
- Collect the magnetic particles from tube 6 and discard them and the tip. The eluate in tube 6 containing the purified DNA is ready to be used or for storage.

- **Store the obtained DNA extract at 4°C or -20°C**

Appendix 3: CTAB-based extraction (EPPO PM7/24 (4))

Two DNA extraction are performed per macerate.
1.5 g of material is homogenized in 7.5 mL of CTAB buffer; 2 test samples of 1 mL for DNA extraction

1. General information

CTAB buffer (EPPO PM7/24 (4)):

CTAB	2.0 g
TRIS (1 M autoclaved solution pH 8.0)	10 mL
EDTA (0.5 M autoclaved solution pH 8.0)	4.0 mL
NaCl (5 M autoclaved solution)	28 mL
PVP-40	1.0 g
Distilled sterile water to	100 mL

Do not autoclave. It is recommended to keep the buffer for no longer than 1 week

2. Method

To perform the analysis, the following protocol has to be performed :

- Weight 1.5 g of material in a bag (e.g.: Bioreba) with 7.5 mL of CTAB buffer and homogenize using appropriate equipment (pneumatic press, hammer …)
- Perform an ultrasonication of bags (1 min at 35-40 kHz)
- Leave bags to soak for at least 15 min under gentle shaking
- Transfer 2 x 1 mL of extract into 2 x 2 mL micro-centrifuge tube
- Heat samples at 65°C for 30 min
- After centrifugation at 16 000 g for 5 min, 1 mL of the supernatant should be transferred to a 2 mL micro-centrifuge tube and 1 mL of chloroform-isoamyl alcohol (24:1) should be added.
- After mixing by inverting, the tube should be centrifuged at 16 000 g for 10 min
- 700 µL of the supernatant should be transferred to a 1.5 mL tube and 490 µL of cold 2-propanol should be added
- After mixing by inverting twice, the tube should be incubated at -20°C for 20 min
- The centrifugation of the samples at 16 000 g for 20 min will allow a pellet to be recovered; the pellet should then be washed with 1 mL of 70% (v/v) ethanol.
- An additional centrifugation at 16 000 g for 10 min followed by decantation in 70% (v/v) ethanol should be performed.
- The sample should be air or vacuum dried (warning : the evaporation of ethanol is long, 2h at 40°C for example)
- The pellet should be resuspended in 100 µL of TE buffer or RNase- and DNase-free water.
- Store the obtained DNA extract at 4°C or -20°C

Appendix 4: Real-time PCR for the specific detection of *Xylella fastidiosa* (Harper *et al*., 2010, erratum 2013 / EPPO PM7/24 (4))

Two amplifications are performed per DNA extraction.

1. Primers and probe

The target sequence is located at the 16S rRNA processing gene rim.

- **Primer XF-F: 5'-CACGGCTGGTAACGGAAGA-3'**
- **Primer XF-R: 5'-GGGTTGCGTGGTGAAATCAAG-3'**
- **Probe XF-P: 5' -6-FAM -TCGCATCCCGTGGCTCAGTCC-BHQ-1- 3'**

2. Master mix

Simplex real-time PCR Harper *et al.* (2010, erratum 2013)

Reagent	working concentration	Volume per reaction (µL)	**Final concentration**
Molecular grade water	N.A.	6.48	**N.A.**
TaqMan™ Fast Universal PCR Master Mix, No AmpErase™ UNG (Applied Biosystems)	2 x	10.0	**1x**
Forward Primer (XF-F)	10 µM	0.60	**0.30 µM**
Reverse Primer (XF-R)	10 µM	0.60	**0.30 µM**
Probe (XF-P)	10 µM	0.20	**0.10 µM**
Molecular grade BSA (non-acetylated) (Invitrogene)	50 µg/µL	0.12	**0.30 µg/µL**
Subtotal		**18.0**	
DNA		**2.0**	
Total		**20**	

3. Real-time PCR conditions:

	Initial denaturation:	95°C for 30 seconds
-	40 cycles consisting of:	94°C for 10 seconds
		62°C for 40 seconds

4. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid amplification of the target organism and target nucleic acid, respectively

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water or ultra-pure water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

5. Interpretation of results

Verification of the controls

- NAC should give no amplification.
- The PAC amplification curves should be exponential.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve and a Ct-value ≤ 38.00.
- A test will be considered negative if it produces a Ct value > 38.
- A test will be considered negative, if it produces no exponential amplification curve.
- Tests should be repeated if any contradictory or unclear results are obtained.

Appendix 5: Duplex real-time PCR for the specific detection of *Xylella fastidiosa* (Harper *et al.*, 2010, erratum 2013 duplexed with Ioos *et al*., 2009 / EPPO PM7/24 (4))

Two amplifications are performed per DNA extraction.

1. Primers and probes

The test was developed by Harper *et al*., 2010 erratum 2013, and duplexed with primers targeting eukaryote DNA developed by Ioos *et al*., 2009 as internal positive control (PIC).

- **Primer XF-F: 5'-CACGGCTGGTAACGGAAGA-3'**
- **Primer XF-R: 5'-GGGTTGCGTGGTGAAATCAAG-3'**
- **Probe XF-P: 5' -6-FAM -TCGCATCCCGTGGCTCAGTCC-BHQ-1- 3'**

Ioos primers and probe were designed in a conserved region of the 18S rDNA throughout a wide range of eukaryotic organisms

- **Primer 18S Uni-F: 5'-GCAAGGCTGAAACTTAAAGGAA-3'**
- **Primer 18S Uni-R: 5'-CCACCACCCATAGAATCAAGA-3'**
- **Probe 18S Uni-P: 5'-Cy5-ACGGAAGGGCACCACCAGGAGT-BHQ-2-3'**

2. Master mix

Duplex real-time PCR Harper *et al*. (2010, erratum 2013) and Ioos *et al*. (2009) (internal control)

Reagent	working concentration	Volume per reaction (µL)	**Final concentration**
Molecular grade water	N.A.	5.08	**N.A.**
TaqMan™ Fast Universal PCR Master Mix, No AmpErase™ UNG (Applied Biosystems)	2 x	10.0	**1x**
Primer XF-F	10 µM	0.60	**0.30 µM**
Primer XF-R	10 µM	0.60	**0.30 µM**
Probe XF-P	10 µM	0.20	**0.10 µM**
Primer 18S Uni-F	10 µM	0.60	**0.30 µM**
Primer 18S Uni-R	10 µM	0.60	**0.30 µM**
Probe 18S Uni-P	10 µM	0.20	**0.10 µM**
Molecular grade BSA (non-acetylated) (Invitrogene)	50 µg/µL	0.12	**0.30 µg/µL**
Subtotal		**18.0**	
DNA		**2.0**	
Total		**20**	

3. Real-time PCR conditions

- Initial denaturation at: 95°C for 30 seconds
- 40 cycles consisting of: 94°C for 10 seconds
  62°C for 40 seconds

4. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid amplification of the target organism and target nucleic acid, respectively

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

5. Interpretation of results

Verification of the controls

- NAC should give no amplification with FAM fluorophore.
- The PAC should be exponential with FAM fluorophore as well as the PIC (Positive Internal Control) with the Cy5 fluorophore.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve with FAM fluorophore and a Ct-value ≤ 38.00.
- A test will be considered negative if it produces a Ct value > 38 with FAM fluorophore.
- A test will be considered negative, if it produces no exponential amplification curve with FAM fluorophore.
- A test will be considered undetermined if FAM Xf target and Cy5 18S target are both negative.
- Tests should be repeated if any contradictory or unclear results are obtained.