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BIOREBA

qPCR *Ralstonia solanacearum* (Rs) set

User Guide

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1. Introduction

1.1 General

This qPCR set enables the simultaneous detection (multiplex) of *Ralstonia solanacearum*, *R. pseudosolanacearum* or *R. syzygii*, and a plant-specific host gene control (IC) with real-time PCR. Bacterial DNA, extracted from various host tissues 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 (Rs: HEX). An internal control (IC) is included for the convenience of the operator. The IC is designed to detect a fragment in the 18S rRNA (18S) 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 Rs set for the detection of *R. solanacearum* species was developed and validated by BIOREBA. The method is based on the primers published by Körner et al., 2017 (1) for the detection of the *R. solanacearum* species complex (RSSC). All species from the RSCC tested so far can be detected, including *R. solanacearum*, *R. pseudosolanacearum* and all three subspecies of *R. syzygii* (*R. syzygii* subsp. *syzygii*, *R. syzygii* subsp. *indonesiensis*, and *R. syzygii* subsp. *celebesensis*).

BIOREBA recommends to homogenize samples using the BIOREBA homogenizer system HOMEX 7 (Art. No. 400007S) and BIOREBA extraction bags "Universal" (Art. No. 430100). 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 Information: *Ralstonia solanacearum*

R. solanacearum (2, 3) is a soil-born bacterial pathogen and a well-known agricultural threat to many plant species worldwide. The bacterium has a broad host range and infects over 250 plant species from more than 50 families, including solanaceous vegetable crops such as tobacco, tomato and potato, but also banana, ginger, mulberry, and ornamental plants like *Pelargonium* species and roses (4).

R. solanacearum typically infects plants through wounds, root tips, or cracks in the lateral roots (5). However, infection can also occur through stem injuries caused by agricultural practices or insect damage, as well as through irrigation water. *R. solanacearum* subsequently colonizes the root xylem, thereby causing a blockage of the vascular system and the water supply of below-ground plant tissue. This typically leads to wilting, yellowing of younger leaves, yield reduction, and ultimately the death of the infected plant (4, 6). A common sign of heavy *R. solanacearum* infection is a milky white bacterial ooze that accumulates at the surface of freshly cut stems, rhizomes, and tubers, which is due to the high R. solanacearum cell densities in the infected tissue (4).

The highly variable *R. solanacearum* strains are grouped within the *R. solanacearum* species complex (RSSC) and were historically classified as races or biovars. However, nowadays, the RSSC species classification is based on four phylotypes (monophyletic cluster of strains revealed by phylogenetic analysis of sequence data) and includes three species: *R. solanacearum* (Phylotpye IIA and IIB), *R. pseudosolanacearum* (Phylotpye I and III), and R. syzygii (Phylotype IV) (7, 8). *R. syzygii* comprises three subspecies: subsp. *syzygii* that causes the Sumatra disease in cloves, subsp. *indonesiensis* with a broad host range, and subsp. *celebesensis*, the causal agent of the blood disease of banana (9).

Due to its lethality, persistence, wide host range, and very broad geographical distribution, *R. solanacearum* is one of the most destructive bacterial plant pathogens worldwide (5). Therefore, bacterial strains of the RSSC are considered priority plant pathogens in many countries around the world and are classified as quarantine organisms in Europe and the USA.

Ralstonia in potatoes

R. solanacearum Phylotype IIB sequevar 1 (IIB-1), formerly referred to as race 3 biovar 2, is the causal agent of potato brown rot, also known as bacterial wilt (6). Brown rot is one of the most destructive diseases of potato and can cause up to 80% yield loss. The first visible symptoms of *R. solanacearum* infection are wilting of single stems, stunting may also occur, as well as dark brown streaks on stem segments above the soil line (4). Eventually, plants fail to recover and become yellow and then necrotic. Infected tubers show brown discoloration and necrosis of the vascular tissue. Further, bacterial ooze may emerge from the eyes and the stolon end of infected tubers, and when it dries, soil can adhere to the tuber, leading to a smutty appearance (4, 6).

Ralstonia in tomatoes

The first symptoms of *R. solanacearum*-induced bacterial wilt in tomato are typically the wilting of the youngest leaves at the ends of branches during the hottest time of the day. As the disease develops, the whole plant shows rapid wilting, whereas the dried leaves stay green (6). Tomato plants are typically infected by *R. solanacearum* (Phylotype IIB-1), but infections by *R. pseudosolanacearum* (Phylotype I) have also been reported in Cambodia in 2019 (10) .

Ralstonia in ginger

Ginger is one of the most important spice crops cultivated in India and several other countries, such as China, Nepal, and Nigeria. *R. pseudosolanacearum* (Phylotype I) causes bacte-

rial wilt of ginger, also referred to as ginger blast or Mahali/green wilt, since the typical symptom is downward drooping of the leaves that at first remain green (11). Affected rhizomes are dark and emit a foul smell. *R. pseudosolanacearum*-infected ginger rhizome pieces that are used as propagative material form the primary source of infection (11). In summer 2023, several outbreaks of *R. pseudosolanacearum* on ginger have been reported in Germany and Switzerland.

Ralstonia in bananas

Bacterial wilt on bananas caused by RSSC species was described more than 150 years ago, and since then Ralstonia infections have been reported in Central and South America, in Southeast Africa, and Southeast Asia (12). Diverse symptoms and banana diseases are caused by different RSSC species. *R. solanacearum* (Phylotype II) infections lead to Bugtok disease in cooking bananas (ABB/BBB type) in the Philippines, whereas in Cavendish (AAA) bananas it leads to the Moko disease. Typical Moko wilt symptoms are rapid yellowing and wilting of leaves, premature fruit ripening, and internal fruit blackening. The banana blood disease (BDB), caused by *R. syzygii* subsp. *celebesensis* (Phylotype IV), mainly occurs in Indonesia, Malaysia and Papua New Guinea, where average yield losses often exceed 35%. BDB leads to yellowing and wilting of leaves, reddish dry rot of the fruit pulp, and a red-brown discoloration of vascular tissue (12).

Ralstonia in roses

In roses, bacterial wilt caused by *R. pseudosolanacearum* (Phylotype I) was first reported in 2016 in the Netherlands (13), and was also found in various rose cultivars in Korea in 2019 (14). The infection route of *R. pseudolanacearum* in rose cultivation has not yet been fully clarified (15). Rose cultivars differ significantly in susceptibility to *R. pseudosolanacearum* (16), and an infection can cause wilting of shoots and flower stalks as well as chlorosis of leaves and branches (15).

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/kit components with an ungloved hand. Keep all components tightly sealed when not in use. Use appropriate laboratory disposable parts. In particular, 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. In order 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 AG will replace the product free of charge or refund the purchase price.

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

2. Intended use

The qPCR Rs set is validated for the simultaneous detection (multiplex) of strains from the *Ralstonia solanacearum* species complex and the host 18S rRNA sequence which serves as internal control (IC) by one-step real-time PCR. Suitable tissues are diverse *R. solanacearum* host plants indluding but not limited to the following plant matrices: potato tubers and leaves, tomato seeds and leaves, pepper seeds and leaves, chilli seeds, banana leaves, rose leaves, watermelon leaves, cucumber leaves, egg plant leaves, ginger rhizomes, tobacco leaves.

3. Format, content, storage conditions and quality

3.1 Set format and contents

Sales Part No.	Product name	Colour of screw cap	Name	Volume
869600	qPCR Rs set 96	Green	Taq Master Mix (2x) Art. No. 830412	1.2 ml
		Clear	Primers/Probes Mix Rs/18S (10x) Art. No. 860100	0.2 ml
		–	Nuclease-free water Art. No. T143.4	1 ml
		–	Rs DNA positive control (PC) Art. No. 860053	30 µl
		–	Plant DNA negative control (NC) Art. No. 870043	30 µl
869200	qPCR Rs set 192	Green	Taq Master Mix (2x) Art. No. 830412	2 x 1.2 ml
		Clear	Primers/Probes Mix Rs/18S (10x) Art. No. 860100	2 x 0.2 ml
		–	Nuclease-free water Art. No. T143.4	2 x 1 ml
		–	Rs DNA positive control (PC) Art. No. 860053	30 µl
		–	Plant DNA negative control (NC) Art. No. 870043	30 µl

3.2 Storage conditions

Store all qPCR components (Taq Master Mix, Primers/Probes Mix Rs/18S, Nuclease-free Water) at -30 °C to -10 °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 pair used in this product specifically amplify a approx. 92 basepair (bp) fragment of the genome of RSSC species. The exact size of the amplified fragment depends on the respective species.

The qPCR Rs set is suitable for detecting RSSC strains from potatoes, ginger, bananas, tomatos, roses and other host plants. All RSSC strains tested so far can be detected (strains from locations in Switzerland, Germany, Netherlands and Indonesia).

No cross reactivity was observed with the following bacteria: *Clavibacter michiganensis* subsp. *sepedonicus*, *Pectobacterium wasabiae*, *P. atrosepticum*, *P. carotovorum* subsp. *brasiliense*, *P. carotovorum* subsp. *carotovorum*, *Dickeya solani*, *Candidatus Arsenophonus phytopathogenicus*, *Candidatus phytoplasma solani*, and *Pseudomonas syringae* subsp.. No negative effect was observed from host matrix plant species (potato, tomato, ginger, banana, rose etc.).

4.2 Sensitivity

The sensitivity of this product is high and was tested with a bacterial suspension of *R. pseudosolanacearum*. The limit of detection ranged from 10^3 to 10^4 CFU/ml of bacteria.

The primers in this kit were developed and optimized for the detection of RSSC strains in seed potato certification using 300 potato tubers per sample:

- Mixed sample (1 infected and 299 healthy): Ct 20.59

Full validation data is 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) 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 samples

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

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

3. 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.

4. 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
Taq Master Mix (2x) Green	10 µl
Primers/Probes Mix Rs/18S (10x) Clear	2 µl
RNA Template	2 – 5 µl
Nuclease-free water	to reach a final reaction volume of 20 µl

Table 1: Preparation of reaction mix

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

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

7. 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.

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	45	95 °C 60 °C	10sec 40 sec

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:

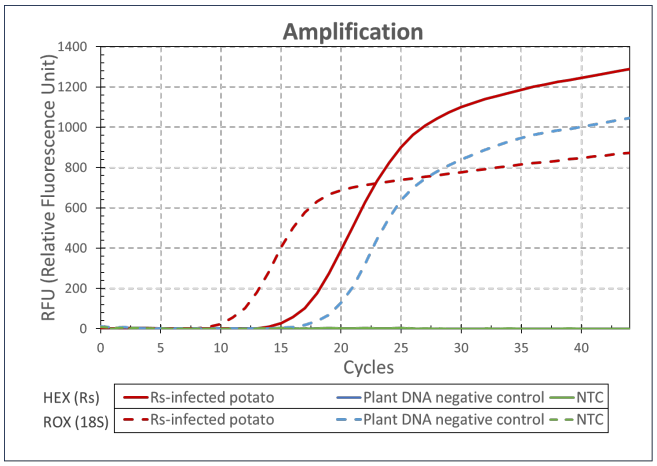
Dye	Pathogen/Control	Max. EX (nm)	Max. EM (nm)
HEX	Rs	535	556
ROX	18S (IC)	576	601

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 curve for HEX (Rs) of a *R. solanacearum*-infected potato sample. No amplification was detected in the Plant DNA negative control and the "no template control" (NTC). Amplification for ROX (18S) could be detected in the Rs-infected potato and in the Plant DNA negative control, but not in the NTC.



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.

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