BIOREBA

A CALIBRE SCIENTIFIC COMPANY

qPCR ARS/PHY/*VER* set

Candidatus Arsenophonus phytopathogenicus (ARS)
Candidatus Phytoplasma solani (PHY)
Verticillium spp. (VER)

User Guide



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1. Introduction

1.1 General

The qPCR ARS/PHY/VER set enables the simultaneous detection (multiplex) of *Candidatus* Phytoplasma solani, *Candidatus* Arsenophonus phytopathogenicus, *Verticillium* spp. and a plant-specific host gene control (IC) with a quadruplex real-time PCR. Bacterial DNA, extracted from a wide range of host matrices (such as potato tubers and leaves, celery roots and beet tuber) is amplified in a qPCR reaction. The amplification of the DNA can be monitored in real time because the specific probe is labeled with a fluorophore (*Ca.* P. solani: FAM; *Ca.* A. phytopathogenicus: HEX; *Verticillium* spp.: Cy5). An internal control (IC) is included to detect a fragment in the cytochrome c oxidase (COX) host gene sequence with a fluorophore-labeled (IC:ROX) specific probe. The control shows whether the extraction of host tissue and the amplification of the DNA worked as intended.

The qPCR set includes a lyophilized qPCR ARS/PHY/VER Lyo Master Mix 2x which comprises the DNA polymerase as well as the primers and probes.

The qPCR ARS/PHY/VER set for the detection of *Ca.* P. solani, *Ca.* A. phytopathogenicus, *Verticillium* spp. was developed and validated by BIOREBA. The method is based on published primers for the detection of *Ca.* P. solani, *Ca.* A. phytopathogenicus, while for the detection of *Verticillium* spp., primers developed by Freihart et al., 2025 (1) were used.

BIOREBA recommends homogenizing samples in extraction bags "Universal" (Art. No. 430100) using the BIOREBA homogenizer system HOMEX 7 (Art. No. 400007S). For the extraction of DNA, it is recommended to use standard DNA extraction protocols established for plant material. Upon request, BIOREBA can recommend an optimal extraction method.

1.2 Stolbur, Syndrome des Basses Richesses (SBR) and *Verticillium* spp.

The combined molecular detection of *Ca.* P. solani (causative agent of Stolbur), *Ca.* A. phytopathogenicus (causative agent of Syndrome des Basses Richesses, SBR), and *Verticillium* spp. is essential for diagnosing decline symptoms in potato tubers and other susceptible crops, where overlapping symptomatology and frequent co-occurrence complicate field level assessment.

Ca. P. solani is a phloem restricted phytoplasma associated with tuber necrosis, growth suppression, and reduced yield in solanaceous hosts. Its epidemiology is tightly linked to insect vectors enabling rapid spread across agroecosystems (2). Because phytoplasmas cannot be cultured, reliable detection depends on PCR based assays targeting conserved genomic regions as shown in Lee et al., 2000 (3) and Hren et al. 2007 (4).

Ca. A. phytopathogenicus, the causal agent of SBR in sugar beet, has gained attention due to its ability to colonize phloem tissues and induce severe reductions in sucrose accumulation. Although historically associated with beet, *Arsenophonus* species have been documented in mixed infections in diverse crops, where they may intensify physiological stress or interact with other phloem colonizing pathogens (5). Their presence in tuber-producing systems, particularly potato, is increasingly recognized: recent field surveys have documented widespread prevalence of *Ca.* A. phytopathogenicus in potato tubers, transmitted by the same planthopper vector (*Pentastiridius leporinus*) responsible for spread in sugar beet, with occurrence closely tied to regions of high vector pressure and to crop rotations that place potato near sugar beet fields (6).

Verticillium spp., especially *Verticillium dahliae* and *Verticillium albo-atrum*, are soil borne vascular wilt pathogens capable of causing chronic decline, wilt, vascular browning, and tuber discoloration in potatoes. Verticillium wilt is often exacerbated under environmental stress or in combination with other pathogens, and its persistence in soil for many years makes it a major threat in potato growing regions as shown in Klosterman et al., 2009 (7). Mixed infections involving *Verticillium* spp. and phloem limited pathogens have been shown to intensify disease severity due to simultaneous impairment of xylem and phloem transport.

Because these organisms affect overlapping tissues, share ecological niches, and may co-infect plants under field conditions, a unified qPCR panel provides a more comprehensive diagnostic framework than single pathogen assays. It improves differential diagnosis, supports early detection, and enables more accurate interpretation of complex symptom profiles in potatoes and other crops.

1.3 Special handling instructions

Perform the tests in an RNase-free work environment. Always wear gloves when handling samples containing DNA/RNA, and the components of the set. Do not touch any set components with an ungloved hand. Keep all components tightly sealed when not in use. 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 lot numbers in the same test procedure. 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.

1.4 Warranty and liability

BIOREBA products are guaranteed to meet the specifications described on the product certificate of analysis and in the user guide, which is included with every shipment. No further warranties are given. If you have any questions about specifications or performance, please contact our administrative office (admin@bioreba.ch).

Our products are for laboratory use only and are not intended for human or animal applications. Should a product fail for reasons other than inappropriate handling or misuse, BIOREBA will replace the product free of charge or refund the purchase price.

BIOREBA shall not be liable for any direct or indirect, special or consequential damage of any kind resulting from the use of our products.

2. Intended use

The qPCR ARS/PHY/VER set is validated for the simultaneous detection (multiplex) of *Ca.* P. solani, *Ca.* A. phytopathogenicus, *Verticillium* spp. and a plant-specific host gene control (COX) which serves as internal control (IC) by real-time PCR.

Suitable tissues are diverse host plants including but not limited to the following plant matrices: potato stolons, leaf and petioles, celery root, grapevine leaf midribs, petioles and roots, sugar beet taproot flesh, petiole bases or leaves, vascular tissue near crown. Symptomatic tissue (showing wilting, yellowing, discoloration, or necrosis) generally yields the highest pathogen DNA concentrations.

3. Format, content, storage conditions and quality

3.1 Set format and contents

		Components		
Sales Part No.	**Product name**	**Colour of screw cap**	**Name**	**Volume**
819601	qPCR ARS/PHY/VER set 96	Green	ARS/PHY/VER Lyo qPCR Master Mix (2x) Art. No. 810101	2x 525 µl
		Black	Reconstitution Buffer for Lyo qPCR Art. No. 831413	1.05 ml
		Red	ARS/PHY/VER qPCR positive control (PC) Art. No. 810054	30 µl
		White	qPCR negative control (NC) Art. No. 830044	30 µl
			Nuclease-free water Art. No. T143.4	1 ml

3.2 Storage conditions

Store all qPCR components (Lyo qPCR Master Mix, positive and negative controls, nuclease-free water) at -20 °C.

3.3 Lot-to-Lot consistency

Quality control of the qPCR set is performed based on predetermined specifications to ensure consistent product quality. See lot-dependent certificate of analysis included with the shipment.

4. Specificity and Sensitivity information

4.1 Specificity

The primer pairs used in this product specifically amplify a 127 basepair (bp) fragment of the genome of *Ca.* P. solani, a 77 bp fragment of the genome of *Ca.* A. phytopathogenicus and a 128-130 bp fragment of the genome of *Verticillium* species. The exact size of the amplified fragment of the latter depends on the respective species.

The qPCR ARS/PHY/VER set is suitable for pathogen detection in potato, sugar beet, celery, grapevine, and other host plants. This broad host range reflects the overlapping epidemiology of the three target pathogens: *Ca.* P. solani and *Ca.* A. phytopathogenicus share *P. leporinus* as a common insect vector and frequently co-occur in potato and sugar beet, while *Ca. P. solani* additionally causes "Bois Noir" in grapevine and Stolbur-related disease in celery and other solanaceous and umbelliferous crops. *Verticillium* spp. independently affects vascular tissue across most of these same hosts, producing overlapping wilt and decline symptomatology that this multiplex assay is designed to differentiate.

16SrXXII-A subgroup *Ca.* P. solani strains were successfully amplified with the qPCR ARS/PHY/VER set. Based on *in silico* analyses, the set recognizes strains from the 16SrXXII-P subgroup. No cross-reactions were observed with the tested strains.

Following *Verticillium* species were amplified using the qPCR ARS/PHY/VER set: *V. dahlia*, *V. longisporum*, *V. tricorpus*, *V. nubilieum*, *V. nonalfaalfa* and *V. albo-atrum*. No cross-reactions were observed.

No negative effect was observed from host matrix plant species (potato tubers and leaves, etc.).

4.2 Sensitivity

The sensitivity of the assay in the host matrix was assessed in potato tubers: one tuber infected with either *Ca.* A. phytopathogenicus, *Ca.* P. solani or *Verticillium* spp. could be detected in a pool of 200 healthy tubers (1:200).

The analytical sensitivity of the assay was determined using a dilution series of the plasmid positive control (PC) with a known copy number per reaction. Because Candidatus species cannot be cultivated, the plasmid PC served as the quantification standard. Copy numbers were converted to genome equivalents using the target-specific gene copy number per genome, which was set individually for each target of this multiplex assay (*Ca.* A. phytopathogenicus, *Ca.* P solani, and *Verticillium* spp.). Full validation data are available online.

5. Materials and equipment required (not provided)

- Nuclease-free filter tips and micropipettes
- Optical grade nuclease-free tubes/plate
- Disposable latex or vinyl gloves
- Thermal cycler for real-time PCR

6. Protocol

Please pay attention to the following points:

- The protocol in this manual must be followed.
- Create an nuclease-free environment by cleaning the bench with 1 % bleach followed by 70 % ethanol.
- Gloves must be worn at all times.
- Use nuclease-free tubes and filter tips.
- Use appropriate eye protection and wear protective clothing.
- To avoid cross-contamination, use separate rooms for
  a) nucleic acid extraction,
  b) preparation of the Master Mix and
  c) qPCR amplification.
- Avoid unnecessary freeze-thaw cycles of the qPCR components.

6.1 DNA extraction

For the extraction of DNA it is recommended to use standard DNA extraction protocols established for plant material. Upon request, BIOREBA can recommend an optimal extraction method.

6.2 Preparation of the qPCR reactions

1. Slowly thaw the set components on ice or at 4 °C. Thereafter, the components should always be kept on ice.

2. Add 525 µl Reconstitution Buffer for Lyo qPCR to the tube with the ARS/PHY/VER Lyo qPCR Master Mix 2x. Resuspend the lyophilized Master Mix by pipetting up and down until the solution is clear.

Only resuspend as many tubes lyophilized Master Mix as required at the time since the Master Mix is most stable in its lyophilized form*.

3. Shake the tubes briefly, and spin down the liquid.

4. To prepare the reaction mix, first determine the number of reactions and then increase the number by 1 or 2 to have enough volume for all reactions.

5. Prepare the reaction mix (without DNA template) by combining the components of the set to reach a final volume of 20 µl per reaction (see Table 1).

Component	Volume
ARS/PHY/VER Lyo qPCR Master Mix 2x	10 µl
DNA Template / PC / NC	2 – 5 µl
Nuclease-free water	to reach a final reaction volume of 20 µl

Table 1: Preparation of reaction mix

6. Add the reaction mix (without DNA template) to each PCR tube or well of an optical-grade PCR plate.

7. Add 2-5 µl DNA template to the reaction mix.

8. Seal the PCR tubes or PCR plates, centrifuge briefly to collect components at the bottom of the PCR tubes or wells. Protect from light before thermocycling.

*Avoid repeated freeze-thaw cycle of the resuspended Lyo qPCR Master Mix. Unused portion can be divided into aliquots and frozen at -20° C for later use.

6.3 Thermal cycling

Place the PCR tubes or PCR plate in a thermocycler. Start cycling according to the program below (Table 2).

Step	Cycles	Temperature	Time
Hot Start	1	95 °C	5 min
Denaturation Annealing/Extension	40	95 °C 60 °C	5 sec 1 min

Table 2: PCR cycling conditions

6.4 Monitoring the PCR amplification

To monitor PCR amplification in real-time, an appropriate thermocycler is required, which can measure the fluorescence of the following fluorophores:

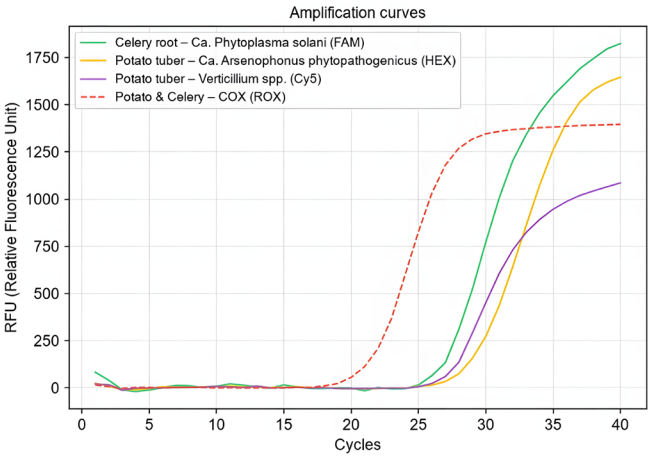
Dye	Pathogen/Control	Max. Ex (nm)	Max. Em (nm)
HEX	*Ca.* A. phytopathogenicus	535	556
FAM	*Ca.* P. solani	495	520
Cy5	*Verticillium* spp.	649	670
ROX	COX (IC)	576	601

Table 3: Fluorophores overview

Please refer to the manufacturer's manual for information on programming the thermocycler, monitoring and evaluation.

6.5 Amplification of typical samples

The graph below shows the amplification curves for FAM (PHY) in a celery root sample infected with *Ca.* P. solani, for HEX (ARS) in a potato tuber sample infected with *Ca.* A. phytopathogenicus and for Cy5 (VER) in a potato tuber sample infected with *Verticillium* spp. For illustration, DNA extracts were pooled before amplification.



Evaluation criteria

In order to distinguish positive from negative samples we recommend taking the following criteria into account:

A) The Ct value
B) The PCR efficiency
C) The delta RFU
(the difference between baseline and final RFU)

The range of values for each of the above can be determined for every channel by means of a dilution series of a known sample.

7. References

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