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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 kit 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*': 10^1 - 10^3
- Analytical sensitivity for '*Ca. P. pyri*': 10^1
- 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 kit 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.

Kit format and content

Article N°	Product name
nPCR AP 96	Nested PCR Apple Proliferation Group 96 tests

Content	96 tests
Direct Master Mix	2x48 tests
Nested Master Mix	2x48 tests
Positive Control	8 tests
Negative Control	8 tests

Storage conditions

This kit 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 kit 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.

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FIRST PCR

Reaction set-up

a) Slowly thaw Direct Master Mix by placing it on ice or at 4°C.
b) Shake briefly Direct Master Mix and spin down the liquid.
c) Add 23 µl of Direct Master Mix (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) 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

a) 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.
b) 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

a) Slowly thaw Nested Master Mix by placing it on ice or at 4°C.
b) Shake briefly Nested Master Mix and spin down the liquid.
c) Add 23 µl of Nested Master Mix (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) 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 1st 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

a) 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.
b) 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 kit).*

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

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Results analysis

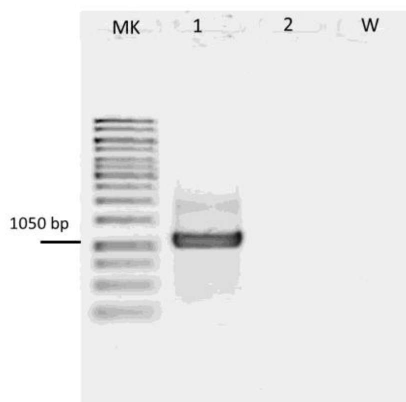
ANALYSIS VALIDATION

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 kit - **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

Characterization of different strains

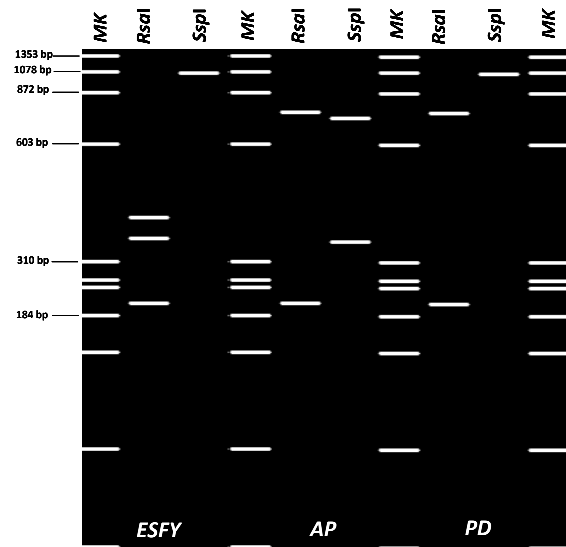
It is possible to distinguish the different phytoplasma strains of Apple Proliferation Group:

- '*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 kit (necessary enzymes not provided in the kit).

- 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 kit.



Special handling instructions

This kit 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 kit. Do not touch any kit 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 kits with different batch numbers in the same test procedure. Do not interchange reagents with other kits. 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 kit components. Do not use reagents after their expiration date.

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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 kit 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

Warranty and Responsibilities

Qualiplante SAS guarantees the buyer exclusively concerning the quality of reagents and of the components used to produce the Kits. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits Qualiplante SAS responsibility to the replacement of the product. No other warranties, of any kind, express or implied-are provided by Qualiplante SAS.
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