

Information pest: phytoplasmas of the Apple proliferation group

Fruit trees of the family *Rosaceae* may be seriously affected by phytoplasmas of the Apple Proliferation group (AP 16SrX group). The AP group includes:

- '*Candidatus Phytoplasma mali*', which causes apple proliferation (AP) that is the most important graft-transmissible and vector-borne disease of apple in the southern half of Europe;
- '*Ca. P. prunorum*', associated with European stone fruit yellows (ESFY). This phytoplasma causes economically important disorders in apricot, Japanese plum and peach;
- and '*Ca. P. pyri*', associated with pear decline (PD) that affect pear mainly in Europe and North America.

Psyllids are the principal vectors of these three phytoplasmas.

The most typical symptom caused by '*Ca. P. mali*' is witches' broom at the end of shoots. Early leaf reddening is a good indication of the presence of this phytoplasma. The most easily recognized symptoms caused by '*Ca. P. pyri*' occur in late summer with the development of premature autumn leaf colour on affected trees. Typical symptoms of '*Ca. P. prunorum*' are reddening and curling of leaves.

Introduction

The PCR AP Group set has been developed by Qualiplante based on a method published in the Appendix 4 of [PM7/62 \(3\) 'Candidatus Phytoplasma mali', 'Ca. P. pyri' and 'Ca. P. prunorum'](#), European and Mediterranean Plant Protection Organization Bulletin (2020) 50 (1), 69–85.

This Nested End-Point PCR method is based on a first amplification of P1/P7 primers (Deng & Hiruki, 1991; Schneider et al., 1995) that permits to confirm the presence of a phytoplasma amplifying the whole length of 16S and intergenic 16S-23S and a small part of 23S rRNA gene. FO1/rO1 primers (Lorenz et al. 1995) are used for second PCR and are specific for 16SrX group phytoplasmas.

Validation data of the method are available from a test performance study realized in 2011 (Euphresco [FruitPhytoInterlab project](#)), where 20 laboratories analysed a total of 30 samples. The performance characteristics obtained are:

- Analytical sensitivity for '*Ca. P. mali*': 10¹ - 10³
- Analytical sensitivity for '*Ca. P. pyri*': 10¹
- Diagnostic sensitivity: 99.3%
- Diagnostic specificity: 97.7%
- Repeatability: no data available
- Reproducibility: Kappa coefficient 0.94

This product should be used only for research purposes.

Intended use

The PCR set is validated for the detection of AP Group, without any distinction, in Nested End-Point PCR.

Suitable tissues are symptomatic leaf mid-vein tissue and/or vascular tissue (phloem) from bark or roots. Analysis on asymptomatic plants is possible but not recommended due to limited experience in EU.

Set format and content

Two sets are available for 24 and 96 tests.

Article N°	Product name
7AP---P2	PCR AP Group set 24
7AP---P9	PCR AP Group set 96

Content	set 24	set 96
Direct Master Mix	24 tests 7AP---P2-DM-	2x48 tests 7AP---P9-DM-
Nested Master Mix	24 tests 7AP---P2-NM-	2x48 tests 7AP---P9-NM-
Positive Control	3 tests 7AP---P2-PC-	8 tests 7AP---P9-PC-
Negative Control	3 tests 7AP---P2-NC-	8 tests 7AP---P9-NC-

Storage conditions

This set can be shipped at room temperature but upon receipt it should be stored immediately at the recommended storage temperature: **from -30 ° C to -10 ° C**.

Avoid prolonged exposure to light and repeated freeze and thaw cycles.

Shelf life

If the set is correctly stored, at constant-temperature freezer, its performance is guaranteed until the expiration date indicated on the tubes label.

Materials and equipment (not provided)

- DNA extraction tools and reagents
- Nuclease-free filter tips and micropipettes
- Optical grade nuclease-free tubes/plate
- Disposable latex or vinyl gloves
- DNA ladder and loading-dye buffer
- PCR thermal cycler
- Agarose gel reagents and apparatus

Nucleic acids extraction

Extract DNA from samples according to your usual protocol. Upon request, Qualiplante can recommend you an extraction method.

FIRST PCR

Reaction set-up

- Slowly thaw **Direct Master Mix** by placing it on ice or at 4°C.
- Shake briefly **Direct Master Mix** and spin down the liquid.
- Add 23 µl of **Direct Master Mix** (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
- Add 2 µl of DNA template to the **Direct Master Mix**. Do not forget to prepare a PCR tube or well of a PCR plate for the **Positive Control** and the **Negative Control**.

Components	Volume/PCR tube or well
DNA template or Positive control or Negative control	2 µl
Direct Master Mix	23 µl
Total Volume / PCR tube or well	25 µl

In order to confirm the absence of any reagent's contamination, we strongly recommend including a no-template control (e.g. DEPC water) in the assay.

Run and thermal cycling

- Seal carefully the PCR tubes or PCR plate. Centrifuge briefly to collect components at the bottom of the PCR tubes or wells of the plate. Protect from light before thermocycling.
- Load the PCR tubes or plate into the PCR thermal cycler and follow the thermal cycling below:

Steps	Temp (°C)	Time	Cycle(s)
Initial denaturation	95°C	2 min	1
Denaturation	95°C	1 min	35
Annealing	50°C	1 min	
Elongation	72°C	3 min	
Final elongation	72°C	10 min	1
Storage	4°C	∞	-

Dilution of 1st PCR products

Using DNase and RNase free PCR tubes, **dilute to 1:30** in sterile water each PCR product for samples, **Positive Control** and **Negative Control** obtained during the previous step.

Shake and spin down each tube.

SECOND PCR

Reaction set-up

- Slowly thaw **Nested Master Mix** by placing it on ice or at 4°C.
- Shake briefly **Nested Master Mix** and spin down the liquid.
- Add 23 µl of **Nested Master Mix** (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
- Add 2 µl of **diluted 1st PCR products** to the **Nested Master Mix**. Do not forget to prepare a PCR tube or well of a PCR plate for the 1st PCR products obtained with **Positive Control** and **Negative Control**.

Components	Volume/PCR tube or well
PCR products from the 1 st PCR diluted to 1:30 (*)	2 µl
Nested Master Mix	23 µl
Total Volume / PCR tube or well	25 µl

(*) See the section "Dilution of 1st PCR products"

Run and thermal cycling

- Seal carefully the PCR tubes or PCR plate. Centrifuge briefly to collect components at the bottom of the PCR tubes or wells of the plate. Protect from light before thermocycling.
- Load the PCR tubes or plate into the PCR thermal cycler and follow the thermal cycling below:

Steps	Temp (°C)	Time	Cycle(s)
Initial denaturation	95°C	2 min	1
Denaturation	95°C	1 min	38
Annealing	50°C	1 min	
Elongation	72°C	2 min	
Final elongation	72°C	8 min	1
Storage	4°C	∞	-

Agarose gel electrophoresis

Prepare an agarose gel at **1% w/v** in **1X-TAE buffer**.

Gel loading:

- load the DNA ladder (for example 2'000 bp DNA step ladder).
- load 10 µl of PCR products from the previous step adding the loading dye buffer (*not provided in the set*).

Run: run the gel electrophoresis for 40-45 minutes at 80V.

Results analysis

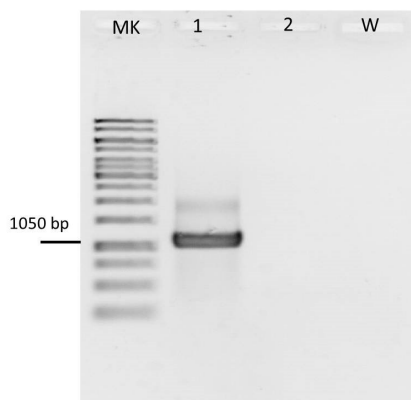
ANALYSIS VALIDATION

The specific product of an AP Group phytoplasma is a **1'050 bp fragment**.

The analysis is validated when:

- ✓ 1 DNA fragment of 1'050 bp is visible in the positive control lane.
- ✓ none DNA fragment are visible in the negative control lane.

The picture below represents a 1X-TAE 1% agarose gel showing the DNA amplification of '*Ca. P. mali*' in an apple leave.



MK: DNA ladder - **1:** AP Group infected sample or **Positive Control** of the set - **2:** Healthy sample or **Negative Control** - **W:** No template control.

RESULTS INTERPRETATION

- ✓ A sample is **positive** when a 1'050 bp DNA specific fragment is present in the PCR reaction; in this case, the sample is infected by a phytoplasma including in the AP Group phytoplasma.
- ✓ A sample is **negative** when no fragment is present in the PCR reaction.

The table below summarizes the results interpretation:

Fragment size 1'050 bp	Interpretation
-	NEGATIVE
✓	POSITIVE AP Group

POSITIVE: infected sample – **NEGATIVE:** healthy sample

Characteriation of different strains

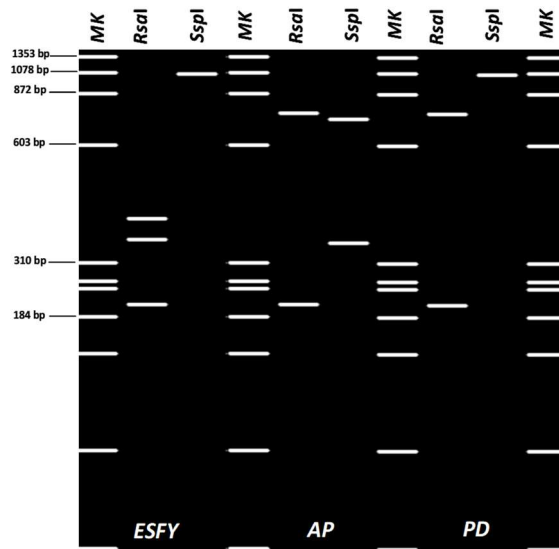
It is possible to distinguish the different phytoplasma strains of Apple Proliferation Group:

- '*Ca. P. mali*',
- '*Ca. P. prunorum*',
- '*Ca. P. pyri*',

by using an enzymatic digestion of the 1'050 bp DNA fragment obtained with the PCR set (necessary enzymes not provided in the set).

- The *RsaI* enzyme permits to distinguish ESFY from AP and PD.
- The *SspI* enzyme permits to distinguish AP from ESFY and PD.

The picture below represents a virtual digestion performed with *RsaI* and *SspI* enzymes on a DNA fragment obtained with the PCR AP set.



Special handling instructions

This set was designed to be used by laboratory staff trained to follow the usual molecular biology precautions. Always perform the tests in a nuclease-free work environment. Always wear gloves when handling samples containing DNA and the components of the set. Do not touch any set components with an ungloved hand. Use appropriate laboratory disposable parts. Use nuclease-free tubes and filter tips to avoid degradation and cross-contamination. Do not use components from sets with different batch numbers in the same test procedure. Do not interchange reagents with other sets. To avoid cross-contamination, use separate rooms for (a) nucleic acids extraction, (b) preparation of the Master Mix and (c) amplification. To avoid cross-contamination and obtain reliable results, it is essential to strictly follow the protocol in this manual. Avoid unnecessary freeze-thaw cycles of the set components. Do not use reagents after their expiration date.

Troubleshooting

Post-PCR data analysis shows no amplification, or amplification plots look grossly abnormal:

Possible causes	Corrective actions
Evaporation of the sample due to inadequate sealing of the plate	Repeat the test using the appropriate tools to seal correctly the plate
Consumables are not appropriate for the method	Repeat the test using consumables recommended by the thermal cycler supplier
The quality of nucleic acid extracted is low	Repeat the extraction step. Ensure that the method of extraction has been performed correctly. In any doubt, please, contact us
Abnormal amplification	Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles

No amplification reaction is observed in the positive control well, while other samples are positive:

Possible causes	Corrective actions
The positive control provided with the set was not added into the reaction well	Repeat the test. If the problem persists, please, contact us

An amplification plot is observed in the negative control well:

Possible causes	Corrective actions
Contamination of the negative control or the Master Mix with target-positive nucleic acid	Repeat the test by applying appropriate quality procedures to prevent contamination. Seal the plate correctly

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