Final Report

Project title (Acronym)
Validation of molecular tests for the detection of tomato brown rugose fruit virus (ToBRFV) in seed of tomato and pepper

Project duration:

Start date:	01-11-2019
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Contents

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2. Short project report

2.1. Short executive summary

Tomato brown rugose fruit virus (ToBRFV) is a relatively recently described tobamovirus (Salem *et al*., 2016) causing problems in tomato and pepper cultivation worldwide (Luria *et al.,* 2017, Salem *et al.,* 2019). ToBRFV has been reported to cause yellow spots, green spots, necrotic lesions and occasional rugose symptoms on tomato fruits, chlorosis and mosaic symptoms on leaves and occasional leaf narrowing (Cambrón-Crisantos *et al*., 2018; Luria *et al*., 2017; Menzel *et al*., 2019). For pepper, stunting of young plants, puckering and yellow mottling of leaves, and misshapen fruits have been reported (Salem *et al.,* 2019). These symptoms render affected fruits non-marketable.

Tobamoviruses are very stable and will remain infectious for long periods of time when present in crop debris, soil and on surfaces. Infectivity on seeds is preserved for up to several years (Dombrovsky and Smith, 2017). ToBRFV is easily transmitted mechanically (via contaminated tools, hands, clothing, direct plant-to-plant contact, and bumble bees) and seed-to-seedling transmission is expected to play a role in the spread of the virus (Levitzky *et al*., 2019, Salem *et al.,* 2021).

As of November 1, 2019, emergency measures (EU) 2019/1615 are in place in the EU to prevent introduction and further spread of ToBRFV. The initial measures have been replaced by Commission Implementing Regulation (EU) 2021/1809. These measures include an obligatory annual survey, and specific requirements for movement and introduction of plants for planting, including seeds, of *Solanum lycopersicum* and its hybrids and *Capsicum* spp. In addition, there is a requirement for testing of at least 20% of the consignments of seeds and plants for planting of *Solanum lycopersicum* and *Capsicum* spp. upon entry into the EU. For consignments from Israel, the required testing rate is even 50% and for seeds from China 100%. Therefore, reliable and harmonised protocols for the detection of ToBRFV in tomato and pepper plants and seeds are needed. In the framework of the EU H2020 research project VALITEST (www.valitest.eu) a test performance study (TPS) has been conducted on leaf and fruit material of tomato and pepper. In addition, however, there is a need for reliable and harmonised test protocols for the detection of ToBRFV in tomato and pepper seeds.

A TPS was organised in the framework of this Euphresco project to compare the suitability of different available methods and tests for the detection of ToBRFV in tomato and pepper seeds. Since the number of tests that can be included in a TPS is limited, comparative experiments were conducted in order to select the most suitable tests for the TPS. The main results of these comparative experiments were that DAS-ELISA was not sensitive enough to reliably detect ToBRFV in tomato seeds with a medium level of the virus. In addition, for molecular tests using guanidine hydrochloride (GH+) buffer for grinding and RNA extraction resulted in a more sensitive detection of ToBRFV in tomato compared to phosphate buffer.

Based on the results of the comparative experiments, the TPS included two real-time RT-PCR tests (ISHI-Veg, 2019; Menzel & Winter, 2021), two end-point RT-PCR tests (Loewe kit, adapted from Rodriguez-Mendoza *et al.,* 2019; Alkowni *et al.,* 2019) and two isothermal amplification tests (Sarkes *et al.,* 2020; Agdia AmplifyRP® kit). GH+ buffer was chosen as the extraction buffer to be used. Twenty-six laboratories worldwide, but mostly from Europe and the Mediterranean region, participated in this study.

The project results showed that the real-time RT-PCR tests (ISHI-Veg, 2019 and Menzel & Winter, 2021) allowed to diagnose ToBRFV in all samples with only a few percent false negative and false positive results. In contrast, end-point RT-PCR and isothermal amplification tests appeared unsuitable for the reliable detection of ToBRFV in tomato and pepper seeds, because they were not sensitive enough. The real-time RT-PCR tests (ISHI-Veg, 2019 and Menzel & Winter, 2021) have been recommended for the detection of ToBRFV in seeds by the European and Mediterranean Plant Protection Organisation (EPPO, 2021) and are currently required for testing of tomato and pepper seeds under EU emergency measures. The TPS

results confirm the suitability of the real-time RT-PCR tests for the detection of ToBRFV in tomato and pepper seeds.

2.2. Project aims

The objective of the project was to validate protocols for the detection of ToBRFV in seeds. Several tests for the detection of ToBRFV have been developed, but harmonised protocols are missing. Validation is essential to gather information on the performance of diagnostic tests and data is preferably substantiated by an inter-laboratory comparison. Hence, this Euphresco project aimed to fill this gap by comparing different DAS-ELISA, real-time RT-PCR, end-point RT-PCR, and isothermal amplification tests for the detection of ToBRFV in seeds of tomato (*Solanum lycopersicum* and its hybrids) and pepper (*Capsicum* spp.).

2.3. Description of the main activities

2.3.1. Comparative experiments (pre-evaluation)

Several methods for the detection of ToBRFV have been developed, including DAS-ELISA, end-point RT-PCR, real-time RT-PCR and isothermal amplification. However, the number of tests that can be included in a TPS is limited. To assess the suitability of the serological and molecular tests developed for the detection of ToBRFV, a comparison was made by the organisers prior to the TPS. The technical report in Appendix 1 gives a detailed description of this comparison.

At the start of this project, four ToBRFV antisera for DAS-ELISA were considered. The experiments included: 1) an assessment of the analytical specificity of each DAS-ELISA by testing leaf material infected with tobamoviruses closely related to ToBRFV and 2) a comparison of a universal extraction buffer (NVWA extraction buffer, see Appendix 1) versus the extraction buffers prescribed by the test suppliers on leaf material infected with ToBRFV. Based on these experiments, the two most promising DAS-ELISAs were evaluated on seed samples with different levels of ToBRFV from naturally infected tomatoes. As samples with a medium level of ToBRFV (Cq values 18-30) were not or only barely detected by both tests (in contrast to the real-time RT-PCR tests performed which provided positive results), it was concluded that the tested DAS-ELISAs were not sensitive enough for ToBRFV detection in tomato seeds.

Multiple end-point and real-time RT-PCR tests for the detection of ToBRFV were identified at the start of the project. Three real-time RT-PCR tests and two conventional RT-PCR tests were selected for comparison of ToBRFV detection in tomato seeds, together with two isothermal amplification tests that were developed in 2020. This experiment also included a comparison between two extraction buffers commonly used for molecular tests, in order to identify the most suitable extraction buffer for the TPS. For the real-time RT-PCR tests, the use of GH+ buffer resulted in a ten times more sensitive detection of ToBRFV compared to phosphate buffer.

2.3.2. Test performance study

Based on the results of the pre-evaluation, the most suitable tests to include in the TPS were selected: the two most sensitive real-time RT-PCR tests (ISHI-Veg 2019; Menzel and Winter 2021), two end-point RT-PCR tests (Loewe kit, adapted from Rodriguez-Mendoza *et al.,* 2019; Alkowni *et al.,* 2019) and two isothermal amplification tests (Sarkes *et al.,* 2020; Agdia AmplifyRP®). GH+ buffer was chosen as the extraction buffer to be used.

Several seed companies provided both ToBRFV-infested and non-infested seed lots of pepper and tomato. Suitable seed lots for this TPS were selected by the organiser based on molecular tests. Samples of 1000 seeds with different concentrations of ToBRFV (high (Cq <18), medium (Cq 18-30)) and ToBRFV-negative samples were prepared. For pepper, seed samples with high ToBRFV levels were not available at the time, thus only samples with medium concentration were used for the TPS.

An invitation for the TPS was circulated within the Euphresco network and through professional contacts. Laboratories inside and outside the Euphresco network, including diagnostic laboratories, laboratories at for-profit companies, and laboratories at public institutions, had the opportunity to express their interest to participate. Twenty-six laboratories indicated interest in participation and joined the TPS. Each laboratory decided for itself which tests to perform. Most laboratories performed real-time RT-PCR tests (20-24 laboratories), followed by end-point RT-PCR tests (9-15 laboratories). Only a few laboratories performed isothermal amplification tests (4-5 laboratories). The technical report in Appendix 1 gives a detailed description of the set-up and results of the TPS.
The instructions and protocols were provided in an instruction booklet. The homogeneity, assigned values and stability of the samples were determined by the TPS organiser, independent of the results of participants. Participants had to use their own disposables and equipment. The resulting data sets were collected by the organiser. Data sets were excluded when >10% of the results was missing or undetermined, controls did not show the expected results, and/or essential aspects of the protocol were not followed. In addition, data sets were excluded when the results differed significantly (i.e., were outliers) from those obtained by other laboratories.
To evaluate the performance of each test, the qualitative results (positive or negative) as returned by the participants were used. The number and percentage of true/false positive and true/false negative results were calculated and based on these numbers relevant performance criteria were calculated (concordance/accuracy, diagnostic sensitivity, diagnostic specificity, false negative rate, false positive rate, positive predictive value, and negative predictive value). Based on these criteria the suitability of the different tests for seed testing was assessed.

2.4. Main results

The results of the pre-evaluation and TPS indicated that out of all methods tested, real-time RT-PCR is the most sensitive for the detection of ToBRFV in both tomato and pepper seeds. The ISHI-Veg and Menzel & Winter tests showed a diagnostic sensitivity and diagnostic specificity of at least 97%. Details on the test results are included in the technical report in Appendix 1.
The pre-evaluation of DAS-ELISAs and TPS results of end-point RT-PCR and isothermal amplification tests showed a high amount of false negative results, especially for pepper seeds. The diagnostic sensitivity of these tests was considerably lower than of any of the real-time RT-PCR tests, with the highest diagnostic sensitivity values for the Agdia isothermal amplification test (tomato 81%, pepper 43%). It should be noted that for the isothermal amplification tests this conclusion was based on only three data sets. For all tests, the number of false positives was very low, resulting in a high diagnostic specificity (>97%).

2.5. Conclusions and recommendations to policy makers

The results of this study showed that the real-time RT-PCR tests (ISHI-Veg and Menzel & Winter) allow the reliable and correct diagnosis of ToBRFV in tomato and pepper seeds with only a few percent false negative and false positive results. In contrast, DAS-ELISA, end-point RT-PCR and isothermal amplification tests as assessed in this study, appeared unsuitable for reliable detection of ToBRFV in tomato and pepper seeds, because they were not sensitive enough. However, for the isothermal amplification tests this conclusion was based on a limited number of data sets.
Based on these results, the recommended tests for the detection of ToBRFV in seeds of tomato and pepper would be the real-time RT-PCR tests developed by ISHI-Veg (2019), and Menzel & Winter (2021). Both real-time RT-PCR tests are already recommended for the detection of ToBRFV in seeds by the European and Mediterranean Plant Protection Organisation (EPPO, 2021), indicating that there is currently no need to adjust the EPPO Diagnostic Protocol. In addition, a recently published real-time RT-PCR test from Abiopep (Bernabé-Orts *et al*., 2021) targeting a different gene than the other real-time RT-PCR tests

was tested by two labs outside the scope of this TPS and may give comparable results, but may require more extensive testing.
Both real-time RT-PCR tests (ISHI-Veg and Menzel & Winter) are currently required for official testing of tomato and pepper seeds according to the most recent version of the EU emergency measures in Commission Implementing Regulation (EU) 2021/1809. Official testing allows to guarantee the absence of ToBRFV prior to import or movement of these seeds into or within the EU, and to test consignments of these seeds at the border control post of first arrival into the EU. Both tests should be used in combination to confirm the presence of ToBRFV in a consignment. The results indicate that there is currently no need to adjust the list of methods prescribed for seed testing. However, additional comparisons with a recently developed real-time RT-PCR that targets a different genome region (Bernabé-Orts *et al*., 2021), can be worth to consider.
To conclude, the results of this TPS indicate that sensitive and reliable tests for the detection of ToBRFV are available to limit further spread of this virus via seeds.

2.6. Benefits from trans-national cooperation

The cooperation of 26 partners from around the world enabled the assessment of multiple ToBRFV tests in different laboratories, which is essential for a TPS. This transnational cooperation has strengthened networks which allowed for sharing of knowledge between project partners and can enable future collaborations.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

None.

4. Open Euphresco data

None.

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Appendix 1 Technical project report

Test Performance Study

"Validation" of molecular tests for the detection of tomato brown rugose fruit virus (ToBRFV) in seed of tomato and pepper

Euphresco 2019-A-327

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1. Introduction

Tomato brown rugose fruit virus (ToBRFV) is a relatively recently described tobamovirus (Salem *et al*., 2016) causing problems in tomato and pepper cultivation worldwide (Luria *et al.,* 2017, Salem *et al.,* 2019). ToBRFV has been reported to cause yellow spots, green spots, necrotic lesions and occasional rugose symptoms on tomato fruits, chlorosis and mosaic symptoms on leaves and occasional leaf narrowing (Cambrón-Crisantos *et al*., 2018; Luria *et al*., 2017; Menzel *et al*., 2019 For pepper, stunting of young plants, puckering and yellow mottling of leaves, and misshapen fruits have been reported (Salem *et al.,* 2019). These symptoms render affected fruits non-marketable. The severity of the reported symptoms varies between studies. For example, the apparent brown rugose symptoms that were reported from Jordan, were only found in one tomato variety in Israel for which the symptom severity was different between growing locations (Luria *et al*., 2017). In the USA, 14 survey samples infected by ToBRFV were all co-infected with pepino mosaic virus (PepMV; Ling *et al*., 2019).This suggests that disease severity may be influenced by the host cultivar, growing conditions and the presence of additional viruses.

ToBRFV is a single-stranded positive-sense RNA virus with a genome size of approximately 6400 nucleotides that consists of four open reading frames which encode two proteins that form the RNA dependent RNA polymerase complex (RdRP), a movement protein (MP) and a coat protein (CP) (Salem *et al*., 2016). Tobamoviruses are very stable and will remain infectious for long periods of time when present in crop debris, soil and on surfaces. Infectivity on seeds is preserved for up to several years (Dombrovsky and Smith, 2017). ToBRFV is easily transmitted mechanically (via contaminated tools, hands, clothing, direct plant-to-plant contact, and bumble bees) and seed-to-seedling transmission is expected to play a role in the spread of the virus (Levitzky *et al*., 2019, Salem *et al.,* 2021).

The use of tomato varieties that are resistant to tobamoviruses has often been a good control strategy to avoid economic damage. The first commercial tomato cultivars resistant to ToBRFV have recently become available. In pepper, L resistance alleles may provide some protection against ToBRFV, but this resistance seems to be compromised at higher temperatures (Luria *et al*., 2017; Fidan *et al*., 2021).

The first confirmed ToBRFV outbreak in Jordan dates back to the spring of 2015 (Salem *et al*., 2016). Shortly afterwards, the presence of ToBRFV was confirmed in Israel (Luria *et al*., 2017) and Mexico (Cambrón-Crisantos *et al.,* 2018; Camacho-Beltran *et al*., 2019). Since 2018, various outbreaks have been reported from across the globe, including China (Yan *et al*., 2019), Greece (EPPO, 2019b), Italy (Panno *et al*., 2019a), Germany (Menzel *et al.,* 2019), the Netherlands (EPPO, 2019a), Palestine (Alkowni *et al.* 2019), Spain (EPPO, 2019c), Turkey (Fidan *et al*., 2019), the United Kingdom (Skelton *et al.,* 2019), the United States (Ling *et al.,* 2019) and multiple (other) European countries (EPPO Reporting Service 2020-2021; https://gd.eppo.int/reporting/).

As of November 1, 2019, emergency measures (EU) 2019/1615 are in place in the EU to prevent introduction and further spread of ToBRFV. The initial measures have been replaced by Commission Implementing Regulation (EU) 2021/1809. These measures include an obligatory annual survey, and specific requirements for movement and introduction of plants for planting, including seeds, of *Solanum lycopersicum* and *Capsicum* spp. In addition, there is a requirement for testing of at least 20% of the consignments of seeds and plants for planting of *Solanum lycopersicum* and *Capsicum* spp. upon entry into the EU. For consignments from Israel the required testing rate is even 50% and for seeds from China 100%. Therefore, reliable and harmonised protocols for the detection of ToBRFV in tomato and pepper plants and seeds are needed. In the framework of the EU H2020 research project VALITEST (www.valitest.eu) a test performance study (TPS) has been conducted on leaf and fruit material of tomato and

pepper. In addition, there is a need for reliable and harmonised test protocols for the detection of ToBRFV in tomato and pepper seeds.

Several tests for the detection of ToBRFV have been developed, but harmonised protocols are missing. Validation is essential for information on the performance of the tests and data are preferably substantiated by an inter-laboratory comparison. Hence, this Euphresco project aims to fill this gap by validating ELISA, RT-PCR, real-time RT-PCR and isothermal amplification tests for the detection of ToBRFV in seeds of tomato (*Solanum lycopersicum*) and pepper (*Capsicum* spp.). The objective of this initiative is to agree on harmonised protocols for detection of ToBRFV in seed. This TPS was organised and coordinated by the National Reference Centre for Plant Health (part of the Netherlands Food and Consumer Product Safety Authority (NVWA)), in collaboration with Naktuinbouw and the Italian Research Centre for Plant Protection and Certification (CREA-DC).

2. Comparative experiments for method and test selection

2.1 Introduction

The aim of the TPS for the detection of ToBRFV in tomato and pepper seeds was to compare the suitability of different methods and tests. Several methods for the detection of ToBRFV have been developed, including DAS-ELISA, end-point RT-PCR, real-time RT-PCR and isothermal amplification. However, the number of tests that can be included in a TPS is limited. This section describes comparative experiments that assessed serological and molecular tests developed for the detection of ToBRFV, in order to select the most suitable tests to include in the TPS.

2.1.1 Serological tests

At the start of this study, four ToBRFV antisera for DAS-ELISA were available. These antisera were developed by Agdia, DSMZ, Loewe and Prime Diagnostics and will be referred to by those names. Each antiserum was developed specifically for the detection of ToBRFV, except for Agdia, which was developed for the detection of tobacco mosaic virus (TMV) but is known to react with ToBRFV as well. Information on test specificity is important for the interpretation of the results. Therefore, Experiment 1 was conducted to assess the analytical specificity of each DAS-ELISA regarding tobamoviruses closely related to ToBRFV. Ideally, a test will only detect the pathogen of interest, in this case ToBRFV.

Experiment 2 was conducted for each test to compare detection of ToBRFV using a universal extraction buffer versus the specific extraction buffer prescribed by the test supplier. To allow a fair comparison between test results all tests to be compared should be performed on exactly the same samples. Since a seed extract can only be prepared once per sample, all tests need to be performed with the same extraction buffer. Extraction buffers of the four DAS-ELISAs differ slightly in composition and, therefore, the effect of the universal buffer on the performance should be known.

Based on the abovementioned experiments, the most promising DAS-ELISAs were selected. To assess their suitability for detection of ToBRFV in seeds, the selected DAS-ELISAs were tested using tomato seed samples with different levels of ToBRFV infection in Experiment 3.

2.1.2 Molecular tests

Multiple end-point RT-PCR and real-time RT-PCR tests for the detection of ToBRFV were available at the start of this study. Based on previous in-house validation studies, five of these tests were selected for an experiment on ToBRFV detection in tomato seeds, together with two isothermal amplification tests that became available in 2020. This experiment also included

a comparison between two extraction buffers commonly used for molecular tests, in order to identify the most suitable extraction buffer for the TPS.

2.2 Materials and Methods

2.2.1 Serological tests

Preparation of infected leaf material (experiment 1 and 2)

Infected leaf material (stored at -80°C) was ground in inoculation buffer (0.02 M phosphate buffered-saline (PBS) containing 2% (w/v) polyvinylpyrrolidone (PVP; MW 10,000) at an approximate ratio of 1:10 (w:v). The virus was mechanically transferred to host plants by gently rubbing the inoculum onto carborundum-dusted leaves. Inoculated plants were grown in a greenhouse at 18-22 °C with supplemental illumination for a daylength of at least 14 hours. Young leaves were harvested when systemic symptoms appeared around 3-4 weeks after inoculation and stored at -80°C.

Homogenisation of plant material

Leaves or seeds were ground in extraction buffer using extraction bags (Bioreba) and a hand-held homogeniser (Bioreba).

Optical density

Optical Density (OD) was measured at 405 nm, for two technical replicates per sample. A threshold for detection was calculated by multiplying the average OD-value of the uninfected leaves or seeds by two.

Experiment 1: analytical specificity

The four DAS-ELISA protocols (Agdia, DSMZ, Loewe and Prime Diagnostics; Appendix 1) were conducted on samples derived from healthy *Solanum lycopersicum* (tomato) leaves and samples derived from maintenance host leaves infected with ToBRFV, two isolates of tobacco mosaic virus (TMV), and tomato mosaic virus (ToMV) (Table 1). Leaf tissue stored at -80°C was ground in a universal extraction buffer (named “NVWA extraction buffer”; Table 2) at an approximate ratio of 1:10 (w:v), hereafter referred to as the undiluted sample. Ten-fold dilutions from 10^{-1} to 10^{-7} of each virus isolate were made in leaf extract of healthy tomato (leaf:buffer ratio 1:10 (w:v)).

Table 1. Virus isolates tested with each DAS-ELISA protocol.

Virus	Collection number	Host plant	Maintenance host
ToBRFV	33610403/33610411	*Solanum lycopersicum*	*Nicotiana occidentalis*-P1
TMV isolate 1	8904669	*Bouvardia*	*Nicotiana tabacum* ‘White burley’
TMV isolate 2	5674012	Unknown	*Nicotiana benthamiana*
ToMV	6184840	Unknown	*Nicotiana occidentalis*-P1

Table 2. Composition and preparation of NVWA extraction buffer.

Buffer	Preparation
PBS buffer (0.02M)	8.0 g NaCl, 0.4 g KH_2PO_4, 5.8 g $Na_2HPO_4.12H_2O$, 0.2 g KCl, add ~800 mL demineralised water, adjust to pH 7.4, add demineralised water to 1000 mL total volume.

NVWA extraction buffer	Add to 1000 mL 0.02 M PBS: 0.5 ml Tween® 20, 20.0 g PVP10, 2.0 g albumin from bovine serum.

Experiment 2: extraction buffer comparison

ToBRFV infected tomato leaves were divided over 50 extraction bags (Bioreba) and ground in 5 ml water with a hand-held homogeniser (Bioreba). Plant sap was collected in one tube to obtain a homogeneous stock and stored at -20˚C. Aliquots of 1 ml plant sap were later freeze dried under vacuum for at least 18 hours in a 5 ml freeze-drying glass vial. The freeze dried samples were stored at 4˚C. For comparison, a vial of freeze dried material (corresponding to approximately 0.2 g of ToBRFV infected leaf material) was mixed with different extraction buffers at a ratio of 1:10 (w:v) and hereafter referred to as the undiluted sample.

To mimic seed matrix conditions, the undiluted sample was mixed with uninfected tomato seed extract in five-fold and ten-fold dilutions from 5^{-1} to 5^{-5} and 10^{-1} to 10^{-5} respectively. Seed extract was prepared by grinding 250 seeds in 10 mL of extraction buffer per extraction bag.

Four DAS-ELISAs (Agdia, DSMZ, Loewe, Prime Diagnostics; Appendix 1) were performed on extract from uninfected seeds, the undiluted sample and serial dilutions thereof using the original extraction buffer (Appendix 1) prescribed by the supplier in comparison to the NVWA extraction buffer (Table 2).

Experiment 3: Testing ToBRFV infected seeds with selected antisera (analytical sensitivity)

Tomato seed samples from three seed lots with different levels of ToBRFV (Table 3) were tested using the DSMZ and Prime Diagnostics DAS-ELISA (Appendix 1) with NVWA extraction buffer (Table 2).

Table 3. Used tomato seed lots with their qualitative ToBRFV levels

Seed lot	ToBRFV level	Cq values*
1	Negative	>32
2	Medium	18-30
3	High	<18

* Cq value range based on multiple samples tested according to the ISHI-Veg protocol.

A seed sample consisted of 1000 seeds and was split into four subsamples of 250 seeds each. Seed extract was prepared by grinding each subsample in 10 mL of extraction buffer. Each subsample was tested individually and in a pooled sample with an equal amount of extract from all four subsamples. In addition, the undiluted sample from experiment 2 was included as a ToBRFV-positive control.

2.2.2 Molecular tests

Sample composition and preparation

Six seed samples were prepared by spiking 999 uninfected *Solanum lycopersicum* (tomato) seeds with one seed that was naturally infected with ToBRFV. Three samples were homogenised in GH+ extraction buffer (Table 4), the other three samples were homogenised in phosphate extraction buffer (0.1 M Na_2HPO_4/KH_2PO_4, pH 7.2). For each sample, two ten-fold dilution series from 10^{-1} to 10^{-6} were obtained by mixing 100 µl of seed extract from infected seeds in 900 µl of seed extract from uninfected seeds. One dilution series was used for RNA extraction, the other dilution series was used directly (as raw extract) for isothermal assays. A negative control sample consisting of 1000 ToBRFV-negative tomato seeds was included for each extraction buffer. RNA extracts from leaf material infected with other tobamoviruses (tomato mosaic virus (ToMV), tobacco mosaic virus (TMV), pepper mild mottle virus (PMMoV),

bell pepper mottle virus (BPeMV) and tobacco mild green mosaic virus (TMGMV)) were included as non-target controls (all homogenised in phosphate buffer).

Table 4. GH+ extraction buffer composition.

Ingredient	Amount	Final concentration
guanidine hydrochloride	573 g	6 M
sodium acetate (4M, pH5.2)	50 mL	0.2 M
EDTA Na_2 $2H_2O$	9.3 g	25 mM
PVP-10	25.0 g	2.5% w/v
Distilled water to	1.0 L	

RNA extraction using GH+ buffer

Each sample of 1000 seeds was incubated with 20 mL of GH+ extraction buffer (Table 4) at room temperature for ~60 min in a grinding bag (BIOREBA XL), followed by homogenisation with an Interscience BagMixer (position 4) for 90 sec after which the dilution series were prepared. One mL of raw extract per dilution was transferred into a 1.5 mL tube and 30 µL Dithiothreitol (5M) was added. After incubation for 15 min at 65 °C at 850 rpm, the material was centrifuged at 16,000 g for 10 min. To extract RNA using the RNeasy Plant Mini Kit (Qiagen), 750 µL of supernatant was used following the manufacturer's instructions. RNA was eluted in 50 µL of RNase-free water prewarmed at 65°C. RNA extract and raw extract were stored at -20°C.

RNA extraction using phosphate buffer

Each sample of 1000 seeds was incubated with 40 mL of 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7.2) at 4 ± 2 °C overnight in a grinding bag (BIOREBA XL), followed by homogenisation with an Interscience BagMixer (position 4) for 90 sec after which the dilution series were prepared. One mL of raw extract per dilution was transferred into a 1.5 mL tube, and spun down at 10,000 g at 4 °C for 10 min. Then, 600 µl of supernatant was mixed with 600 µl of RLT buffer (without β-mercaptoethanol) and used for RNA extraction using the RNeasy Plant Mini kit (Qiagen), following the manufacturer's instructions. RNA was eluted with 50 µL of RNase-free water prewarmed at 65°C. RNA extract and raw extract were stored at -20°C.

Molecular tests

Multiple molecular tests for the detection of ToBRFV were available. Generic tobamovirus RT-PCR tests such as Letschert *et al.* (2002), Levitzky *et al.* (2019), Li *et al.* (2018), and Menzel *et al.* (2019) were excluded from this test because they also detect other tobamovirus species. Based on previous in-house validation studies (Valitest, CREA-DC) the ToBRFV specific tests described by Ling *et al.* (2019) and Panno *et al.* (2019a) were excluded due to poor analytical specificity (cross-reactions) (Anthoine *et al.,* 2020). The test described by Luria *et al.* (2017) was excluded as other tests show better analytical sensitivity and analytical specificity (EPPO 2021).

The following tests were included:

- Real-time RT-PCR (using 2 µl RNA): ISHI-Veg (2019), Panno *et al.* (2019b), Menzel and Winter (2021)
- End-point RT-PCR (using 2 µl RNA): Loewe Biochemica GmbH using primers from Rodriguez-Mendoza *et al.* (2019), Alkowni *et al.* (2019)

- Isothermal amplification: AmplifyRP® XRT for ToBRFV, employing recombinase polymerase amplification (RPA) technology (Agdia) (using 10 µl RNA and 10 µl raw seed extract)
- Loop-mediated isothermal amplification (LAMP) colorimetric reaction (Sarkes *et al.*, 2020) (using 1 µl RNA and 1 µl raw seed extract).

Each (diluted) sample was tested in triplicate, negative controls were tested in duplicate and non-target controls were included as single samples.

2.3 Results and discussion

This section describes the results of the comparative experiments described above, in order to decide which tests to include in the TPS for ToBRFV detection in seed of tomato and pepper.

2.3.1 Serological tests

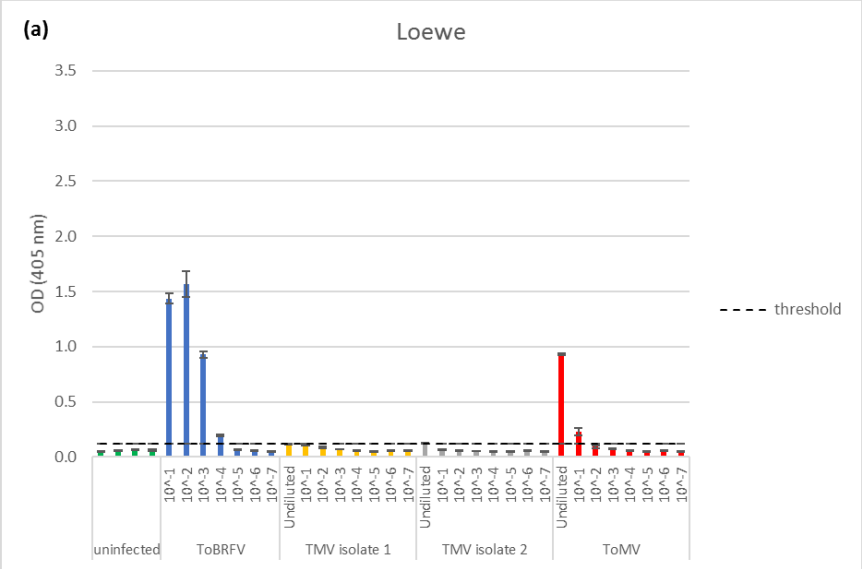
Experiment 1: analytical specificity

To assess analytical specificity each DAS-ELISA was performed on leaf material infected with the virus species of interest, i.e. ToBRFV, and the closely related tobamoviruses TMV (two different isolates) and ToMV (Table 1). Uninfected tomato leaf material was included as a negative control and to determine the detection threshold per test.

ToBRFV was detected up to a dilution of 10^{-4} (Loewe, DSMZ, Prime Diagnostics) or 10^{-5} (Agdia) (Fig. 1). Cross-reactions were observed for Loewe with ToMV (up to 10^{-1}), for Agdia with all tested isolates (generally up to 10^{-5}), for DSMZ with all tested isolates (depending on the isolate ranging from undiluted up to 10^{-3}) and for Prime Diagnostics with TMV isolate 1 (undiluted) and ToMV (up to 10^{-3}).

Since Agdia was developed to detect TMV, it was not surprising that the TMV isolates were clearly detected. In addition, ToBRFV and ToMV were also clearly detected. Agdia was the most sensitive test for all virus isolates. However, since the average OD-value of uninfected leaf material was also higher for Agdia compared to the other tests (0.09 versus 0.06-0.07), this also resulted in a higher calculated detection threshold.

Based on these results, Loewe, DSMZ and Prime Diagnostics reacted most strongly to ToBRFV itself although reactions with related tobamoviruses were observed for all four tests,. Agdia on the other hand reacted similarly to all tested virus isolates.



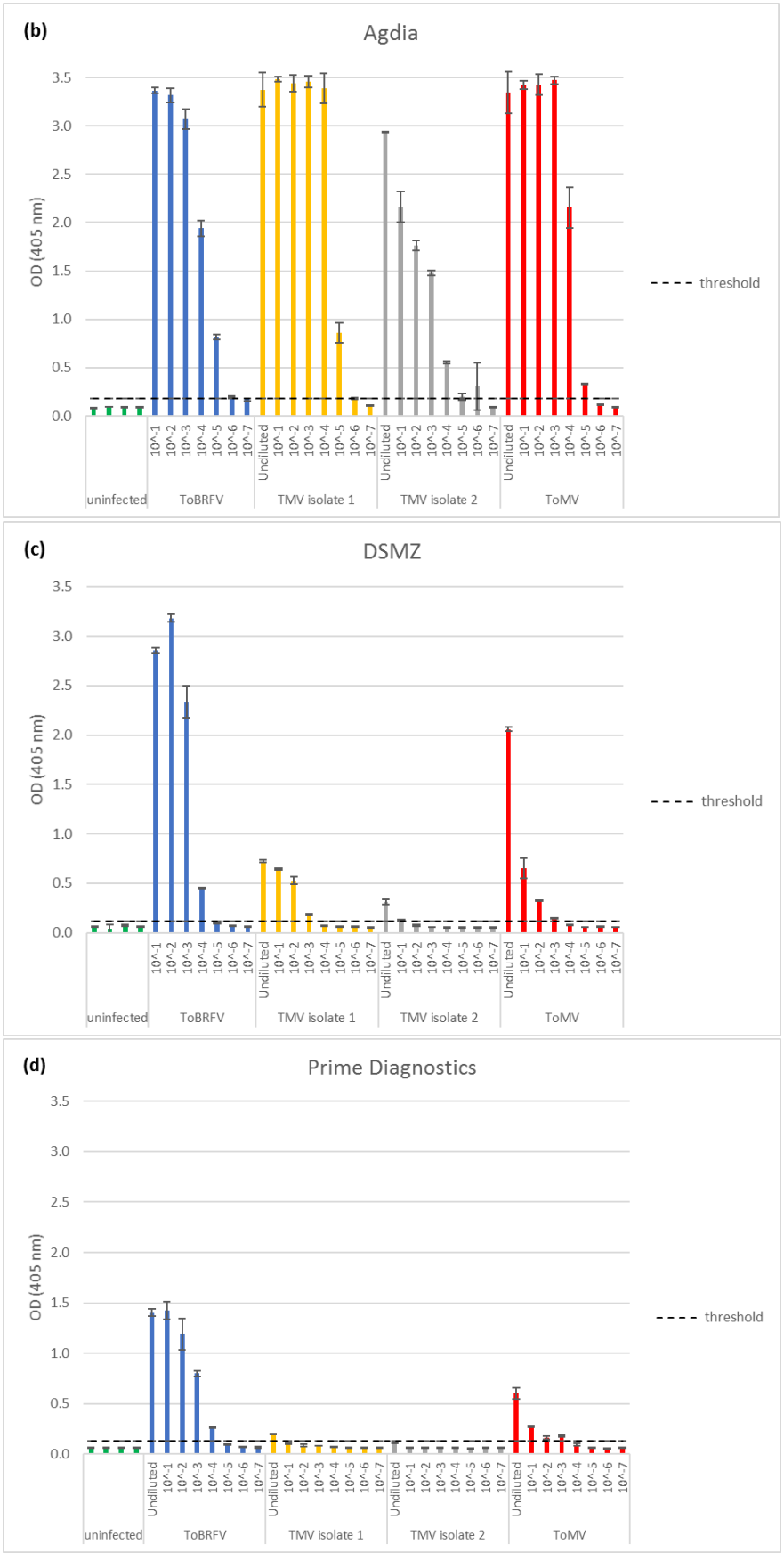


Figure 1. Average DAS-ELISA OD-values of samples measured in two technical replicates after 2 hours of incubation (a) Loewe (b) Agdia (c) DSMZ (d) Prime Diagnostics. Samples consisted of extract from uninfected tomato leaves, and serial dilutions of ToBRFV (blue), TMV isolate 1 (yellow), TMV isolate 2

(grey) and ToMV (red) infected leaf material in uninfected tomato leaf extract. Error bars indicate the standard deviation. The detection threshold was calculated by multiplying the average OD-value of uninfected samples by two.

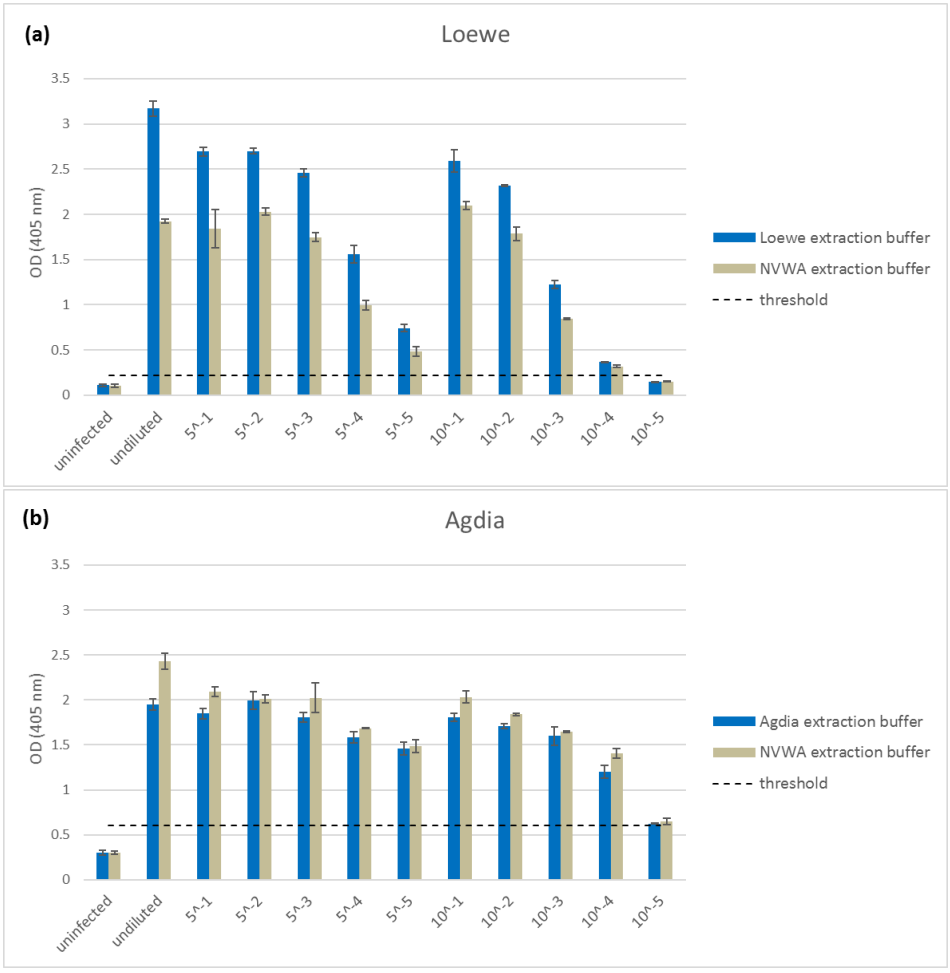
Experiment 2: Extraction buffer comparison

Each DAS-ELISA was performed on extract from non-ToBRFV infected seeds, ToBRFV infected leaf material and dilutions thereof in uninfected seed extract, thereby comparing the original extraction buffer (Appendix 1) prescribed by the supplier to the NVWA extraction buffer (Table 2).

OD-values measured using the Loewe protocol were much lower when the NVWA extraction buffer was used compared to Loewe extraction buffer (Fig. 2a). For the other protocols, the effect of the extraction buffer on resulting OD-values was less prominent (Fig. 2b-d).

For Loewe, Agdia, and DSMZ the highest dilution (10^{-5}) resulted in OD-values below or very close to the threshold (Fig. 2a-c). Prime Diagnostics was the most sensitive, as all dilutions resulted in OD-values clearly above the threshold (Fig. 2d).

Based on these results the Loewe antiserum was considered less suitable for this TPS, because of its clearly lower OD-values with the (universal) NVWA extraction buffer. Even though qualitative outcomes (detection versus no detection) were similar to the antisera of Agdia and DSMZ, outcomes are expected to deviate at lower concentrations of ToBRFV.



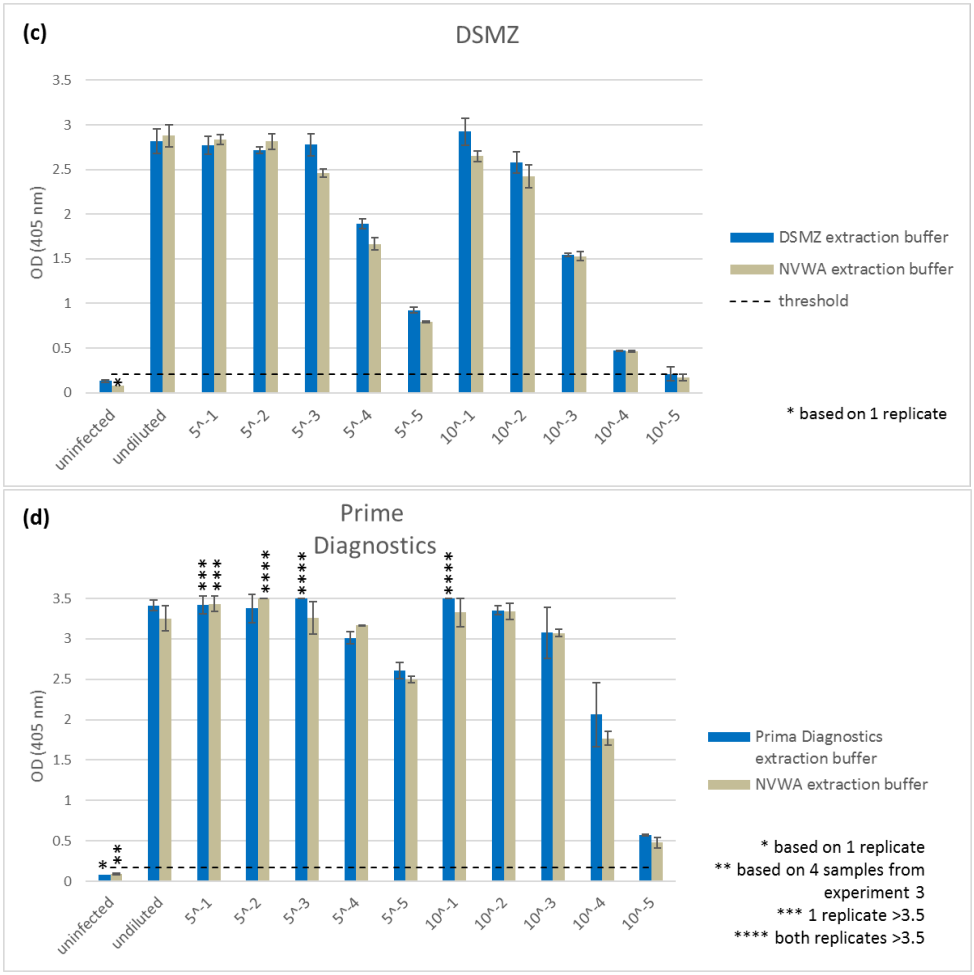


Figure 2. Average DAS-ELISA OD-values of samples measured in two technical replicates prepared with the original extraction buffer (blue) versus the NVWA extraction buffer (brown) (a) Loewe, after 2 hours of incubation (b) Agdia, after 1 hour of incubation (c) DSMZ, after 1 hour of incubation (d) Prime Diagnostics, after 1 hour of incubation. Samples consisted of extract from uninfected seeds, extract from ToBRFV-infected leaf material and two serial dilutions from 5^{-1} to 5^{-5} and from 10^{-1} to 10^{-5} in extract from non-ToBRFV infected seeds. Error bars indicate the standard deviation. For samples with OD-values >3.5, an OD-value of 3.5 was used to calculate the average value and standard deviation. The threshold for detection was calculated by multiplying the average OD-value of uninfected seeds by two.

Experiment 3: testing ToBRFV infected seeds with selected antisera (analytical sensitivity)

Based on experiment 1, Agdia was considered less suitable for the TPS than the other tests due to its strong cross-reactions with tobamoviruses other than ToBRFV. Based on experiment 2, Loewe was considered less suitable than the other tests, as the use of the NVWA extraction buffer clearly lowered its sensitivity. Therefore, DSMZ and Prime Diagnostics were selected for experiment 3, in which ToBRFV infected seeds were tested. Each sample was split into four subsamples of 250 seeds each. Detection of ToBRFV in one subsample is sufficient to indicate infection of the sample. The highly infected samples were barely detected by DSMZ (Fig. 3a) and clearly detected by Prime Diagnostics (Fig. 3b). The medium infected samples were not or only barely detected by both tests (Fig. 3), whereas real-time RT-PCR tests performed on samples from the same seed lot resulted in clear exponential curves with Cq values from 18 to 30 (Table 3). In conclusion, experiment 3 indicated that the tested DAS-ELISAs are not sensitive enough for ToBRFV detection in tomato seeds.

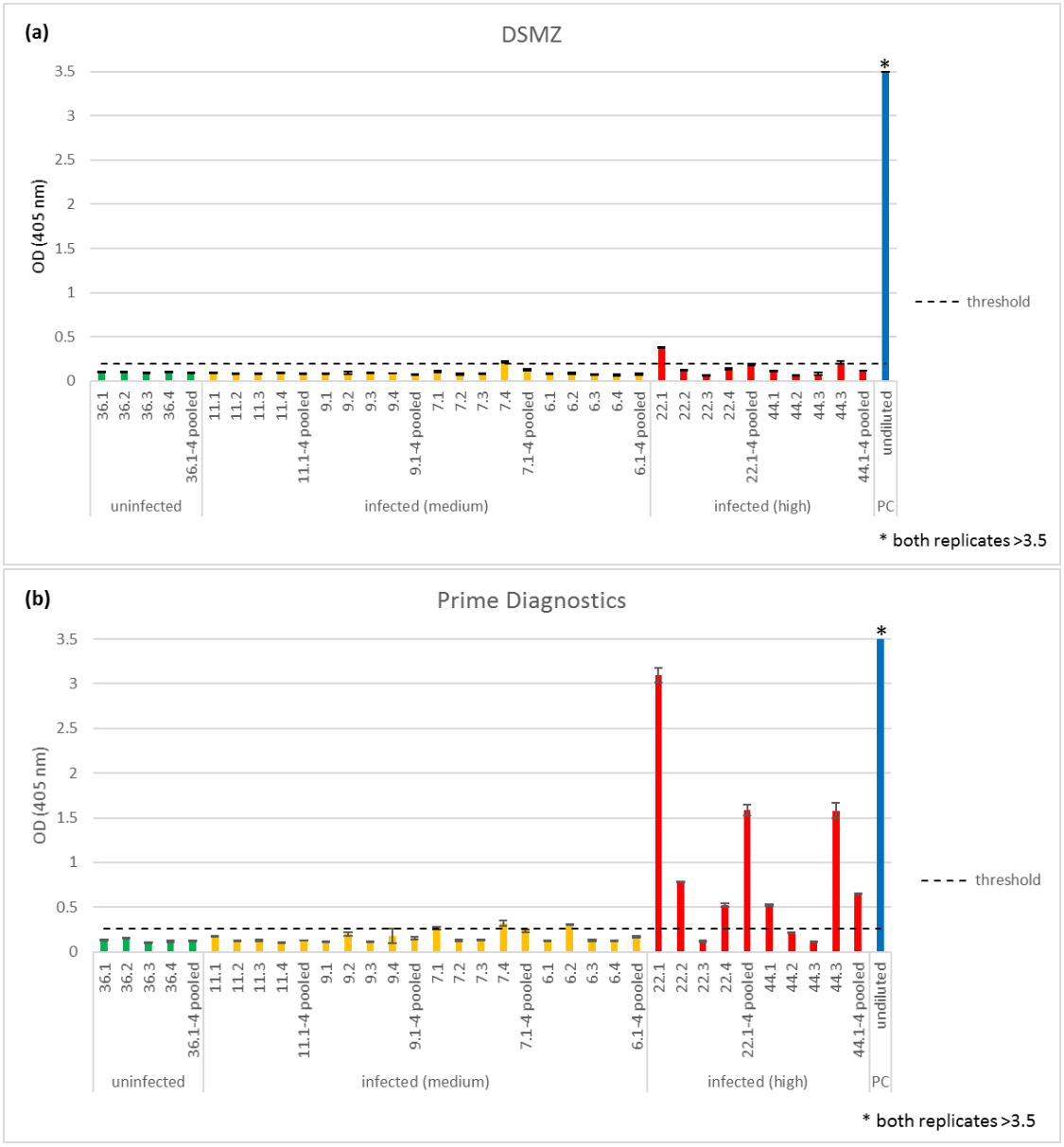


Figure 3. Average DAS-ELISA OD-values of samples measured in two technical replicates (a) DSMZ, after 1 hour of incubation (b) Prime Diagnostics, after 1 hour of incubation. Samples consisted of extract from non-ToBRFV infected seeds (green: 1 sample), extract from ToBRFV infected seeds (yellow: medium level of infection; 4 samples), extract from ToBRFV infected seeds (red: high level of infection; 2 samples) and extract from ToBRFV infected leaf material (blue: positive control (PC)). Each seed sample was split into four subsamples that were measured independently and in a pooled sample combining extract from all subsamples. Error bars indicate the standard deviation. For samples with OD-values >3.5, an OD-value of 3.5 was used to calculate the average value and standard deviation. The threshold for detection was calculated by multiplying the average OD-value of uninfected subsamples by two.

2.3.2 Molecular tests

Seven molecular tests were compared to determine their suitability for the detection of ToBRFV in seeds. In order to identify the most suitable extraction buffer for the TPS, this comparison was performed by using two different extraction buffers commonly used for molecular tests, phosphate and GH+ buffer.

Total RNA extract

Total RNA extract from GH+ buffer resulted in lower average Cq values for the real-time RT-PCR tests, with an average difference of around one cycle compared to phosphate buffer and the detection of one additional 10X dilution (Table 5). For the other molecular tests, both extraction buffers gave similar qualitative results regarding the lowest dilution to be detected.

The real-time RT-PCR tests from Menzel and Winter (2019) and ISHI-Veg (2019) detected the target up to a dilution of 10^{-4} for phosphate buffer and up to a dilution of 10^{-5} for GH+ buffer. The test from Panno *et al*. (2019) detected the target up to the 10^{-3} dilution for phosphate buffer and 10^{-4} for GH+ buffer. The cut-off value to determine whether a sample was positive or negative was placed at the highest dilution where the amplification curve overlapped with the prior dilution.

Both endpoint RT-PCR tests (Loewe and Alkowni *et al*., 2019) detected the target up to the 10^{-2} dilution. The isothermal amplification-based tests detected the target up to a dilution of 10^{-4} and 10^{-3} using RPA and LAMP methods, respectively. It should be noted that 10 µl of RNA was used for the isothermal tests, whereas 2 µl of RNA was used for the PCR tests.

Leaf material infected with non-target tobamoviruses and the negative control consisting of non-ToBRFV infected seeds tested negative in all tests.

Raw seed extract

When raw seed extract was used for the isothermal assays, only the RPA assay was able to detect ToBRFV up to a 10^{-1} dilution and only using phosphate extraction buffer. The lack of detection when using GH+ buffer could be caused by interferance with the colorimetric LAMP reaction and RPA reaction.

Table 5. Results of comparison of molecular tests on tomato seeds spiked with ToBRFV per test and extraction buffer.

			Real-time RT-PCR (2 µl RNA)			Endpoint RT-PCR (2 µl RNA)		RPA		Colorimetric LAMP	
		Rep.	Menzel & Winter, 2019	ISHI-Veg, 2019	Panno et al., 2019b	Loewe	Alkowni et al., 2019	Agdia (10 µl RNA)	Agdia (10 µl raw extract)	Sarkes et al, 2020 (1 µl RNA)	Sarkes et al, 2020 (1 µl raw extract)
Phosphate	NC	2	38.5 / neg	neg / neg	neg / neg	neg	neg	neg	neg	neg	neg
	undiluted	3	19.9 ± 0.4	21.9 ± 0.4	25.2 ± 0.5	pos	pos	pos	pos	pos	neg
	10^{-1}	3	23.46 ± 0.4	25.6 ± 0.5	28.4 ± 0.7	pos	pos	pos	pos	pos	neg
	10^{-2}	3	26.9 ± 0.5	28.4 ± 0.4	31.7 ± 0.5	pos	pos	pos	neg	pos	neg
	10^{-3}	3	29.6 ± 0.5	31.2 ± 0.6	34.5 ± 1.1	neg	neg	pos	neg	pos	neg
	10^{-4}	3	32.7 ± 0.4	32.7 ±0.7	35.6 ± 0.5	neg	neg	pos	neg	neg	neg
	10^{-5}	3	33.7 ± 0.7	33.4 ± 0.9	neg	neg	neg	neg	neg	neg	neg
	10^{-6}	3	34.4 ± 0.1	34.8 ± 1.0	neg	neg	neg	neg	neg	neg	neg
GH+	NC	2	38.7 / 37.6	36.1 / neg	37.82 / neg	neg	neg	neg	neg *	neg	neg *
	undiluted	3	18.2 ± 1.2	19.9 ± 0.8	22.8 ± 0.8	pos	pos	pos	neg *	pos	neg *
	10^{-1}	3	21.0 ± 0.4	23.3 ± 1.0	26.4 ± 0.7	pos	pos	pos	neg *	pos	neg *
	10^{-2}	3	25.1 ± 1.0	25.9 ± 0.9	28.9 ± 0.6	pos	pos	pos	neg *	pos	neg *
	10^{-3}	3	28.9 ± 0.9	30.7 ± 1.7	32.5 ± 0.4	neg	neg	pos	neg *	pos	neg *
	10^{-4}	3	31.4 ± 0.2	32.7 ± 0.5	34.0 ± 0.4	neg	neg	pos	neg *	neg	neg *
	10^{-5}	3	34.0 ± 0.7	34.5 ± 1.0	35.1 ± 1.2	neg	neg	neg	neg *	neg	neg *
	10^{-6}	3	33.1 ± 0.4	35.6 ± 1.2	neg	neg	neg	neg	neg *	neg	neg *
Phosphate**	ToMV	1	neg	neg	neg	neg	neg	neg	not tested	neg	not tested
	TMV	1	neg	neg	neg	neg	neg	neg	not tested	neg	not tested
	PMMoV	1	neg	neg	neg	neg	neg	neg	not tested	neg	not tested
	BPeMV	1	neg	neg	neg	neg	neg	neg	not tested	neg	not tested
	TMGMV	1	neg	neg	neg	neg	neg	neg	not tested	neg	not tested

2.4 Conclusion

The goal of these comparative experiments was to select the most suitable methods and tests to include in the TPS for detection of ToBRFV in tomato and pepper seeds.

Out of four antisera for DAS-ELISA, Agdia showed strong cross-reactions with tobamoviruses other than ToBRFV, and Loewe was not robust when a universal extraction buffer was used. The two remaining DAS-ELISAs, DSMZ and Prime Diagnostics, were tested on tomato seeds infected with different levels of ToBRFV. Because these tests were not sensitive enough to reliably detect ToBRFV in medium infected tomato seed samples, it was decided not to include DAS-ELISA in the TPS.

Three real-time RT-PCR tests, two conventional RT-PCR tests and two isothermal amplification tests were performed on serial dilutions of RNA from ToBRFV infected tomato seeds, using two different extraction buffers: phosphate and GH+. For the real-time RT-PCR tests on tomato seeds, the use of GH+ buffer resulted in a 10 times more sensitive detection of ToBRFV compared to phosphate buffer. Therefore GH+ buffer was chosen as the extraction buffer to be used in the TPS.

All molecular tests detected ToBRFV up to dilutions of 10^{-2} to 10^{-4}, though it must be noted that the isothermal amplification tests were performed on 10 µl of RNA instead of 2 µl. The isothermal amplification tests were also performed on raw seed extract, but this resulted in less sensitive or no detection of ToBRFV. To limit the number of tests included in the TPS, the real-time RT-PCR developed by Panno *et al.* (2019b) was excluded because of its lower sensitivity in comparison to the other two real-time RT-PCR tests.

3. Test performance study

3.1 Materials and Methods

3.1.1 Participant and test selection

An invitation was circulated within the Euphresco network and through professional contacts. Laboratories inside and outside the Euphresco network, including diagnostic laboratories, private laboratories at commercial companies, and laboratories at public institutions, had the opportunity to express their interest to participate, which 26 laboratories did (Table 6). Each laboratory was assigned a unique code (P01-P26) to anonymise the results. There was no selection for participation, but participants had to indicate their expertise (Appendix 2) for the methods performed.

Table 6. List of participating laboratories.

Partner	Name of the participating laboratory	Country
2	Naktuinbouw	The Netherlands
3	Council for Agronomic Research and the analysis of Bioeconomy (CREA-DC)	Italy
4	Flanders Research Institute for Agriculture, Fisheries and Food (ILVO)	Belgium
5	French Agency for Food, Environmental and Occupational Health and Safety (ANSES)	France
6	Julius Kühn-Institut (JKI)	Germany
7	Plant Protection and Inspection Services (PPIS)	Israel
8	Austrian Agency for Health and Food Safety (AGES)	Austria
9	All Russian Plant Quarantine center (VNIIKR)	Russia
10	National Institute of Biology (NIB)	Slovenia
11	Eurofins Plant Pathology Laboratory (Eurofins)	France
12	The French Variety and Seed Study and Control Group (GEVES)	France
13	Hazera seeds Ltd	Israel

14	University of Palermo (UNIPA)	Italy
15	BASF	The Netherlands
16	Enza Zaden	The Netherlands
17	Ministry for Primary Industries (PHEL-MPI)	New Zealand
18	Spanish Plant Breeders Association (ANOVE)	Spain
19	Croatian Agency for Agriculture and Food (HAPIH)	Croatia
20	Agdia EMEA	France
21	Fera Science Ltd	United Kingdom
22	Instituto Nacional de Investigação Agrária e Veterinária (INIAV)	Portugal
23	Canadian Food Inspection Agency (CFIA)	Canada
24	Centro Nacional de Referencia Fitosanitaria del SENASICA (SENASICA)	Mexico
25	Bactochem	Israel
26	Microlab	Israel
27	Hylabs	Israel

The methods and tests included in this TPS (Table 7, Appendix 3) were selected based on the experimental results described in section 2. Each laboratory decided for itself which tests to perform. Most laboratories performed real-time RT-PCR tests, followed by end-point RT-PCR tests. Only a few laboratories performed the isothermal amplification tests. Outside of the scope of this TPS, a third real-time PCR from Abiopep (Bernabé-Orts *et al*., 2021; Appendix 4) was included by two labs and results are included in this report as well.

*Table 7. Methods and tests selected for the TPS. **Bold and underlined**: abbreviated names used in this report.*

Real-time RT-PCR	End-point RT-PCR	Isothermal amplification
Test adapted from **ISHI-Veg** (2019)	**Loewe** kit (adapted from Rodríguez-Mendoza *et al.* (2019)	LAMP (colorimetric reaction): based on **Sarkes** *et al.* (2020)
Test adapted from Menzel and Winter (**M&W**) (2021)	Test adapted from **Alkowni** *et al.* (2019)	**Agdia** AmplifyRP® Isothermal Amplification (RPA)

3.1.2 Seed sample preparation

Seed samples consisted of tomato (sample set 1) and pepper (sample set 2). Sample types consisting of samples with different levels of ToBRFV (high, medium) and ToBRFV-negative samples were prepared (Table 8). For pepper, insufficient ToBRFV infected seeds were available to include samples with a high level of ToBRFV. Each sample type was prepared from different seed batches as indicated in Appendix 5. All ToBRFV-negative samples were found to contain at least one other tobamovirus, with the exception of the “pepper neg1” samples (Appendix 5).

Table 8. Number of samples per sample type.

Seed sample set	ToBRFV level				Total # samples
	high	medium	neg1*	neg2*	
1: tomato	4	20	5	1	30
2: pepper	NA	5	4	1	10

*neg: no ToBRFV present

Each sample consisted of 1000 seeds packaged in an individual bag. For pepper, samples were provided as two individually packaged subsamples (A and B) of 500 seeds each, which had to be pooled for further processing either before or after grinding, depending on the grinding procedure.

Samples were randomised per set, with several ToBRFV-negative samples manually placed next to samples with high levels of ToBRFV as a control for cross-contamination. Sample set 1 (tomato) included one labelled ToBRFV-negative isolation control (NIC; ToBRFV negative) and one labelled positive isolation control (PIC; high level of ToBRFV). Sample set 2 (pepper) included one labelled NIC (ToBRFV negative), while a PIC (high level of ToBRFV) was not available. All other samples were coded without an identity label to ensure blind testing. A sample decoding table is provided in Appendix 6.

Homogeneity, assigned values and stability of the samples were determined by the TPS organiser, independent of the results of participants.

3.1.3 Homogeneity test

Ten samples of each sample type (Table 8), except for “neg2” samples, were randomly selected and tested according to the TPS instructions for the ISHI-Veg protocol in week 2, 2021. Tomato seeds were soaked for 30-60 minutes and ground using an Interscience Bagmixer. Pepper seeds were prepared using a Genogrinder.

3.1.4 Assigned values

The assigned value or expected result of each sample type was based on the presence or absence of ToBRFV infected seeds in the sample, i.e. the true ToBRFV status of the seeds (Appendix 5). In addition, three samples of each sample type, except for “neg2” samples, were tested according to the TPS instructions for all six test protocols in week 7, 2021. Tomato seeds were soaked for 30-60 minutes and ground using an Interscience Bagmixer. Pepper seeds were prepared using a Genogrinder.

3.1.5 Stability test

One complete sample set was tested according to the TPS instructions for the ISHI-Veg protocol in week 18, 2021, one week after the deadline for submission of the results by the participants. Tomato seeds were soaked for 30-60 minutes and ground using an Interscience Bagmixer. Pepper seeds were prepared using a Genogrinder.

3.1.6 TPS instructions

Instructions and protocols (Appendix 3) were provided in an instruction booklet. Participants had to use their own disposables and equipment. Primers, probes, reagents and a positive amplification control (PAC) had to be ordered according to instructions. For each sample, participants were asked to provide qualitative test results and Cq values (real-time RT-PCR), gel images (end-point RT-PCR) or images (colorimetric reaction). Results, deviations from the recommended protocols and expertise of the participating laboratory were reported using an Excel Form.

3.1.7 TPS timeline

Samples were dispatched on March 15, 2021 via postal carrier. Upon receipt, participants were asked to return a sample receipt form stating the date of receipt and condition of the samples. The deadline for the submission of results was May 1, 2021.

3.1.8 Performance criteria

To evaluate the performance of each test-matrix combination, the qualitative results submitted by the participants were used. The number and percentage of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) results were calculated based on the assigned value of each sample according to Table 9. Undetermined results (UND) were excluded from the calculation of performance criteria. The performance criteria defined in Table 10 were calculated per test. Data sets were excluded if >10% of the results was missing or undetermined, controls did not show the expected results, and/or essential aspects of the protocol were not followed. In addition, data sets were excluded when results differed significantly from those obtained by other laboratories.

Table 9. Definition of true positive (positive agreement), true negative (negative agreement), false positive (positive deviation) and false negative (negative deviation) samples.

		Assigned value	
		Positive	**Negative**
Participant's result	**Positive**	True Positive (TP)	False Positive (FP)
	Negative	False Negative (FN)	True Negative (TN)

Table 10. Definition and calculation of the assessed performance criteria.

Performance criterium	Definition	Calculation
Concordance/Accuracy (ACC)	Degree of agreement between the test result and the assigned value	(TP + TN) / total number of samples
Diagnostic sensitivity (DSE)	Estimate of the ability of the test to detect the target	TP / (TP + FN)
Diagnostic specificity (DSP)	Estimate of the ability of the test not to detect the non-target	TN / (TN + FP)
False negative rate	Type I error	1 - DSE
False positive rate	Type II error	1 - DSP
Positive predictive value	Probability that samples with a positive test result are truly infected	TP / (TP + FP)
Negative predictive value	Probability that samples with a negative test result are truly uninfected	TN / (TN + FN)

3.2 Results and discussion

3.2.1 Assigned values, homogeneity and stability

Assigned values were based on the known presence or absence of ToBRFV infected seeds in the sample and corresponded perfectly with the outcomes of the real-time RT-PCR tests (Appendix 7). Even though most of the used seed batches contained tobamoviruses other than ToBRFV (Appendix 5), ToBRFV-negative samples tested negative in all six tests (Appendix 7). Therefore, these other tobamoviruses (at least at the tested levels) were not detected by any of the ToBRFV tests and the seeds were considered as fit for purpose. The "tomato neg2" sample was not included in these tests, but the later performed stability test, although based on one sampe, showed a negative result for this negative control as well (Table 11).

The homogeneity test showed that the different sample types were quite homogeneous with a maximum standard deviation of Cq 3.5, and each individual sample corresponding to its assigned value (Table 11). The stability test showed the same qualitative outcomes for all sample types as in the homogeneity test 16 weeks earlier, even though Cq values were slightly higher at the later time point (Table 11).

Table 11. Assigned value, average Cq value and standard deviation per sample type for the homogeneity and the stability test (ISHI-Veg test).

		Homogeneity test (week 2, 2021)					Stability test (week 18, 2021)				
	Assigned value	FAM		VIC		Positive/ total	FAM		VIC		Positive / total
Sample type		Ave	SD	Ave	SD		Ave	SD	Ave	SD	
Tomato high	Positive	16.7	1.5	15.6	1.5	10/10	16.9	1.6	17.9	1.8	4/4
Tomato medium	Positive	23.7	3.3	23.1	3.5	10/10	26.4	3.6	27.1	3.4	20/20
Tomato neg1	Negative	35.4*	2.1	39.0*	1.3	0/10	35.0	2.9	35.8	2.5	0/5
Tomato neg2	Negative	NA	NA	NA	NA	NA	32.9**	NA	33.9**	NA	0/1
Pepper medium	Positive	26.2	0.3	27.0	0.4	10/10	29.4	0.4	29.2	0.4	5/5
Pepper neg1	Negative	34.8*	2.8	38.8*	1.9	0/10	38.6	1.8	39.6	0.9	0/4
Pepper neg2	Negative	NA	NA	NA	NA	NA	40.0 **	NA	39.4**	NA	0/1

Positive/total: number of positive reactions/total number of reactions, * Mainly aspecific curves, ** based on 1 sample.

3.2.2 Collected data

One laboratory (P08) was unable to obtain the TPS samples from customs and could therefore not participate. The majority of participants received the samples within several days. Thirteen laboratories could not meet the deadline for the submission of results (May 1, 2021), often due to COVID-19 related reasons. The latest results were received on July 9, 2021. Late submission and/or delayed performance of the tests did not seem to affect the quality of the results, as several data sets that were submitted after the deadline showed 100% concordance for one or more tests.

3.2.3 Data exclusion

All laboratories had at least one year of experience with the methods and tests that they performed for the TPS. One laboratory had no traceability or QA in place, but showed relatively high concordance levels. Therefore, no data sets were excluded based on reported expertise (Appendix 2).

Data sets were excluded (based on the qualitative results submitted by the participants) when >10% of the results was missing or undetermined, controls did not show the expected results and/or essential aspects of the protocol were not followed. Remaining datasets of which results differed significantly (i.e. were outliers) from results obtained by other laboratories were excluded as well (Figure 4, Table 12).

A few participants used different kits/enzymes than prescribed for the TPS for some of the tests (ISHI-Veg: 2 + 1 unreported, M&W: 1 + 1 unreported, Alkowni: 2). The results of only one test produced with a different kit were outliers. Therefore, those different kits/enzymes that did not produce outlier results were not considered to represent essential aspects of the protocol and the produced results were all included in the analysis.

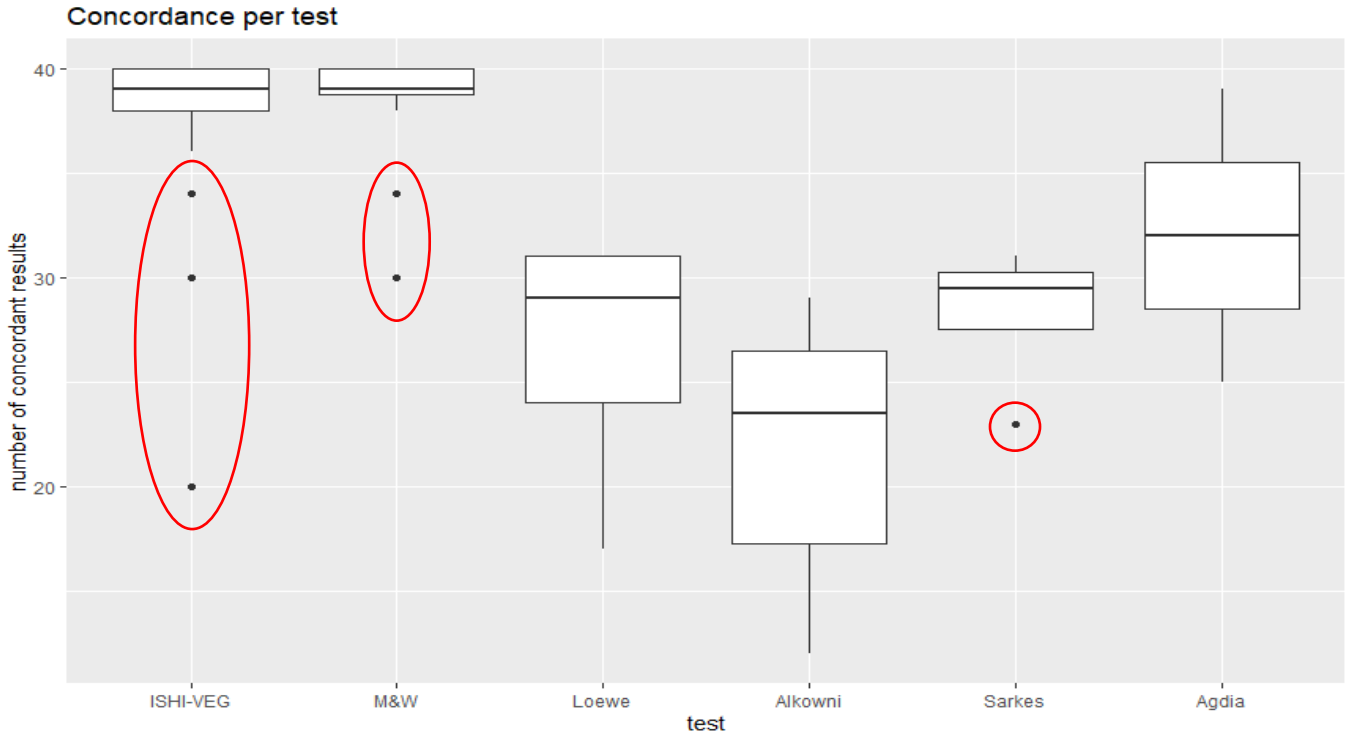


Figure 4. Box and whisker plot of the number of concordant results (true positives + true negatives) per test (data sets with >10% missing or undetermined results, non-concordant controls or non-compliance with essential aspects of the protocol are already excluded). Outlier data sets excluded from the analysis are encircled in red. The Abiopep test is not depicted as this test was not part of the TPS and was performed by only two labs.

Table 12. Number of participants per test, excluded data sets and number of remaining data sets included in the analysis described in this report.

Test	# Participants	Excluded data sets (reason for exclusion)	# Data sets included in TPS analysis
Real-time RT-PCR (ISHI-VEG)	24	P01 (protocol deviation) P05 (>10% undetermined results) P16 (outlier) P19 (protocol deviation) P21 (outlier) P23 (non-concordant controls) P25 (>10% undetermined results) P26 (outlier)	16
Real-time RT-PCR (M&W)	20	P02 (non-concordant controls) P05 (>10% undetermined results) P23 (non-concordant controls) P24 (outlier) P25 (>10% undetermined results) P26 (outlier)	14
End-point RT-PCR (Loewe)	9		9
End-point RT-PCR (Alkowni)	15	P15 (protocol deviation)	14
Isothermal amplification LAMP (Sarkes)	5	P16 (protocol deviation) P22 (outlier)	3
Isothermal amplification RPA (Agdia)	4	P01 (protocol deviation)	3
Real-time RT-PCR (Abiopep, outside scope TPS, Appendix 4)	2		2

3.2.4 Test performance comparison

ACC, DSE and DSP

Test performance was compared using the qualitative results submitted by participants. Undetermined results (UND) were excluded from the calculation of performance criteria (Table 10). Overall, the real-time RT-PCR tests showed a much better performance for the criteria assessed than the end-point RT-PCR and isothermal amplification tests (Table 13). Detailed results for each sample type per test are shown in Appendix 8. Note that the expected results (assigned values) were based on the true health status of the seeds and not on the expected results per method, in order to determine the most suitable method-test combination for the detection of ToBRFV in seeds.

The accuracy (ACC), diagnostic sensitivity (DSE) and diagnostic specificity (DSP) of both TPS real-time RT-PCR tests (ISHI-Veg and Menzel & Winter) was at least 97.7% for tomato and 96.3% for pepper. A third real-time RT-PCR outside of the scope of this TPS (Abiopep, (Bernabé-Orts *et al*., 2021), showed slightly lower values (at least 95.0% for tomato and 88.9% for pepper), but this was based on only two data sets. For the Abiopep test the results presented here were produced using the TaqMan® RNA-to-Ct™ 1-Step Kit. Additionally, two other kits (KAPA and AgPath, Appendix 4) were both tested once and produced comparable results to the TaqMan kit. Additional comparisons with this Abiopep test can be worth to consider, because it targets a different genome region (RNA-dependent RNA polymerase gene) than the ISHI-Veg test (movement protein and coat protein genes) and the Menzel & Winter test (coat protein gene) which could be advantageous in some situations.

The ACC and DSE of end-point RT-PCR tests (Loewe and Alkowni) were much lower than for real-time RT-PCR tests, due to a high number of false negative (FN) results, especially for pepper (Table 13, Fig. 5). Almost no ToBRFV was detected in pepper seeds with a medium level of ToBRFV, whereas for tomato seeds with a medium level of ToBRFV, the virus was detected at a rate of only 57.8% (TP) in the best case (Loewe, Appendix 8). Tomato seeds with a high level of ToBRFV produced FN results in 5.6-17.9% of the cases for Loewe and Alkowni respectively, whereas all other tests reliably detected ToBRFV when present at a high level.

The ACC and DSE of isothermal amplification tests were intermediate between the results of real-time RT-PCR tests and end-point RT-PCR tests (Table 13, Fig. 5). Performance criteria were comparable for Sarkes and Agdia for tomato, but for pepper Agdia was able to detect more samples with a medium level of ToBRFV, though only at a rate of 40% (Appendix 8). Both tests only reliably detected a high level of ToBRFV in tomato seeds. It should be noted that these results were based on only three valid data sets, so these values could be heavily influenced by the participant's proficiency.

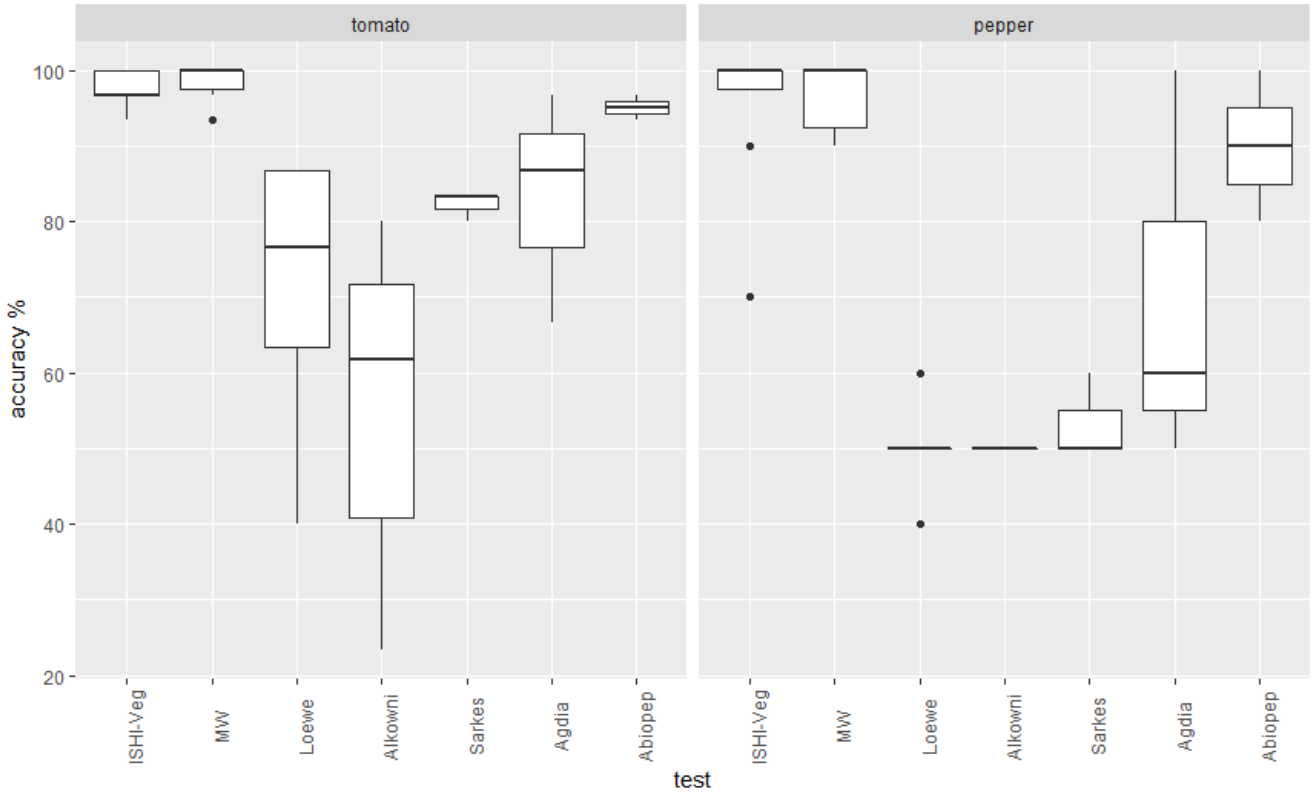
DSP was at least 95.7% for all tests due to the overall absence of false positives (FP).

Table 13. Overview of the TPS results. Excluded data sets (Table 12) not taken into account.

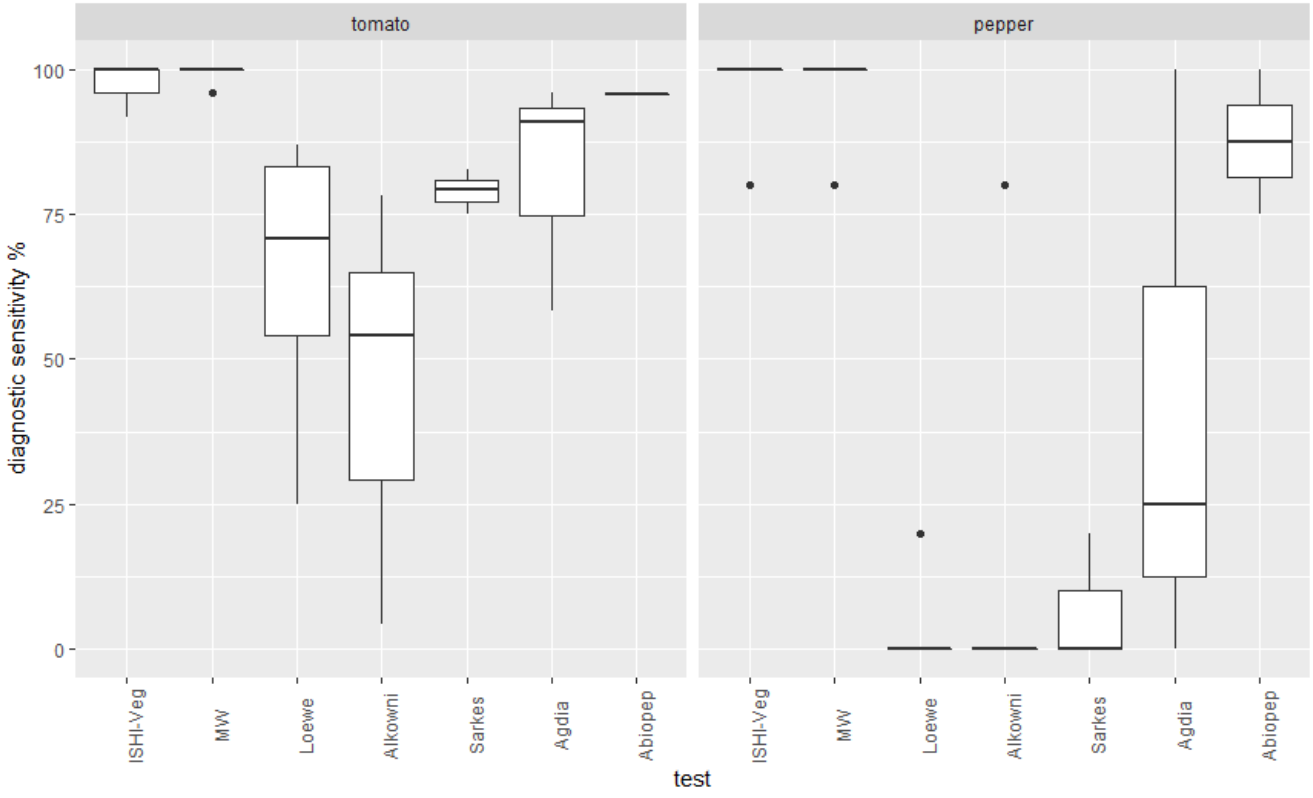
	Method - test - matrix combination													
	real-time RT-PCR				end-point RT-PCR				isothermal amplification				real-time RT-PCR	
	ISHI-Veg		M&W		Loewe		Alkowni		Sarkes		Agdia		Abiopep*	
Diagnostic parameter	**tomato**	**pepper**	**tomato**	**pepper**	**tomato**	**pepper**	**tomato**	**pepper**	**tomato**	**pepper**	**tomato**	**pepper**	**tomato**	**pepper**
Total nr of data sets	16	16	14	14	9	9	14	14	3	3	3	3	2	2
Total nr of data points	480	160	420	140	270	90	420	140	90	30	90	30	60	20
TP	376	78	333	68	138	1	147	4	56	1	57	6	45	8
TN	93	76	82	68	54	44	83	66	18	15	18	15	12	10
FP	2	2	1	0	0	1	1	3	0	0	0	0	0	0
FN	8	2	3	2	77	44	184	66	15	14	13	8	2	1
UND	1	2	1	2	1	0	5	1	1	0	2	1	1	1
TP %	78.3%	48.8%	79.3%	48.6%	51.1%	1.1%	35.0%	2.9%	62.2%	3.3%	63.3%	20.0%	75.0%	40.0%
TN %	19.4%	47.5%	19.5%	48.6%	20.0%	48.9%	19.8%	47.1%	20.0%	50.0%	20.0%	50.0%	20.0%	50.0%
FP %	0.4%	1.3%	0.2%	0.0%	0.0%	1.1%	0.2%	2.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
FN %	1.7%	1.3%	0.7%	1.4%	28.5%	48.9%	43.8%	47.1%	16.7%	46.7%	14.4%	26.7%	3.3%	5.0%
UND %	0.2%	1.3%	0.2%	1.4%	0.4%	0.0%	1.2%	0.7%	1.1%	0.0%	2.2%	3.3%	1.7%	5.0%
Concordant	469	154	415	136	192	45	230	70	74	16	75	21	57	18
Non-concordant	10	4	4	2	77	45	185	69	15	14	13	8	2	1
Concordance/Accuracy (ACC)	97.7%	96.3%	98.8%	97.1%	71.1%	50.0%	54.8%	50.0%	82.2%	53.3%	83.3%	70.0%	95.0%	90.0%
Diagnostic sensitivity (DSE)	97.9%	97.5%	99.1%	97.1%	64.2%	2.2%	44.4%	5.7%	78.9%	6.7%	81.4%	42.9%	95.7%	88.9%
Diagnostic specificity (DSP)	97.9%	97.4%	98.8%	100.0%	100.0%	97.8%	98.8%	95.7%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
False negative rate	2.1%	2.5%	0.9%	2.9%	35.8%	97.8%	55.6%	94.3%	21.1%	93.3%	18.6%	57.1%	4.3%	11.1%
False positive rate	2.1%	2.6%	1.2%	0.0%	0.0%	2.2%	1.2%	4.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Positive predictive value	99.5%	97.5%	99.7%	100.0%	100.0%	50.0%	99.0%	57.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Negative predictive value	92.1%	97.4%	96.5%	97.1%	41.2%	50.0%	31.0%	50.0%	54.5%	51.7%	58.1%	65.2%	85.7%	90.9%

* outside of the TPS scope.

(a) Accuracy



(b) Diagnostic sensitivity



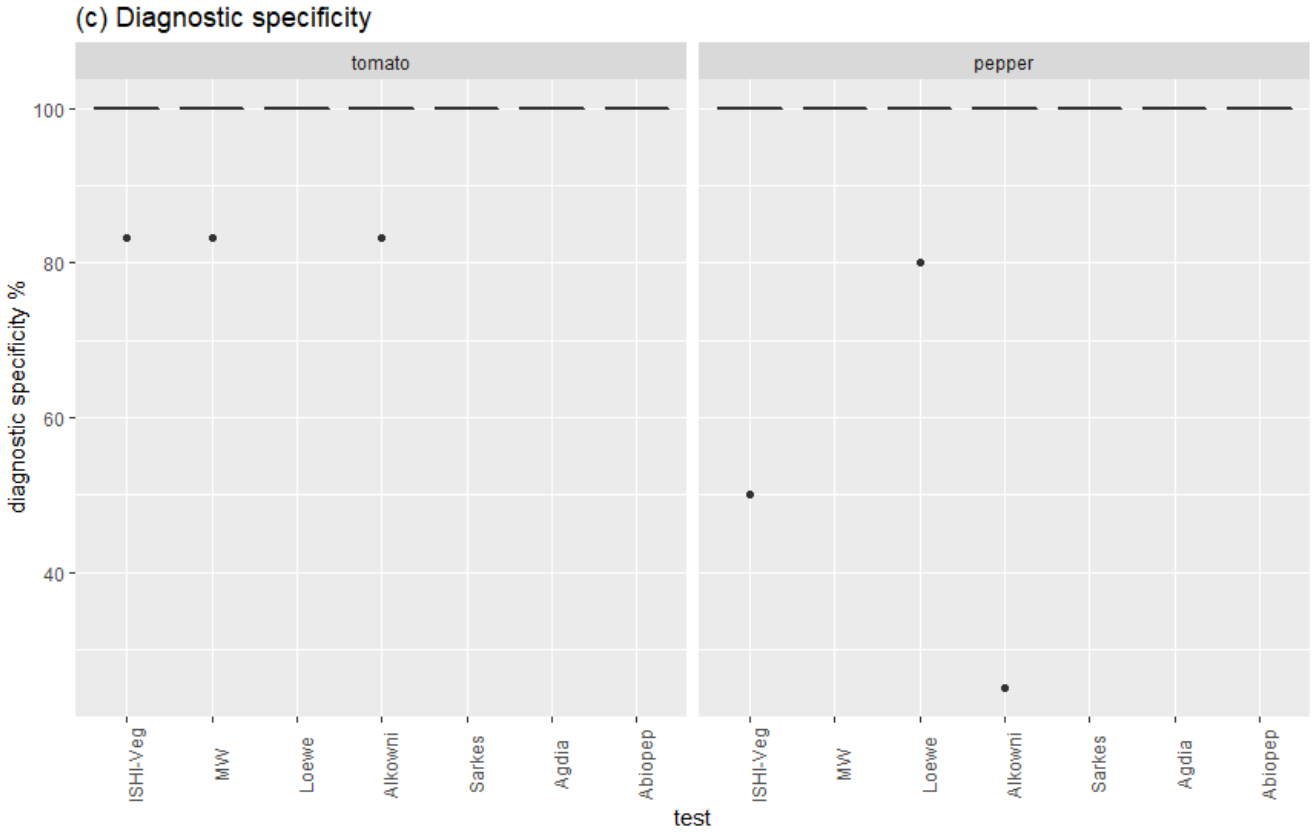


Figure 5. Box and whisker plots of accuracy (ACC), diagnostic sensitivity (DSE) and diagnostic specificity (DSE) per test and crop.

False positive results

A few FP results were reported from real-time RT-PCR and end-point RT-PCR tests (exact numbers per sample type can be found in Table 13, Appendix 8). It cannot be excluded that these FP results were due to cross-reactions when other tobamoviruses were present in the ToBRFV negative samples (Appendix 5). Another explanation could be cross-contamination with ToBRFV positive samples. However, FP only concerned a few per cent of the ToBRFV negative samples.

Matrix effect

For all tests, ToBRFV detection appeared more difficult in pepper than in tomato seeds, although this effect was minimal for the real-time RT-PCR tests (Table 13, Fig. 5). Data from the TPS organiser showed that when ToBRFV negative pepper seeds were spiked with the same amount of infected tomato seeds as for highly infected tomato samples, the resulting Cq values were about 1 Cq higher than for tomato (Appendix 7, Table A7.2), indicating that the pepper matrix (slightly) inhibits the real-time RT-PCR. As mentioned above, this matrix effect was much larger for the end-point RT-PCR and isothermal amplification tests. Possibly, the larger amplicon size used in the end-point RT-PCR and isothermal amplification tests compared to real-time RT-PCR tests inhibits the reaction in a pepper seed matrix.

Cut-off values real-time RT-PCR

The combined quantitative results of all laboratories that performed one or more real-time RT-PCR tests (Appendix 9) showed that Cq values sometimes overlap between samples with a medium level of ToBRFV and negative samples. This illustrates that assigning a common Cq value cut-off is not feasible. As the Cq cut-off value depends on equipment, material and chemistry it needs to be verified by each laboratory itself when implementing the test. Therefore, only the qualitative interpretation of the test result was taken into account for the test performance comparison.

4. Conclusion

The results from this TPS and its preliminary experiments indicated that out of all methods tested here, real-time RT-PCR is the most sensitive method for the detection of ToBRFV in both tomato and pepper seeds. The ISHI-Veg and Menzel & Winter tests showed a diagnostic sensitivity and diagnostic specificity of at least 97%. The Abiopep test showed a slightly lower diagnostic sensitivity (tomato 96%, pepper 89%), but this test was not part of the TPS and only based on two data sets.

DAS-ELISA (comparative experiments), end-point RT-PCR and isothermal amplification all resulted in a high number of false negative results, especially for pepper seeds. The diagnostic sensitivity of these tests was considerably lower than of any of the real-time RT-PCR tests, with the highest diagnostic sensitivity values for the Agdia test (tomato 81%, pepper 43%). It should be noted that for the isothermal amplification tests this conclusion was based on only three data sets.

For all tests, the number of false positives was low, resulting in a high diagnostic specificity. It cannot be excluded that the few false positive results that were reported from real-time RT-PCR and end-point RT-PCR tests were due to cross-reactions with other tobamoviruses or cross-contamination with ToBRFV positive samples, but this is unlikely to play a large role since it only concerned a few per cent of the ToBRFV negative samples.

In conclusion, the results of this study showed that the real-time RT-PCR tests (ISHI-Veg and Menzel & Winter) reliably and correctly indicated the presence or absence of ToBRFV in all samples with only a few percent false negative and false positive results. In contrast, DAS-ELISA, end-point RT-PCR and isothermal amplification tests as assessed in this study, appeared unsuitable for reliable detection of ToBRFV in tomato and pepper seeds, because they are not sensitive enough. Both real-time RT-PCR tests have been recommended for the detection of ToBRFV in seeds by the European and Mediterranean Plant Protection Organisation (EPPO, 2021) (PM 7/146) and are currently required for testing of tomato and pepper seeds in the EU emergency measures. The TPS results confirm the suitability of these tests for the detection of ToBRFV in tomato and pepper seeds. Additional comparisons with a recently developed real-time RT-PCR that targets a different genome region (Bernabé-Orts *et al*., 2021), can be worth to consider.

5. Acknowledgement

We thank the companies that provided seed lots for this TPS. Without their cooperation, this study would not have been possible. We thank Agdia for providing AmpliFire® machines. Many thanks to Natasa Mehle for critically reading the report and to our NVWA colleagues from the Molecular Biology group for advice.

6. Disclaimer

The results presented in this report only reflect this specific case study and the associated performance results of the commercial reagents at the time when they were included in the study.

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Appendix 1 – DAS-ELISA protocols (comparative studies)

Loewe

LOEWE® Standard Complete Kit for DAS-ELISA for 480 Tests

Assay Principle

During the first step of the assay the surface of a microtiter plate is coated with the antigen-specific coating-antibody (IgG). When an antigen-containing sample is added during the second step, the antigen binds to the immobilized IgG, forming an antibody-antigen complex. This complex reacts with the enzyme-labelled antibody-AP-conjugate (Conjugate) during the third step by forming a double-antibody sandwich. During the fourth step the coupled alkaline phosphatase (AP) reacts with the substrate 4-nitrophenylphosphate in an enzymatic reaction, resulting in yellow coloured 4-nitrophenol as product. This colour development can be evaluated visually or measured in a spectrophotometer at 405 nm after 1 and 2 hours.

Content of the Kit

Coating Antibody Solution (IgG)	0.5 ml
Antibody-AP-conjugate Solution (Conjugate)	0.5 ml
Positive Control Lyophilisate	1 x 10 tests or 2 x 5 tests
Negative Control Lyophilisate	1 x 10 tests or 2 x 5 tests
Coating Buffer Powder	1 Liter
Wash Buffer Powder	1 x 5 Liter
Conjugate/Sample Buffer Powder	2 x 1 Liter
Substrate Buffer Solution	25 ml 5 x concentrate
Substrate	5 tablets á 20 mg
Tween ™ 20	5 ml
High-Binding ELISA Plate with 1 sealing tape	5 pieces

Handling and Storage of Antibodies and Controls

The antibodies must be kept refrigerated (ca. 4°C) upon receipt. Once opened, we recommend using the reagents within 5 months. Our DAS-ELISA reagents are standardized for use at a dilution of 1:200 and a test volume of 200 µl/well. Following dilution scheme can be used for preparation of the IgG or Conjugate working solutions:

Nr. of wells to be filled	Stock Solution from original vial	Buffer IgG in Coating Buffer Conjugate in Conjugate/Sample Buffer
1x8 (1 stripe)	8.5 µl	1.7 ml
4x8	34 µl	6.8 ml
96 (1 plate)	100 µl	20 ml
480 (5 plates)	500 µl	100 ml

Positive and Negative Controls must be kept refrigerated. Once dissolved as indicated on the Certificate of Analysis, it is advised to aliquot the controls and store them frozen until use. Repeated freezing and thawing should be avoided as it can result in loss of activity, especially with Positive Controls.

Analytical data and other product specifications can be derived from the *product specification sheet* that is included with each kit.

LOEWE®

Buffer Preparation

Coating Buffer	Dissolve the content of the sachet in ca. 900 ml distilled water, adjust pH to 9.6 and fill up to 1 l. Store refrigerated until use. Keep frozen in glass bottles for long-term storage.
Wash Buffer	Dissolve content of the sachet in 5 l of distilled water. Add 2.5 ml of Tween ™ 20, adjust pH to 7.4. Store refrigerated. Keep frozen for long-term storage.
Conjugate/Sample Buffer	Dissolve content of the sachet in ca. 900 ml water and adjust pH to pH 7.4 with sodium hydroxide. Add 0.5 ml Tween ™ 20 and fill up to 1 l. If desired 0.01% sodium azide can be added. Store refrigerated for not longer than 1 week. We recommend freezing aliquots and use the buffer as fresh as possible.
Substrate Buffer	Dilute 25 ml of 5 x-concentrate with ca. 100 ml of water. Adjust pH to 9.8 with HCl before filling up to 125 ml. Store refrigerated. Keep frozen in glass bottles for long-term storage.
Substrate Solution	Dissolve 1 substrate tablet in 20 ml diluted substrate buffer (1x) **immediately prior to use.**

Please note: Our buffer formulations **do not contain sodium azide.**

Assay Procedure

Steps	Dilution of Reagents	Add (per well)	Incubate	at	Wash*
1. Application of coating-antibody (IgG)	Dilute IgG **1:200** from original vial in Coating Buffer	0.2 ml	4 h (Cover with sealing tape)	37°C	4 x
2. Sample application	Prepare samples at a **1:20** dilution in Sample Buffer, if not stated otherwise in the product certificate. (Dilute LOEWE® Positive or Negative Controls **in 2.1 ml** Sample Buffer, if not specified otherwise.)	0.2 ml	over night (Cover with sealing tape)	4°C	4 x
3. Application of antibody-AP-conjugate	Dilute AP-conjugate **1:200** from original vial in Conjugate Buffer	0.2 ml	4 h (Cover with sealing tape)	37°C	4 x
4. Enzymatic assay	Substrate solution	0.2 ml	1 - 2 h	room temp.	-

***Washing:** After each incubation step, the reagents are removed with Wash Buffer by four washing cycles using an automated washer.

Evaluation

After 1 and 2 hours of substrate incubation, evaluate the reaction visually or read photometrically at 405 nm. We strongly advise to add the positive and negative controls to each plate for verification of a strong positive and a low negative reaction. To determine potential background of healthy plants, add fresh non-infected extracts of the tested species and tissue at the same dilution to each plate. However, the positive/negative threshold needs to be determined by the user, as it depends on many factors, such as plant species and its physiological conditions (e.g. tissue type, age).

LOEWE®

Instructions for ELISA: Standard Double Antibody Sandwich Assay (DAS-ELISA)

Assay Principle

During the first step of the assay the surface of a microtiter plate is coated with the antigen-specific coating-antibody (IgG). When an antigen-containing sample is added during the second step, the antigen binds to the immobilized IgG, forming an antibody-antigen complex. This complex reacts with the enzyme-labelled antibody-AP-conjugate during the third step by forming a double-antibody sandwich. During the fourth step the alkaline phosphatase (AP) reacts with the substrate 4-nitrophenylphosphate in an enzymatic reaction, resulting in yellow coloured 4-nitrophenol as product. This colour development can be evaluated visually or measured in a spectrophotometer at 405 nm after 1 and 2 hours.

Handling and Storage of the Reagents

Our DAS-ELISA reagents are standardized for use at a dilution of 1:200 and a test volume of 200 µl/well. The products must be kept refrigerated (ca. 4°C) upon receipt. Once opened, we recommend using the reagents within 5 months.

Assay procedure

Steps	Dilution of Reagents	Add (per well)	Incubate	at	Wash*
1. Application of coating-antibody (IgG)	Dilute IgG **1:200** from original vial in Coating Buffer	0.2 ml	4 h	37°C	4 x
2. Sample application:	Prepare samples at a **1:20** dilution in Sample Buffer, if not stated otherwise in the product certificate. (Dilute LOEWE® **positive or negative controls in 2.1 ml** Sample Buffer, if not specified otherwise.)	0.2 ml	over night	4°C	4 x
3. Application of antibody-AP-conjugate	Dilute AP-conjugate **1:200** from original vial in Conjugate Buffer	0.2 ml	4 h	37°C	4 x
4. Enzymatic assay	Substrate Solution	0.2 ml	1 - 2 h	room temp.	-

***Washing:** After each incubation step, the reagents are removed with Wash Buffer by four washing cycles using an automated washer.

Buffer Formulations

Coating Buffer	1.59 g Na_2CO_3 2.93 g $NaHCO_3$	Dissolve in distilled water and fill to 1 l. Adjust pH 9.6. (Store refrigerated)
Wash Buffer	8.0 g NaCl 2.9 g Na_2HPO_4 x 12 H_2O 0.2 g KH_2PO_4 0.2 g KCl 0.5 ml Tween 20	Dissolve in distilled water and fill to 1 l. Adjust pH 7.2 - 7.4. (Store refrigerated)
Conjugate/Sample Buffer for sample preparation and conjugate dilution	Ingredients for Wash Buffer formulation (see above) *and add*: 20 g polyvinyl pyrrolidone (K10-K40) 2 g bovine serum albumin 0.1 g NaN_3 (only if desired)	Dissolve in distilled water and fill to 1 l. Adjust pH 7.4. (Store refrigerated for no longer than 1 week. We recommend freezing aliquots and using the buffer solution as fresh as possible.)
Substrate Buffer	97 ml diethanolamine 0.2 g $MgCl_2$ x 6 H_2O	Dissolve in distilled water and fill to 1 l. Adjust pH to 9.8 with 1 N HCl.
Substrate Solution	1 mg/ml 4-nitrophenylphosphate-di-Na-salt in substrate buffer	Prepare this solution immediately prior to use!

Evaluation

We strongly advise to add positive and negative controls to the plate. To determine potential background of healthy plants, fresh non-infected extracts of the tested species, should be added to the plate. The positive/negative threshold needs to be determined by the user, as it depends on many factors, such as plant species and its physiological conditions (e.g. tissue type, age).

User Guide: DAS-ELISA Reagent Set

General User Guide • GEB / ECI • Alkaline Phosphatase

Test Principle, Intended Use and Limitations

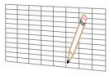
This product is intended for the qualitative detection of the target analyte via a direct, double antibody sandwich protocol known as DAS-ELISA. Upon successful completion of the test, samples containing the target analyte will turn yellow, due to the alkaline phosphatase enzyme label, while negatives will remain colorless. Visit the product webpage for information regarding host reactions, cross-reactions, or other limitations.

Handling Information

Antibodies should be stored refrigerated (2 - 8 °C) between uses. All test materials should be warmed to room temperature (18 - 30 °C) before use. For materials provided please see the product webpage. The buffers necessary to run this assay can be purchased as buffer pack ACC 00111. Do not store 1X buffers for more than one day.

Safety

Agdia recommends reading all relevant SDS sheets before using assay components: http://docs.agdia.com/DataSheets.aspx.



Test Preparation

1. Visit the product webpage to view buffer formulations, logsheet, and other documents.
2. Record lot numbers of materials to be used in the test using the logsheet.
3. Prepare a humid box by lining an airtight container with a wet paper towel.
4. Mix both concentrated and diluted antibodies thoroughly before each use.

Scan for buffer formulations

Prepare Capture Antibody

1. Prepare the capture antibody (CAB) in a non-binding container, such as Agdia's sample cups (ACC 00960).
2. Dilute the thoroughly-mixed CAB, per the dilution on the label, in 1X carbonate coating buffer (see example). You will need 100 µL of diluted CAB per well; a full plate will need 10 mL.

   Example: (Wells Used 16 x 100 µL) ÷ 200† = 8 µL Capture Antibody
   †Bottle dilution will be either 100 or 200
3. Thoroughly mix and pipette 100 µL of diluted CAB into each testwell of the provided high-bind microtiter plate.
4. Incubate plate in the humid box for either 4 hours at room temperature (18 - 30 °C) or overnight at 2 - 8 °C.
5. Coated plates should be used within 24 hours.

Positive and Negative Control Preparation

1. Use General Extract Buffer (GEB) to hydrate fresh controls, according to label, at least five minutes before use.
2. Recap and mix thoroughly.
3. Use of frozen or aliquoted controls comes with increased stability risks and may not match expected O.D. values.

Sample Preparation and Plate Loading

1. Sample symptomatic tissue if possible. Other plant parts may be tested, including asymptomatic tissue.
2. At the time of testing, grind and dilute the samples at a 1:10 ratio with GEB.

   Example: 0.3 g plant tissue, extracted with 3 mL of GEB.
3. Empty coated plate contents and wash 3 times with 1X PBST.
4. Tap plate dry using lint-free paper towel.
5. Dispense 100 µL of the extracted samples, positive control, negative control, and extraction buffer into the plate following your logsheet.
6. Incubate plate in the humid box for either 2 hours at room temperature or overnight at 2 - 8 °C.

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Prepare Enzyme Conjugate

1. Prepare the enzyme conjugate (ECA) in a non-binding container, such as Agdia's sample cups (ACC 00960).
2. Dilute the thoroughly-mixed ECA, per the dilution on the label, in 1X ECI buffer (see example). You will need 100 µL of diluted ECA per well; a full plate will need 10 mL.

   Example: (Wells Used 16 x 100 µL) ÷ 200† = 8 µL Enzyme Conjugate

   †Bottle dilution will be either 100 or 200
3. Wash the sample from the plate 8 times using 1X PBST.
4. Tap plate dry using lint-free paper towel.
5. Thoroughly mix and pipette 100 µL of diluted ECA into each testwell.
6. Incubate plate in the humid box for 2 hours at room temperature.

Prepare Substrate

1. Add 1 PNP substrate tablet per 5 mL of 1X PNP substrate buffer into a dedicated container and keep in the dark until use. You will need 100 µL of diluted PNP solution per well; a full plate will need 10 mL. Ensure tablets are dissolved before use.
2. Wash the ECA from the plate 8 times using 1X PBST.
3. Tap plate dry using lint-free paper towel.
4. Pipette 100 µL of dissolved PNP solution into each testwell.
5. Incubate, protected from light, for 1 hour at room temperature.

Interpreting Results

1. Visually inspect wells and remove bubbles, if present. Measure O.D. values with a spectrophotometer at 405 nm or 405 nm with a 650 nm blank.
2. The test is valid if the positive and negative control O.D. results meet expected values (see Certificate of Analysis).
3. Sample interpretations should be performed on a case-by-case basis. Plant tissue interactions with ELISAs can vary greatly between plant species and even varieties. Certain healthy tissues can cause an elevated or higher than normal O.D. value. In this case, a healthy sample(s) of the same species or variety is needed to determine the healthy average.
4. Generally, positive and negative thresholds can be determined by using 2 times the healthy average. Any samples with an O.D. value higher than 2 times the healthy average are positive, and samples with an O.D. value below 2 times the healthy average are negative. An alternative method for threshold calculations is the healthy average plus 3 times the standard deviation of the healthy sample set.

Method 1	*Healthy Avg.*	*0.105*	*2 x Healthy Avg.*	*0.210*
	Sample 1	*0.355 (Positive)*	*Sample 2*	*0.190 (Negative)*

Method 2	*Healthy Avg.*	*0.105*	*Std. Dev.*	*0.030*	*Healthy Avg. + 3 x Std. Dev.*	*0.195*
	Sample 1	*0.355 (Positive)*	*Sample 2*	*0.190 (Negative)*		

5. Positive O.D. values indicate the presence of the target pathogen (or in some cases, a closely related pathogen). Visit the product webpage to see if any other pathogens are known to cross-react with this test. As with all diagnostic tools, Agdia recommends confirming all results with a secondary detection method before making any economic decisions (ex: discarding plants due to positive test results, etc.).

Warranty

Agdia reagents are warrantied for performance issues that arise from manufacturer defect. See product packaging for relevant expiration dates. Agdia's return policy can be found at www.agdia.com/customer-support/return-policy.

Additional Information

If you would like more information on how to run ELISA, please see Agdia's FAQ section, http://www.agdia.com/customer-support/frequent-questions-and-troubleshooting. For further documentation, including this user guide, buffer formulations, and a logsheet, please see Agdia's specific product webpages. For answers to your technical questions, please contact us at techsupport@agdia.com.

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User Guide: Buffer Formulations

Buffers from Scratch

Instructions

Prepare buffers fresh each day. Storing 1X buffers for extended periods comes with risks such as precipitation, growth, or contamination which can have an effect on test performance. For those willing to assume this risk Agdia has recommended storage conditions and preservatives where possible.

Chemicals from different vendors can vary in purity level and lot-to-lot consistency, which can affect test results. You assume this risk when purchasing chemicals separately to make buffers per these formulations.

Pre-mixed buffer packs may also be purchased from Agdia. Buffer packs are recommended since Agdia evaluates suppliers to
reduce the risk of inconsistent test performance attributed to variation in raw material quality.

Safety

Agdia recommends reading all relevant SDS sheets.

Carbonate Coating Buffer (CCB) (1X) 1. Used to dilute capture antibodies. 2. Dissolve components in 800 mL of water[1]. 3. Adjust pH to the range of 9.5 - 9.7. 4. Adjust volume to 1000 mL with water[1]. 5. **Optional**: Add sodium azide (Sigma S2002) at a rate of 0.2 g per liter (0.02 %) and store at 2 - 8 ºC.	Sodium carbonate (anhydrous) Sodium 1.59 g bicarbonate 2.93 g Water[1] 1000 mL
General Extract Buffer (GEB) (1X) 1. GEB is used to grind and dilute samples. 2. Dissolve components in 800 mL of 1X PBST. 3. Adjust pH to the range of 7.2 - 7.8. 4. Adjust volume to 1000 mL with 1X PBST. 5. **Optional**: Add sodium azide (Sigma S2002) at a rate of 0.2 g per liter (0.02 %) and store at 2 - 8 ºC.	**Sodium sulfite (anhydrous)** 1.30 g Polyvinylpyrrolidone (PVP) MW 24-40,000 20.00 g Powdered egg (chicken) albumin, Grade II 2.00 g TWEEN® 20 20.00 g 1X PBST 1000 mL
ECI Buffer (1X) 1. ECI is used to dilute enzyme conjugate antibodies. 2. Dissolve components in 800 mL of 1X PBST. 3. Adjust pH to the range of 7.2 - 7.6. 4. Adjust volume to 1000 mL with 1X PBST. 5. **Optional**: Add sodium azide (Sigma S2002) at a rate of 0.2 g per liter (0.02 %) and store at 2 - 8 ºC.	Bovine serum albumin (BSA) 2.00 g Polyvinylpyrrolidone (PVP) MW 24-40,000 1X 20.00 g PBST 1000 mL
PNP Substrate Buffer (1X) 1. **PNP substrate buffer is used with PNP substrate tablets to make** an active substrate for alkaline phosphatase ELISA systems. 2. Dissolve components in 800 mL of water[1]. 3. Adjust pH to the range of 9.7 - 9.9 with hydrochloric acid. 4. Adjust volume to 1000 mL with water[1]. 5. **Optional**: Add sodium azide (Sigma S2002) at a rate of 0.2 g per liter (0.02 %) and store at 2 - 8 ºC.	Magnesium chloride hexahydrate 0.10 g Diethanolamine 97.00 mL Water[1] 1000 mL

[1]Use deionized or similar purity water.

PBST Buffer (Wash Buffer) (1X)

1. **PBST buffer is used to wash ELISA plates.**
2. Dissolve components in 800 mL of water[1].
3. Adjust pH to the range of 7.2 - 7.6.
4. Adjust volume to 1000 mL with water[1].
5. **Optional**: Store at 18 - 30 ºC. Sodium azide is not recommended.

Sodium chloride	8.00 g
Sodium phosphate, dibasic (anhydrous) Potassium	1.15 g
phosphate, monobasic (anhydrous) Potassium chloride	0.20 g
TWEEN® 20	0.20 g
Water[1]	0.50 g
	1000 mL

PBST Buffer (Stock Solution) (20X)

1. **20X PBST buffer, Stock Solution, can be stored and diluted** to 1X PBST as needed.
2. Dissolve components in 800 mL of water[1].
3. Adjust volume to 1000 mL with water[1].
4. Store at 18 - 30 ºC. Sodium azide is not recommended. Shelf life is one year.

Preparing 1X PBST from 20X PBST

5. Dilute 1 volume of 20X PBST concentrate with 19 volumes of deionized or similar purity water before use.

   Example: To prepare 1000 mL of 1X PBST, mix 50 mL of 20X PBST concentrate with 950 mL of water.

6. Adjust the pH to the range of 7.2 to 7.6.
7. **Optional**: Store at 18 - 30 ºC. Sodium azide is not recommended.

Sodium chloride	160.00 g
Sodium phosphate, dibasic (anhydrous) Potassium	23.00 g
phosphate, monobasic (anhydrous) Potassium chloride	4.00 g
TWEEN® 20	4.00 g
Water[1]	10.00 g
	1000 mL

PBS Buffer (1X)

1. **PBS Buffer is used as a solvent in MPBS.**
2. Dissolve components in 930 mL of water[1].
3. Adjust pH to the range of 7.3 - 7.5.
4. Adjust volume to 1000 mL with water[1].
5. **Optional**: Store at 18 - 30 ºC. Sodium azide is not recommended.

Sodium phosphate, dibasic (anhydrous) Potassium	1.15 g
chloride	0.20 g
Potassium phosphate, monobasic (anhydrous) Sodium	0.20 g
chloride	8.00 g
Water[1]	1000 mL

Indirect Sample Extraction Buffer (IEB) (1X)

1. IEB is used to grind and dilute samples.
2. Dissolve components in 800 mL of water[1].
3. Adjust pH to the range of 9.5 - 9.7.
4. Adjust volume to 1000 mL with water[1].
5. **Optional**: Add sodium azide (Sigma S2002) at a rate of 0.2 g per liter (0.02 %) and store at 2 - 8 ºC.

Sodium carbonate (anhydrous) Sodium	1.59 g
bicarbonate	2.93 g
Polyvinylpyrrolidone (PVP) MW 24-40,000 Water[1]	20.0 g
	1000 mL

MEB Buffer (1X)

1. MEB is used to grind and dilute samples.
2. Dissolve components in 200 mL of 1X PBST.
3. Stir for 30 minutes.
4. Adjust pH to the range of 7.2 - 7.8.
5. **Optional**: Store at 2 - 8 ºC. Sodium azide is not recommended.

TWEEN® 20	1.25 g
Nonfat dried milk 1X	1.00 g
PBST	200 mL

ECM Buffer (1X)

1. ECM is used to dilute enzyme conjugate antibodies.
2. Dissolve components in 100 mL of 1X PBST.
3. Stir for 30 minutes.
4. Adjust pH to the range of 7.2 - 7.6.
5. **Optional**: Store at 2 - 8 ºC. Sodium azide is not recommended.

Nonfat dried milk 1X	0.40 g
PBST	100 mL

MPBS Buffer (1X) (BRA Blocking Buffer)

1. MPBS is used to block Bacterial Reagent Set (BRA) ELISA plates.
2. Dissolve components in 18 mL of 1X PBS.
3. Stir for 30 minutes.
4. Adjust pH to the range of 7.2 - 7.6.
5. Adjust volume to 20 mL with 1X PBS.
6. **Optional**: Add sodium azide (Sigma S2002) at a rate of 0.2 g per liter (0.02 %) and store at 2 - 8 ºC.

Nonfat dried milk 1X	1.00 g
PBS	20 mL

MPBST Buffer (1X) (BRA Antibody Diluent)

1. MPBST is used to dilute enzyme conjugate antibodies.
2. Dissolve components in 20 mL of 1X PBST.
3. Stir for 30 minutes.
4. Adjust pH to the range of 7.2 - 7.6.
5. **Optional**: Add sodium azide (Sigma S2002) at a rate of 0.2 g per liter (0.02 %) and store at 2 - 8 ºC.

Nonfat dried milk 1X	0.50 g
PBST	20 mL

Leibniz-Institut
DSMZ-Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH

Double Antibody Sandwich ELISA (DAS-ELISA)

Our ELISA reagents are optimised using greiner bio-one microplates, medium binding. Before opening the tubes containing coating antibody (IgG) and IgG-AP- Conjugate please spindown all the liquid by a short centrifugation (approx. 3000rpm for a few seconds).

1. Dilute specific antibody in coating buffer (recommended dilution see delivery note and tube); i.e. 20µl in 20 ml buffer at a recommended dilution of 1:1000 or 40µl in 20 ml buffer at a recommended dilution of 1:500. Add 200µl to each well of the microtiter plate.

2. Cover the plates and incubate at 37 °C for 2- 4 h.

3. Wash plate with PBS-Tween using wash bottle, soak for a few minutes and repeat washing two times. Blot plates by tapping upside down on tissue paper.

4. Extract samples 1:20 (w/v) in extraction buffer. Add 200 µl aliquots of the test sample to duplicate wells.

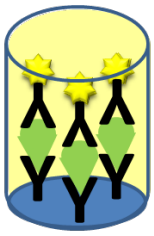
5. Cover the plates and incubate overnight at 4 °C.

6. Wash three times as in step 3.

7. Add 200 µl enzyme conjugate, recommended dilution is given in the delivery note, in conjugate buffer.

8. Cover the plates and incubate at 37 °C for 2- 4 hours.

9. Wash three times as in step 3.



10. Add 200 µl aliquots of freshly prepared substrate (1 mg /ml para- nitrophenyl- phosphate in substrate buffer) to each well.

11. Cover the plate and incubate at 37°C for 30-60 min, or as long as necessary to obtain clear reactions.

12. Assess results by:

a) Visual observation
b) Spectrophotometric measurement of absorbance at 405 nm

Reference
Clark, M. F. and Adams. A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483

Buffers used in ELISA

1. ***Coating buffer (pH 9.6)***

   1.59 g sodium carbonate (Na_2CO_3)
   2.93 g sodium bicarbonate ($NaHCO_3$)
   0.20 g sodium azide (NaN_3)
   Dissolve in 900 ml H_2O, adjust pH to 9.6 with HCl and make up to 1 l.

2. ***PBS (pH 7.4) phosphate buffered saline***

   8.0 g sodium chloride (NaCl)
   0.2 g monobasic potassium phosphate (KH_2PO_4)
   1.15 g dibasic sodium phosphate (Na_2HPO_4)
   0.2 g potassium chloride (KCl)
   0.2 g sodium azide (NaN_3)
   Dissolve in 900 ml H_2O, adjust pH to 7.4 with NaOH or HCl and make up to 1 l.

3. ***PBS-Tween (PBST)***

   PBS + 0.5 ml Tween 20 per liter

4. ***Sample extraction buffer (pH 7.4)***

   PBST + 2% PVP (e.g. Serva PVP-15 polyvinyl pyrrolidone)

5. ***Sample extraction buffer (pH 8.5) for Begomoviruses***

   0.05 M Tris containing 0.06 M sodium sulfite, pH 8.5

6. ***Conjugate buffer***

   PBST + 2% PVP + 0.2% egg albumin (e.g. Sigma A-5253)

7. ***Substrate buffer***

   97 ml diethanolamine
   600 ml H_2O
   0.2 g sodium azide (NaN_3)
   Adjust to pH 9.8 with HCl and make up to 1 liter with H_2O

Buffers can be stored at 4 ° C for at least 2 months. Warm to room temperature before use.

primediagnostics

Protocol

Double antibody sandwich (DAS) ELISA

Our reagents are optimized for use in DAS-ELISA using certified NUNC-Immuno Plates Maxisorp F96 and operating with a working volume of 200 µl per well.
The incubations are performed in a tightly closed humid box. During incubation the plates are covered with a lid or tape.
For washing: empty the wells and soak the wells 15 seconds with washing buffer while shaking. Repeat this 3 – 5 times. Remove any liquid by blotting the plates on paper towels.

Buffers and chemicals:

- **Coating buffer:** **1.59 gr Na_2CO_3, 2.94 gr $NaHCO_3$, pH 9.6**
  Add demineralized water to 1000 ml total volume.

PBS 0.01 M: **8.18 gr NaCl, 0.15 gr KCl, 0.27 gr KH_2PO_4,**
1.42 gr Na_2HPO_4 x 2 H_2O, pH 7.4.
Add demineralized water to 1000 ml total volume.

Washing buffer (PBST): **0.05 % Tween-20 in PBS 0.01 M**

Extraction buffer (SEB): **0.2% egg ovalbumine (grade II), 2% PVP40,**
0.05% Tween-20 and 0.05% NaN_3 in PBS 0.01 M

Substrate buffer: **97 ml diethanolamine, pH 9.8**
Add demineralized water to 1000 ml total volume.

Substrate: **15 mg paranitrophenylphosphate (pNPP) in 20 ml substrate buffer**

Procedure:

	Incubation buffer	Incubation time and temperature	Concentration of reagent 1000x
1. Coating	Coating buffer	Overnight at 4°C or 3h at 37°C	diluted coating antibody 10x diluted positive control
2. Controls and samples	Extraction buffer	Overnight at 4°C	
3. Conjugate	Extraction buffer	Overnight at 4°C or 3h at 37°C 30	1000x diluted AP-conjugate
4. Substrate	Substrate buffer	minutes at room temperature	0.75 mg/ml pNPP

Sample preparation:
Prepare a sample extract in an appropriate buffer and test the extract without further dilution. It is recommended to test a 10 times dilution of the extract as well.
We recommend using negative controls existing of healthy plant extracts originating from the appropriate host of the pathogen as well as an internal control existing of SEB.
The positive control produced by Prime Diagnostics is a qualitative control and can be used as an internal control for the assay only. We recommend using an in-house positive control aswell.

Remarks:

- **A sample is positive if the ratio (OD_{405} sample/OD_{405} healthy plant extract) is at least 2.**
- **The use of other dilutions for the reagents will cause differences in reactivity, specificity, selectivity and detection limits.**
- **Lower reaction volumes will cause higher detection levels and lower** ratio's**. Different**
- **incubation times and temperatures will cause differences in sensitivity and background reactions.**
- **If the buffers are to be stored for more than 1 week it is recommendable to add Sodium Azide in a final concentration of 0.05%.**

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Appendix 2 – Expertise of participating laboratories

Table A2.1. Reported expertise of each participating laboratory (coded).

		P01	P02	P03	P04	P05	P06	P07	P08	P09	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25	P26
Technical expertise for ToBRFV or other mechanically transmitted quarantine viruses (e.g. PSTVd) (routine analyses, method development, publications, participation in conferences etc.)	**nr of years**	35	40	18	15	5	3	4		8	12	8	10	20	8	20	10	15	5	2	1	4	5	20	10	10	4
Expertise in extraction of viruses from seeds	**nr of years**	35	40	7	15	5	3	4		8	7	8	10	10	8	15	10	15	7	1	1	4	10	5	1	10	4
	nr of total samples analyzed	>1000	>1000	>1000	>1000	>100	>1000	>1000		>1000	>100	>30	>1000	>30	>100	>1000	>1000	>1000	>1000	>100	<30	>100	>1000		>30	>1000	>100
	validation data submitted to EPPO database or other publications (Yes/No)	Yes	No	No	Yes	No	No	No		yes	No	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	No
Expertise in the use of end-point RT-PCR	**nr of years**		20	18	15					8	12	10		20	12	15	20			10	5	6	8	15	18		
	nr of total samples analyzed		>1000	>1000	>1000						>1000	>100		>100	>1000	>1000	>1000			>1000	>100	>100	>1000	>1000	>1000		
	validation data submitted to EPPO database or other publications (Yes/No)		No	Yes	Yes					Yes	No	No		No	Yes	No	Yes			No	No	No	Yes	Yes	Yes		
Expertise in the use of real-time RT-PCR	**nr of years**	10	20	12	12	5	3	4		8		5	10	10	10	15	15	10	7	10	1	5	8	18	2	10	4
	nr of total samples analyzed	>1000	>1000	>1000	>1000	>100	>1000	>1000		>1000		>30	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000		>100	>1000	>100
	validation data submitted to EPPO database or other publications (Yes/No)	Yes	No	Yes	Yes	No	No	No		Yes		No	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	No
Expertise in the use of isothermal RT-PCR	**nr of years**	10												5	7	1	10						1				
	nr of total samples analyzed	>1000												>30	>100	>30	>1000						<30				
	validation data submitted to EPPO database or other publications (Yes/No)	yes												Yes	Yes	No	No						No				
Authorized by the national competent authority to work with ToBRFV	**Yes/No**	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes
Previous participation in TPS or PT	**Yes/No**	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes
Traceability in place / QA in place	**Yes/No**	Yes	Yes	Yes	Yes	Yes	Yes	Yes		No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ISO 17025 accredited	**Yes/No**	Yes	Yes	Yes	Yes	Yes	Yes	Yes		No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No	No	under accredi-tation	No	Yes	Yes

Appendix 3 – TPS protocols

1 – Seed preparation and RNA extraction

Note: To keep the workload manageable please work with one sample set at the same time.

Consumables/chemicals

Note: wear a pair of safety glasses while working with GH+ buffer

Table A1. GH+ buffer (prepare fresh)

Ingredient	Amount	Final concentration
guanidine hydrochloride	573 g	6 M
sodium acetate (4M, pH5.2)	50 mL	0.2 M
EDTA Na_2 $2H_2O$	9.3 g	25 mM
PVP-10	25.0 g	2.5% w/v
Distilled water to	1.0 L	

Grinding bags (e.g. BIOREBA Extraction bags Universal-Long (Art. No. 470100), or equivalent depending on your seed milling system)
Dithiothreitol (DTT, 5M)
RNeasy Plant Mini Kit (Qiagen Cat. No. 74904)
96-100% ethanol
1.5 mL tubes (DNase, RNase free)

Protocol

IMPORTANT: Perform a single extraction per sample.

Note 1: the grinding method depends on your experience and equipment. If you have no experience with grinding seeds, we recommend to work very carefully and grind by hand. Grinding bags may get pierced by the seeds, therefore it is safer to grind the seeds first, repair the bag if necessary and add buffer after grinding. Tables A2 and A3 list the preferred methods for tomato and pepper seeds.

Note 2: only use an automated homogeniser if you have experience in using an automated homogeniser for virus extraction from seeds (grinding time is critical, grinding too short may result in not extracting enough virus, grinding too long can lead to degradation of the virus due to heat).

Note 3: grinding by hand may take several minutes per sample.

Note 4: 20 mL of GH+ buffer needs to be added to tomato seeds, while 40 mL of GH+ buffer needs to be added to pepper seeds because of their larger size.

Grinding tomato seeds:

1. Transfer 1000 seeds (one sample) to a grinding bag (or tube if you have a system for tubes).

2. Grind until all seeds are clearly cracked open and crushed (see Figure A1), using a hand-held homogeniser, pneumatic press, hammer or an automated homogeniser (see Table A2 for preferred grinding methods). Add 20 mL of GH+ buffer during or after grinding. If GH+ buffer is added after grinding, make sure everything is well mixed before proceeding to step 3. Optional: soak the seeds in GH+ buffer for 30-60 minutes at room temperature prior to grinding.

Table A2. Overview of preferred methods for grinding tomato seeds.

	Grinder	Remark
Primary choice	Hand-held homogeniser (Bioreba)	
	Pneumatic press	
	Hammer	
	Homex 6 (Bioreba)	
	Genogrinder/seed shaker	
	Stomacher	Soak seeds first 30-60 min, then grind for 90 sec
Secondary choice	Retsch	Cross contamination risk high, every sample needs its own bucket
	Ultra-Turrax	Cross contamination risk high, every sample needs its own rod

Grinding pepper seeds:

1. Transfer both subsamples of 500 seeds to the same grinding bag (or tube if you have a system for tubes), except when using the Genogrinder/seed shaker (see Table A3).
2. Grind until all seeds are clearly cracked open and crushed (see Figure A1), using a hand-held homogeniser, pneumatic press, hammer or an automated homogeniser (see Table A3 for preferred grinding methods). Add 40 mL of GH+ buffer during or after grinding. If GH+ buffer is added after grinding, make sure everything is well mixed before proceeding to step 3.

Table A3. Overview of preferred methods for grinding pepper seeds.

	Grinder	Remark
Primary choice	Hand-held homogeniser (Bioreba)	
	Pneumatic press	
	Hammer	
	Homex 6 (Bioreba)	
	Genogrinder/seed shaker	Grind each subsample of 500 seeds dry for 7 min at 1500 rpm, then add 20 ml of GH+ buffer per subsample. At step 3: centrifuge the tubes 1 min at 5000 rpm, then pool the two subsamples by combining 2 X 500 µl of seed extract from each subsample into a 1.5 mL tube.
Secondary choice	Retsch	Cross contamination risk high, every sample needs its own bucket
	Ultra-Turrax	Cross contamination risk high, every sample needs its own rod

After grinding (tomato and pepper seeds):

3. Transfer 1 mL of seed extract into a 1.5 mL tube and add 30 µL 5 M DTT.
4. Incubate for 15 min at 65 °C at 850 rpm.

Optional: store seed extracts on ice for use on the same day or at -20°C for use on a different day.

5. Centrifuge at 16,000 g at 4°C for 10 minutes

RNA isolation (RNeasy Plant Mini Kit*)
*centrifugation at 4°C (suitable for RNA extraction from tomato and pepper seeds)

1. Transfer 750 µl of the seed extract to the Qiashredder-spin-column (lila).
2. Centrifuge at 16,000 g at 4°C for 2 minutes.
3. Prefill a 1.5 ml Eppendorf tube with 300 µl absolute ethanol (>95%).
4. Transfer 600 µl of the flowthrough to the eppendorf tube with ethanol and mix by pipetting.
5. Transfer 700 µl of the extract/ethanol mix to the RNeasy-mini-column (pink).
6. Centrifuge at 16,000 g at 4°C for 1 minute.
7. Transfer filter to a new clean collection tube (not included in the kit) and at 700 µl RW1-washbuffer.
8. Centrifuge at 16,000 g at 4°C for 30 seconds.
9. Transfer filter to a new clean collection tube (not included in the kit) and at 500 µl RPE-wash buffer.
10. Centrifuge at 16,000 g at 4°C for 30 seconds.
11. Repeat step 9 and centrifuge at 16,000 g at 4°C for 2 minutes.
12. Transfer filter to a new clean 1.5 ml Eppendorf tube (included in the kit) and add 50 µl RNase free water directly to the filter.
13. Leave at RT for 1 minute and centrifuge at 8,000 g at 4°C for 1 minute.
14. Add another 50 µl RNase free water directly to the filter. Repeat step 13.

Store RNA extracts at -20°C until use as a template for the PCR tests. To prevent RNA degradation, keep the number of freeze/thaw cycles for the RNA extracts as low as possible, by performing PCR tests on the same day or by freezing multiple aliquots.

Reporting results

The exact method used should be reported in the TPS results Excel form

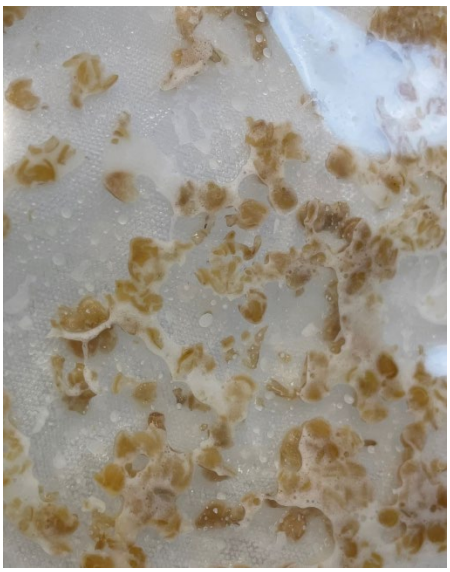


Figure A1. After grinding, all seeds should be cracked open and crushed. This photo shows tomato seeds ground in buffer using a hand-held homogeniser.

2 - Real-time RT-PCR – adapted from ISHI-Veg (2019)

Consumables/chemicals

Primers and probes (Table A2.1) (Thermo Fisher Scientific) (ordered by participant)
TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific, cat no. 4392938) (ordered by participant)
The participant has to use its own disposables and equipment.

Table A2.1 Primers, probes and their sequences

Name	Sequence (5' to 3')
CaTa28 Fw	GGT GGT GTC AGT GTC TGT TT
CaTa28 Pr	6FAM - AGA GAA TGG AGA GAG CGG ACG AGG - BHQ1
CaTa28 Rv	GCG TCC TTG GTA GTG ATG TT
CSP1325 Fw	CAT TTG AAA GTG CAT CCG GTT T
CSP1325 Pr	VIC - ATG GTC CTC TGC ACC TGC ATC TTG AGA - BHQ1
CSP1325 Rv	GTA CCA CGT GTG TTT GCA GAC A

Controls

Tomato NIC (sample 1) and PIC (sample 2), pepper NIC (sample 40) (processed by the participant using the RNA extraction protocol)

NAC: molecular grade water

PAC: RNA ordered from DSMZ by participant (follow DSMZ protocol to recover nucleic acids and dissolve in 100 µl of molecular grade water)

Experimental protocol

Dilute primers and probes to a working concentration of 10 µM. Prepare the real-time RT-PCR mix according to Table A2.2, and run the amplification program according to Table A2.3. Each sample is to be analysed in duplicate (i.e. two PCR wells per sample). Include NIC, PIC, NAC and PAC as control samples. Store all RNA extracts and controls at -20°C after use.

Table A2.2 Real-time RT-PCR mix

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Molecular grade water	-	4.3	-
CaTa28 Fw	10 µM	0.6	0.3 µM
CaTa28 Pr	10 µM	0.4	0.2 µM
CaTa28 Rv	10 µM	0.6	0.3 µM
CSP1325 Fw	10 µM	0.6	0.3 µM
CSP1325 Pr	10 µM	0.4	0.2 µM
CSP1325 Rv	10 µM	0.6	0.3 µM
TaqMan® RT-PCR Mix	2X	10	1X
TaqMan® RT Enzyme Mix	40X	0.5	1X
RNA	-	2	-
Total volume	-	20	-

Table A2.3 Amplification program

Step	Temp (°C)	Time	No. of Cycles
Reverse transcription	48	15 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60	1 min	

Interpretation of results

The specific products for ToBRFV will generate two exponential amplification curves.

Verification of the controls:
- NIC and NAC must generate no amplification.
- PIC and PAC must generate two exponential amplification curves.

If these conditions are met:
- A sample test will be considered positive if both duplicates generate exponential amplification curves with a Cq value below or equal to an appropriate cut-off*.
- A sample test will be considered negative if both duplicates generate no exponential amplification curves, or curves with a Cq value above an appropriate cut-off.
- Tests should be repeated if contradictory or unclear results are obtained.

*It should be noted that a preliminary cut-off value of 32 has been indicated in ISF-ISHI-Veg (2020). As a Cq cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

IMPORTANT: Non-specific (non-exponential) curves should be considered negative.

Reporting results

Cq values generated with the real-time RT-PCR protocol as well as qualitative results (positive, negative or undetermined) and used cut-off values should be reported in the TPS results Excel form. A copy of the real-time RT-PCR report with amplification curves and Cq values should be added.

3 - Real-time RT PCR – adapted from Menzel & Winter (2021)

Consumables/chemicals

Primers and probe (Table A3.1) (Thermo Fisher Scientific) (ordered by participant)
TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher scientific, cat no. 4392938) (ordered by participant)
The participant has to use its own disposables and equipment.

Table A3.1 Primers, probes and their sequences

Name	Sequence (5' to 3')
ToBRFVqs1	CAA TCA GAG CAC ATT TGA AAG TGC A
ToBRFVp1	6FAM – ACA ATG GTC CTC TGC ACC TG- BHQ1
ToBRFVqas2	CAG ACA CAA TCT GTT ATT TAA GCA TC

Controls

Tomato NIC (sample 1) and PIC (sample 2), pepper NIC (sample 40) (processed by the participant using the RNA extraction protocol)
NAC: molecular grade water
PAC: RNA ordered from DSMZ by participant (follow DSMZ protocol to recover nucleic acids and dissolve in 100 µl of molecular grade water)

Experimental protocol

Dilute primers and probes to a working concentration of 10 µM. Prepare the real-time RT-PCR mix according to Table A3.2, and run the amplification program according to Table A3.3. Each sample is to be analysed in duplicate (i.e. two PCR wells per sample). Include NIC, PIC, NAC and PAC as control samples. Store all RNA extracts and controls at -20°C after use.

Table A3.2 Real-time RT-PCR mix

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Molecular grade water	-	5.8	-
ToBRFVqs1	10 µM	0.6	0.3 µM
ToBRFVp1	10 µM	0.5	0.25 µM
ToBRFVqas2	10 µM	0.6	0.3 µM
TaqMan® RT-PCR Mix	2X	10	1X
TaqMan® RT Enzyme Mix	40X	0.5	1X
RNA	-	2	-
Total volume	-	20	-

Table A3.3 Amplification program

Step	Temp (°C)	Time	No. of Cycles
Reverse transcription	48	15 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60	1 min	

Interpretation of results

The specific product for ToBRFV will generate an exponential amplification curve.

Verification of the controls:
- NIC and NAC must generate no amplification.
- PIC and PAC must generate an exponential amplification curve.

If these conditions are met:
- A sample test will be considered positive if both duplicates generate exponential amplification curves with a Cq value below or equal to an appropriate cut-off*.
- A sample test will be considered negative if both duplicates generate no exponential amplification curves, or curves with a Cq value above an appropriate cut-off.
- Tests should be repeated if contradictory or unclear results are obtained.

*It should be noted that a preliminary cut-off value of 35 has been indicated in the test description established for the VALITEST test performance study. As a Cq cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

IMPORTANT: Non-specific (non-exponential) curves should be considered negative.

Reporting results

Cq values generated with the real-time RT-PCR protocol as well as qualitative results (positive, negative or undetermined) and used cut-off values should be reported in the TPS results Excel form. A copy of the real-time RT-PCR report with amplification curves and Cq values should be added.

4 – RT-PCR: Loewe kit: adapted from Rodríguez-Mendoza *et al*. (2019)

Consumables/chemicals

ToBRFV complete RNA PCR reaction kit (Loewe, Cat. No. 09175C/100) (ordered by participant)
The participant has to use its own disposables and equipment.

Controls

Tomato NIC (sample 1) and PIC (sample 2), pepper NIC (sample 40) (processed by the participant using the RNA extraction protocol)
NAC: molecular grade water
PAC: RNA ordered from DSMZ by participant (follow DSMZ protocol to recover nucleic acids and dissolve in 100 µl of molecular grade water)

Experimental protocol

Prepare the RT-PCR mix and run the amplification program according to the manufacturer's instructions included with the kit. Each sample is to be analysed in duplicate (i.e. two PCR wells per sample). Include NIC, PIC, NAC and PAC as control samples. Store RNA extracts and controls at -20°C after use.

Interpretation of results

The specific product for ToBRFV amplification will generate a single band of 475 bp.

Verification of the controls:

- NIC and NAC must be negative.
- PIC and PAC must generate a single band of the right size.

If these conditions are met:

- A sample test will be considered positive if both duplicates generate a band of the right size.
- A sample test will be considered negative if both duplicates generate no band.
- Tests should be repeated if contradictory or unclear results are obtained. If the result is contradictory the second time as well (one out of two duplicates generates a band of the right size), then the test should be interpreted as positive.

Reporting results

Qualitative results (positive, negative or undetermined) should be reported in the TPS results Excel form and labelled gel images should be added.

5 – RT-PCR: adapted from Alkowni *et al*. (2019)

Consumables/chemicals

Primers (Table A5.1) (Thermo Fisher Scientific) (ordered by participant)
OneStep RT-PCR kit (Qiagen, cat no. 210212) (ordered by participant)
The participant has to use its own disposables and equipment.

Table A5.1 Primers and their sequences

Name	Sequence (5' to 3')
ToBRFV-F	AAT GTC CAT GTT TGT TAC GCC
ToBRFV-R	CGA ATG TGA TTT AAA ACT GTG AAT

Controls

Tomato NIC (sample 1) and PIC (sample 2), pepper NIC (sample 40) (processed by the participant using the RNA extraction protocol)

NAC: molecular grade water

PAC: RNA ordered from DSMZ by participant (follow DSMZ protocol to recover nucleic acids and dissolve in 100 µl of molecular grade water)

Experimental protocol

Dilute primers to a working concentration of 10 µM. Prepare the RT-PCR mix according to Table A5.2, and run the amplification program according to Table A5.3. Each sample is to be analysed in duplicate (i.e. two PCR wells per sample). Include NIC, PIC, NAC and PAC as control samples. Store RNA extracts and controls at -20°C after use.

Table A5.2 RT-PCR mix

Reagent	Working concentration	Volume per reaction (µl)	Final concentration

Molecular grade water	-	11.6	-
ToBRFV-F	10 µM	0.4	0.2 µM
ToBRFV-R	10 µM	0.4	0.2 µM
OneStep RT-PCR Buffer (Qiagen)	5X	4	1X
dNTP mix (10 mM each)	10 mM	0.8	0.4 mM
OneStep RT-PCR Enzyme mix (Qiagen)	-	0.8	-
RNA	-	2	-
Total volume	-	20	-

Table A5.3 Amplification program

Step	Temp (°C)	Time	No. of Cycles
Reverse transcription	50	30 min	1
Initial denaturation	95	15 min	1
Denaturation	94	30 sec	35
Annealing	58	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	1

Interpretation of results

The specific product for ToBRFV amplification will generate a single band of 563 bp.

Verification of the controls:
- NIC and NAC must be negative.
- PIC and PAC must generate a single band of the right size.

If these conditions are met:
- A sample test will be considered positive if both duplicates generate a band of the right size.
- A sample test will be considered negative if both duplicates generate no band.
- Tests should be repeated if contradictory or unclear results are obtained. If the result is contradictory the second time as well (one out of two duplicates generates a band of the right size), then the test should be interpreted as positive.

Reporting results

Qualitative results (positive, negative or undetermined) should be reported in the TPS results Excel form and labelled gel images should be added.

6 – Colorimetric LAMP: adapted from Sarkes *et al*. (2020)

Consumables/chemicals

Primers (Table A6.1) (Thermo Fisher Scientific) (ordered by participant)

WarmStart® Colorimetric LAMP 2X Master Mix (NEB, cat no. M1800S) (ordered by participant)

The participant has to use its own disposables and equipment.

Table A6.1 Primers and their sequences

Name	Sequence (5' to 3')
F3	TTG GAG TCT TAG ATG TTG CG
B3	GGA CAC CGT CAA CTA GGA
FIP(F1c+F2)	CCT TCT CCA ACT GTC GCA AGT CAC ATG CTA GGA AGT ACC AC
BIP(B1c+B2)	CCG TGA GTT CTG AGT CAA TGG TTG AGG CTC ACC ATC TCT TAA
LoopF	CTC CAT GCT CAT CAT ACT CCA A
LoopB	GCT CAG AAC ACT GAG GAG ATT

Controls

Tomato NIC (sample 1) and PIC (sample 2), pepper NIC (sample 40) (processed by the participant using the RNA extraction protocol)

NAC: molecular grade water

PAC: RNA ordered from DSMZ by participant (follow DSMZ protocol to recover nucleic acids and dissolve in 100 µl of molecular grade water)

Experimental protocol

Dilute primers to a working concentration of 10 µM. Prepare the LAMP reaction mix according to Table A6.2. Mix by pipetting and check that reaction solutions have a bright pink color, which indicates initial high pH required for a successful pH-LAMP reaction. Incubate samples according to Table A6.3. Each sample is to be analysed in duplicate (i.e. two PCR wells per sample). Include NIC, PIC, NAC and PAC as control samples. Store all RNA extracts and controls at -20°C after use.

Table A6.2 RT-PCR mix

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Molecular grade water	-	0.5	-
F3	10 µM	0.5	0.2 µM
B3	10 µM	0.5	0.2 µM
FIP(F1c+F2)	10 µM	4	1.6 µM
BIP(B1c+B2)	10 µM	4	1.6 µM
LoopF	10 µM	1	0.4 µM
LoopB	10 µM	1	0.4 µM
WarmStart Colorimetric LAMP Master Mix	2X	12.5	
RNA	-	1	-
Total volume	-	25	-

Table A6.3 Amplification program

Step	Temp (°C)	Time	No. of Cycles

Loop-mediated isothermal amplification	65	30 min	1

Interpretation of results

Positive reactions will turn yellow after incubation, while negative reactions will remain pink (Figure A6).

Verification of the controls:
- NIC and NAC must be pink.
- PIC and PAC must be yellow.

If these conditions are met:
- A sample test will be considered positive if both duplicates have turned orange or yellow.
- A sample test will be considered negative if both duplicates have remained pink.
- Tests should be repeated if contradictory or unclear results are obtained. If the result is contradictory the second time as well (one out of two duplicates has turned yellow), then the test should be interpreted as positive.

Figure A6. Colour of negative and positive reactions. Negative reactions will remain pink and positive reactions will turn yellow (can also be orange in case of a weak reaction).

Reporting results

Qualitative results (positive, negative or undetermined) should be reported in the TPS results Excel form and labelled photos of the tubes should be added.

7 – AmplifyRP isothermal amplification by Agdia

Consumables/chemicals

AmplifyRP® kit (Agdia) (ordered by participant).

The participant has to use its own disposables. An AmpliFire® machine can be used (provided by Agdia).

Controls

Tomato NIC (sample 1) and PIC (sample 2), pepper NIC (sample 40) (processed by the participant using the RNA extraction protocol; Appendix 1)

NAC: molecular grade water

PAC: RNA ordered from DSMZ by participant (follow DSMZ protocol to recover nucleic acids and dissolve in 100 µl of molecular grade water)

Experimental protocol

Follow the protocol according to the manufacturer's instructions included with the kit, with some minor modifications:

- Start at step 3.

- At step 4, use 10 µl of RNA instead of sample extract.

Each sample is to be analysed in duplicate (i.e. two PCR wells per sample). Include NIC, PIC, NAC and PAC as control samples. Store all RNA extracts and controls at -20°C after use.

Interpretation of results

The specific product for ToBRFV will generate an exponential amplification curve.

Verification of the controls:
- NIC and NAC must generate no amplification.
- PIC and PAC must generate an exponential amplification curve.

If these conditions are met:
- A sample test will be considered positive if both duplicates generate exponential amplification curves with a Cq value below or equal to an appropriate cut-off*.
- A sample test will be considered negative if both duplicates generate no exponential amplification curves, or curves with a Cq value above an appropriate cut-off.
- Tests should be repeated if contradictory or unclear results are obtained.

*As a Cq cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

Reporting results

Qualitative results (positive, negative or undetermined) should be reported in the TPS results Excel form. A copy of the raw data (can be exported from the AmpliFire® fluorometer as a CSV file) should be added.

Appendix 4 – Abiopep protocol (Bernabé-Orts *et al.*, 2021)*

*Performed by two labs outside the scope of the TPS

Consumables/chemicals

Primers and probes (Table A4.1)

One of the following enzyme mixes:

- TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher scientific) (Table A4.2, Table A4.3)

- AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems) (Table A4.4, Table A4.5)

- KAPA PROBE FAST Universal One-Step qRT-PCR kit (KAPA BIOSYSTEMS) (Table A4.6, Table A4.7)

Table A4.1 Primers, probes and their sequences

Name	Sequence (5' to 3')
AB-620 Fw	CAGATGTGTCGTTGGTCAGAT
AB-621 Rev	CATCACTACGGTGTAATACTTC
AB-622 Pr	FAM-CGTAGCTTTGTCAAGGCATACCCAAA-BHQ

Controls

Tomato NIC (sample 1) and PIC (sample 2), pepper NIC (sample 40) (processed by the participant using the RNA extraction protocol)

NAC: molecular grade water

PAC: RNA ordered from DSMZ by participant (follow DSMZ protocol to recover nucleic acids and dissolve in 100 µl of molecular grade water)

Experimental protocol

Dilute primers and probes to a working concentration of 10 µM. Prepare the real-time RT-PCR mix and run the amplification program according to the Tables belonging to the enzymes of choice (Taqman, Agpath or KAPA) below. Each sample is to be analysed in duplicate (i.e. two PCR wells per sample). Include NIC, PIC, NAC and PAC as control samples. Store all RNA extracts and controls at -20°C after use.

Table A4.2 RT-PCR mix TaqMan® RNA-to-Ct™ 1-Step Kit

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Molecular grade water	-	6.3	-
AB-620 Fw	10 µM	0.4 µL	0.2 µM
AB-621 Rev	10 µM	0.4 µL	0.2 µM
AB-622 Pr	10 µM	0.4 µL	0.2 µM
TaqMan® RT-PCR Mix	2X	10	1X
TaqMan® RT Enzyme Mix	40X	0.5	1X
RNA	-	2 µL	-
Total volume	-	20 µL	-

Table A4.3 Amplification program TaqMan® RNA-to-Ct™ 1-Step Kit

Step	Temp (°C)	Time	No. of Cycles
Reverse transcription	48	15 min	1

Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60	1 min	

Table A4.4 RT-PCR mix AgPath-ID™ One-Step RT-PCR Reagents

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Nuclease free water (AgPath)	-	2 µL	-
AB-620 Fw	10 µM	0.2 µL	0.2 µM
AB-621 Rev	10 µM	0.2 µL	0.2 µM
AB-622 Pr	10 µM	0.2 µL	0.2 µM
RT-PCR buffer (Agpath)	2X	5 µL	1X
RT-PCR enzyme (Agpath)	25X	0.4 µL	1X
RNA	-	2 µL	-
Total volume	-	10 µL	-

Table A4.5 Amplification program AgPath-ID™ One-Step RT-PCR Reagents

Step	Temp (°C)	Time	No. of Cycles
Reverse transcription	48	10 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	45
Annealing	60	1 min	

Table A4.6 RT-PCR mix KAPA PROBE FAST Universal One-Step qRT-PCR kit

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Molecular grade water	-	6.0	-
AB-620 Fw	10 µM	0.4 µL	200.0 nM
AB-621 Rev	10 µM	0.4 µL	200.0 nM
AB-622 Pr	10 µM	0.4 µL	200.0 nM
KAPA PROBE FAST qPCR mix	2X	10.0 µL	1X
ROX HIGH	50X	0.4 µL	1X
KAPA RT MIX	50X	0.4 µL	1X
RNA	-	2 µL	-
Total volume	-	20 µL	-

Table A4.7 Amplification program KAPA PROBE FAST Universal One-Step qRT-PCR kit

Step	Temp (°C)	Time	No. of Cycles
Reverse transcription	42	5 min	1
Initial denaturation	95	3 min	1
Denaturation	95	3 sec	40
Annealing	60	30 sec	

Interpretation of results

The specific products for ToBRFV will generate an exponential amplification curve.

Verification of the controls:

- NIC and NAC must generate no amplification.
- PIC and PAC must generate an exponential amplification curve.

If these conditions are met:

- A sample test will be considered positive if both duplicates generate exponential amplification curves with a Cq value below or equal to an appropriate cut-off*.
- A sample test will be considered negative if both duplicates generate no exponential amplification curves, or curves with a Cq value above an appropriate cut-off.
- Tests should be repeated if contradictory or unclear results are obtained.

***** As a Cq cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

Appendix 5 – Seed batch and sample composition

Table A5.1. Tobamovirus detection in seed batches used for this TPS based on Illumina sequencing data.

Seed batch	Tobamovirus detected
Tomato A	tomato mosaic virus
Tomato B	tomato brown rugose fruit virus tomato mosaic virus
Tomato C	tomato mosaic virus tomato mottle mosaic virus*
Pepper A	**
Pepper B	***
Pepper C	pepper mild mottle virus

* low coverage (<20X). **pepper mild mottle virus detected, but this was probably due to sequencing contamination. *** 302 nucleotides of ToBRFV detected. Viral sequences from seeds with Cq values >23 cannot be reliably detected using this method. This batch had a Cq value of >26. Based on the real-time RT-PCR tests tomato brown rugose fruit virus is known to be present.

*Table A5.2. Composition of each sample type, based on Table A5.1. * possibly.*

Sample type	Composition	Expected tobamovirus
Tomato high	995 seeds (tomato A, untreated) + 5 seeds (tomato B, untreated)	tomato brown rugose fruit virus tomato mosaic virus
Tomato medium	997 seeds (tomato A, untreated) + 3 seeds (tomato B, treated 5 min with HCl)	tomato brown rugose fruit virus tomato mosaic virus
Tomato neg1	1000 seeds (tomato A, untreated)	tomato mosaic virus
Tomato neg2	1000 seeds (tomato C, untreated)	tomato mosaic virus tomato mottle mosaic virus*
Pepper medium	800 seeds (pepper A, untreated) + 200 seeds (pepper B, untreated)	tomato brown rugose fruit virus
Pepper neg1	1000 seeds (pepper A, untreated)	
Pepper neg2	1000 seeds (pepper C, untreated)	pepper mild mottle virus

Appendix 6 - Sample decoding table

Table A6.1. ToBRFV level per sample.

Sample set	Sample No.	ToBRFV level	Control (labelled for participants)
1: tomato	1	neg1	NIC
1: tomato	2	high	PIC
1: tomato	3	neg1	
1: tomato	4	medium	
1: tomato	5	medium	
1: tomato	6	medium	
1: tomato	7	medium	
1: tomato	8	medium	
1: tomato	9	medium	
1: tomato	10	medium	
1: tomato	11	medium	
1: tomato	12	high	
1: tomato	13	neg2	
1: tomato	14	medium	
1: tomato	15	medium	
1: tomato	16	neg1	
1: tomato	17	medium	
1: tomato	18	medium	
1: tomato	19	medium	
1: tomato	20	medium	
1: tomato	21	medium	
1: tomato	22	high	
1: tomato	23	high	
1: tomato	24	neg1	
1: tomato	25	medium	
1: tomato	26	medium	
1: tomato	27	medium	
1: tomato	28	neg1	
1: tomato	29	medium	
1: tomato	30	medium	
2: pepper	31	neg2	
2: pepper	32	medium	
2: pepper	33	medium	
2: pepper	34	neg1	
2: pepper	35	neg1	
2: pepper	36	medium	
2: pepper	37	medium	
2: pepper	38	neg1	
2: pepper	39	medium	
2: pepper	40	neg1	NIC

Neg1 or neg2: no ToBRFV present.

Appendix 7 – Assigned values

Table A7.1. Qualitative and if applicable quantitative (Cq value) outcome of three samples per sample type.

Sample type	Subsample	Real-time RT-PCR			End-point RT-PCR		Isothermal amplification	
		ISHI-Veg		M&W	Loewe	Alkowni	Sarkes	Agdia
		FAM	VIC	FAM				
Tomato high	1	16.49	17.37	15.63	positive	positive	positive	positive
	2	16.89	17.56	16.17	positive	positive	positive	positive
	3	14.79	15.63	14.22	positive	positive	positive	positive
Tomato medium	1	24.76	26.87	25.15	positive	negative	positive	positive
	2	24.34	25.05	23.35	positive	negative	positive	positive
	3	23.2	24.16	22.59	positive	negative	positive	positive
Tomato neg1	1	35.87	35.75	37.16	negative	negative	negative	negative
	2	40	36.77	36.87	negative	negative	negative	negative
	3	40	36.85	36.8	negative	negative	negative	negative
Pepper medium	1	26.71	26.12	25.02	negative	negative	negative	negative
	2	27.03	26.58	25.22	negative	negative	positive	negative
	3	26.2	25.95	24.7	negative	negative	positive	positive
Pepper neg1	1	40	36.87	33.6	negative	negative	negative	negative
	2	36.04	36.82	40	negative	negative	negative	negative
	3	36.72	40	34.6	negative	negative	negative	negative
Pepper neg2	1	40	40	35.78	negative	negative	negative	negative
	2	40	40	34.76	negative	negative	negative	negative
	3	40	40	40	negative	negative	negative	negative

Samples were tested according to the TPS instructions for all six test protocols in week 7, 2021. Assigned values corresponded to the outcomes of the most sensitive tests, the real-time RT-PCR tests. Green: positive, red: negative. Tomato neg2 was not tested.

Table A7.2. Test results of the organiser for 995 pepper seeds spiked with 5 tomato seeds containing ToBRFV (seed batch tomato B, untreated, Appendix 5).

Sample type	Subsample	Real-time RT-PCR			End-point RT-PCR		Isothermal amplification	
		ISHI-Veg		M&W	Loewe	Alkowni	Sarkes	Agdia
		FAM	VIC	FAM				
Pepper high (spiked with tomato seed)	1	17.29	17.39	16.14	negative	positive	positive	positive
	2	15.96	16.03	14.62	positive	positive	positive	positive
	3				positive	positive	positive	positive
		19.56	19.78	18.37				

ToBRFV negative pepper seeds were spiked with the same amount of tomato seeds containing ToBRFV as for “tomato high” samples. These samples were not used in the TPS, but showed that “pepper high” samples resulted in an average Cq value per real-time RT-PCR that was >1 higher than for “tomato high” samples (Table A7.1).

Appendix 8 – Overview of TPS results per test

Real-time RT-PCR ISHI-VEG (16 participants)

Sample type		nr of samples	TP	TN	FP	FN	UND	TP (%)	TN (%)	FP (%)	FN (%)	UND (%)	Concordant	Non-concordant	Concordant (%)	Non-concordant (%)
tomato	high	64	64	0	0	0	0	100.0%	0.0%	0.0%	0.0%	0.0%	64	0	100.0%	0.0%
tomato	medium	320	312	0	0	8	0	97.5%	0.0%	0.0%	2.5%	0.0%	312	8	97.5%	2.5%
tomato	neg1	80	0	79	0	0	1	0.0%	98.8%	0.0%	0.0%	1.3%	79	1	98.8%	1.3%
tomato	neg2	16	0	14	2	0	0	0.0%	87.5%	12.5%	0.0%	0.0%	14	2	87.5%	12.5%
tomato total		480	376	93	2	8	1	78.3%	19.4%	0.4%	1.7%	0.2%	469	11	97.7%	2.3%
pepper	medium	80	78	0	0	2	0	97.5%	0.0%	0.0%	2.5%	0.0%	78	2	97.5%	2.5%
pepper	neg1	64	0	61	1	0	2	0.0%	95.3%	1.6%	0.0%	3.1%	61	3	95.3%	4.7%
pepper	neg2	16	0	15	1	0	0	0.0%	93.8%	6.3%	0.0%	0.0%	15	1	93.8%	6.3%
pepper total		160	78	76	2	2	2	48.8%	47.5%	1.3%	1.3%	1.3%	154	6	96.3%	3.8%

Real-time RT-PCR Menzel & Winter (14 participants)

Sample type		nr of samples	TP	TN	FP	FN	UND	TP (%)	TN (%)	FP (%)	FN (%)	UND (%)	Concordant	Non-concordant	Concordant (%)	Non-concordant (%)
tomato	high	56	56	0	0	0	0	100.0%	0.0%	0.0%	0.0%	0.0%	56	0	100.0%	0.0%
tomato	medium	280	277	0	0	3	0	98.9%	0.0%	0.0%	1.1%	0.0%	277	3	98.9%	1.1%
tomato	neg1	70	0	69	0	0	1	0.0%	98.6%	0.0%	0.0%	1.4%	69	1	98.6%	1.4%
tomato	neg2	14	0	13	1	0	0	0.0%	92.9%	7.1%	0.0%	0.0%	13	1	92.9%	7.1%
tomato total		420	333	82	1	3	1	79.3%	19.5%	0.2%	0.7%	0.2%	415	5	98.8%	1.2%
pepper	medium	71	68	0	0	2	1	95.8%	0.0%	0.0%	2.8%	1.4%	68	3	95.8%	4.2%
pepper	neg1	55	0	54	0	0	1	0.0%	98.2%	0.0%	0.0%	1.8%	54	1	98.2%	1.8%
pepper	neg2	14	0	14	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	14	0	100.0%	0.0%
pepper total		140	68	68	0	2	2	48.6%	48.6%	0.0%	1.4%	1.4%	136	4	97.1%	2.9%

End-point RT-PCR Loewe (9 participants)

Sample type		nr of samples	TP	TN	FP	FN	UND	TP (%)	TN (%)	FP (%)	FN (%)	UND (%)	Concordant	Non-concordant	Concordant (%)	Non-concordant (%)
tomato	high	36	34	0	0	2	0	94.4%	0.0%	0.0%	5.6%	0.0%	34	2	94.4%	5.6%
tomato	medium	180	104	0	0	75	1	57.8%	0.0%	0.0%	41.7%	0.6%	104	76	57.8%	42.2%
tomato	neg1	45	0	45	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	45	0	100.0%	0.0%
tomato	neg2	9	0	9	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	9	0	100.0%	0.0%
tomato total		270	138	54	0	77	1	51.1%	20.0%	0.0%	28.5%	0.4%	192	78	71.1%	28.9%
pepper	medium	45	1	0	0	44	0	2.2%	0.0%	0.0%	97.8%	0.0%	1	44	2.2%	97.8%
pepper	neg1	36	0	36	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	36	0	100.0%	0.0%
pepper	neg2	9	0	8	1	0	0	0.0%	88.9%	11.1%	0.0%	0.0%	8	1	88.9%	11.1%
pepper total		90	1	44	1	44	0	1.1%	48.9%	1.1%	48.9%	0.0%	45	45	50.0%	50.0%

End-point RT-PCR Alkowni (14 participants)

Sample type		nr of samples	TP	TN	FP	FN	UND	TP (%)	TN (%)	FP (%)	FN (%)	UND (%)	Concordant	Non-concordant	Concordant (%)	Non-concordant (%)
tomato	high	56	46	0	0	10	0	82.1%	0.0%	0.0%	17.9%	0.0%	46	10	82.1%	17.9%
tomato	medium	280	101	0	0	174	5	36.1%	0.0%	0.0%	62.1%	1.8%	101	179	36.1%	63.9%
tomato	neg1	70	0	70	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	70	0	100.0%	0.0%
tomato	neg2	14	0	13	1	0	0	0.0%	92.9%	7.1%	0.0%	0.0%	13	1	92.9%	7.1%
tomato total		420	147	83	1	184	5	35.0%	19.8%	0.2%	43.8%	1.2%	230	190	54.8%	45.2%
pepper	medium	70	4	0	0	66	0	5.7%	0.0%	0.0%	94.3%	0.0%	4	66	5.7%	94.3%
pepper	neg1	56	0	52	3	0	1	0.0%	92.9%	5.4%	0.0%	1.8%	52	4	92.9%	7.1%
pepper	neg2	14	0	14	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	14	0	100.0%	0.0%
pepper total		140	4	66	3	66	1	2.9%	47.1%	2.1%	47.1%	0.7%	70	70	50.0%	50.0%

Isothermal amplification Sarkes (3 participants)

Sample type		nr of samples	TP	TN	FP	FN	UND	TP (%)	TN (%)	FP (%)	FN (%)	UND (%)	Concordant	Non-concordant	Concordant (%)	Non-concordant (%)
tomato	high	12	12	0	0	0	0	100.0%	0.0%	0.0%	0.0%	0.0%	12	0	100.0%	0.0%
tomato	medium	60	44	0	0	15	1	73.3%	0.0%	0.0%	25.0%	1.7%	44	16	73.3%	26.7%
tomato	neg1	15	0	15	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	15	0	100.0%	0.0%
tomato	neg2	3	0	3	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	3	0	100.0%	0.0%
tomato total		90	56	18	0	15	1	62.2%	20.0%	0.0%	16.7%	1.1%	74	16	82.2%	17.8%
pepper	medium	15	1	0	0	14	0	6.7%	0.0%	0.0%	93.3%	0.0%	1	14	6.7%	93.3%
pepper	neg1	12	0	12	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	12	0	100.0%	0.0%
pepper	neg2	3	0	3	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	3	0	100.0%	0.0%
pepper total		30	1	15	0	14	0	3.3%	50.0%	0.0%	46.7%	0.0%	16	14	53.3%	46.7%

Isothermal amplification Agdia (3 participants)

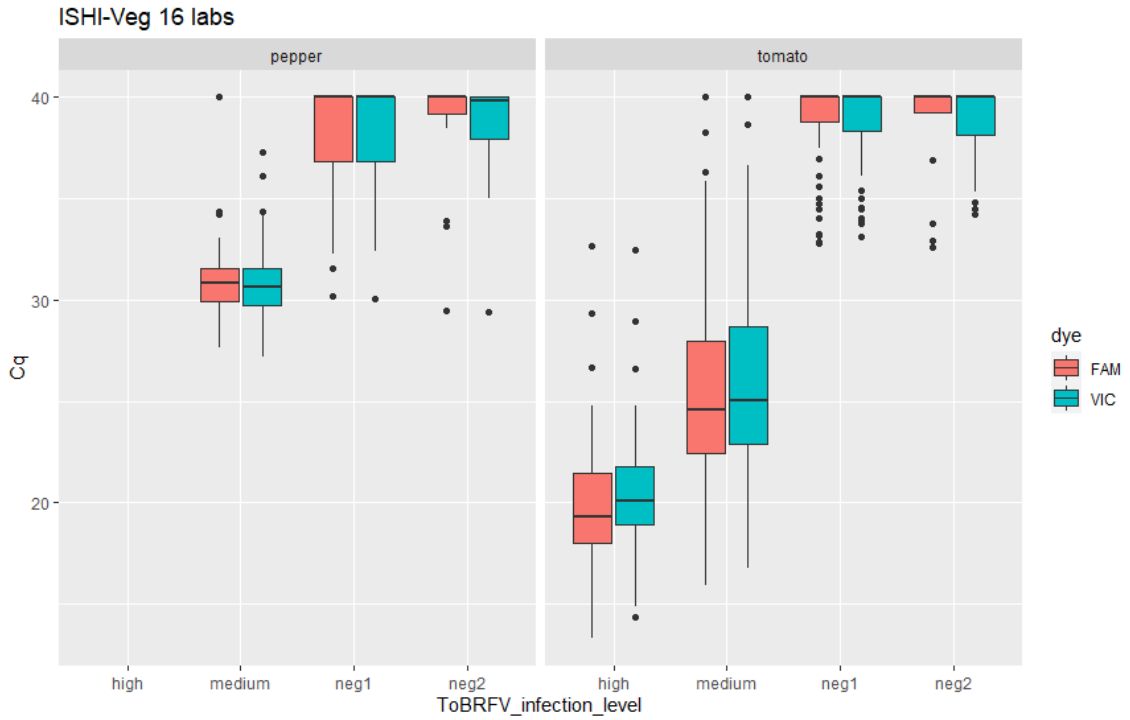
Sample type		nr of samples	TP	TN	FP	FN	UND	TP (%)	TN (%)	FP (%)	FN (%)	UND (%)	Concordant	Non-concordant	Concordant (%)	Non-concordant (%)
tomato	high	12	12	0	0	0	0	100.0%	0.0%	0.0%	0.0%	0.0%	12	0	100.0%	0.0%
tomato	medium	60	45	0	0	13	2	75.0%	0.0%	0.0%	21.7%	3.3%	45	15	75.0%	25.0%
tomato	neg1	15	0	15	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	15	0	100.0%	0.0%
tomato	neg2	3	0	3	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	3	0	100.0%	0.0%
tomato total		90	57	18	0	13	2	63.3%	20.0%	0.0%	14.4%	2.2%	75	15	83.3%	16.7%
pepper	medium	15	6	0	0	8	1	40.0%	0.0%	0.0%	53.3%	6.7%	6	9	40.0%	60.0%
pepper	neg1	12	0	12	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	12	0	100.0%	0.0%
pepper	neg2	3	0	3	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	3	0	100.0%	0.0%
pepper total		30	6	15	0	8	1	20.0%	50.0%	0.0%	26.7%	3.3%	21	9	70.0%	30.0%

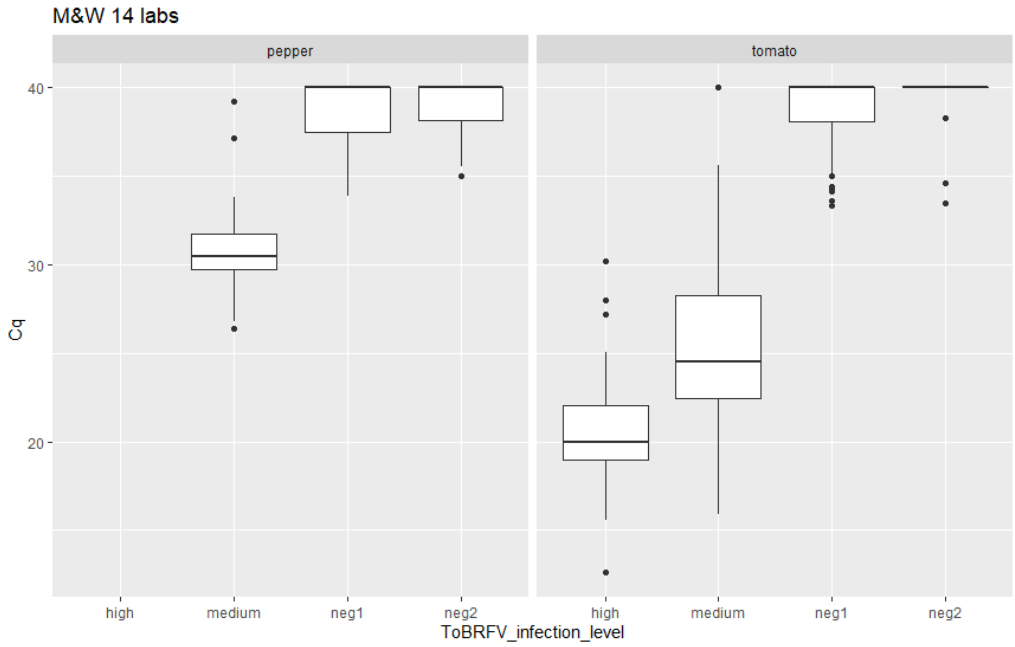
Real-time RT-PCR Abiopep (2 participants) * outside of TPS scope

Sample type		nr of samples	TP	TN	FP	FN	UND	TP (%)	TN (%)	FP (%)	FN (%)	UND (%)	Concordant	Non-concordant	Concordant (%)	Non-concordant (%)
tomato	high	8	8	0	0	0	0	100.0%	0.0%	0.0%	0.0%	0.0%	8	0	100.0%	0.0%
tomato	medium	40	37	0	0	2	1	92.5%	0.0%	0.0%	5.0%	2.5%	37	3	92.5%	7.5%
tomato	neg1	10	0	10	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	10	0	100.0%	0.0%
tomato	neg2	2	0	2	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	2	0	100.0%	0.0%
tomato total		60	45	12	0	2	1	75.0%	20.0%	0.0%	3.3%	1.7%	57	3	95.0%	5.0%
pepper	medium	10	8	0	0	1	1	80.0%	0.0%	0.0%	10.0%	10.0%	8	2	80.0%	20.0%
pepper	neg1	8	0	8	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	8	0	100.0%	0.0%
pepper	neg2	2	0	2	0	0	0	0.0%	100.0%	0.0%	0.0%	0.0%	2	0	100.0%	0.0%
pepper total		20	8	10	0	1	1	40.0%	50.0%	0.0%	5.0%	5.0%	18	2	90.0%	10.0%

TP: true positive, TN: true negative, FP: false positive, FN: false negative, UND: undetermined.

Appendix 9 – Quantitative results real-time RT-PCR tests





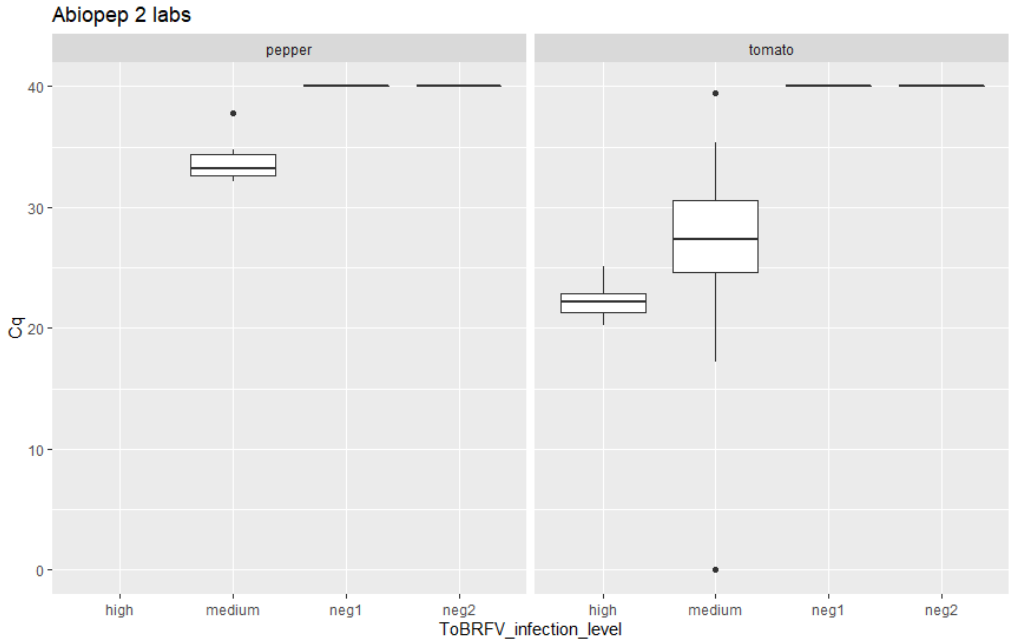


Figure A9.1. Box and whisker plots of Cq values reported from each real-time RT-PCR per sample type and crop. A Cq value of 40 indicates non-specific/negative/non-exponential curves.