

Methods in  
Molecular Biology 1302

Springer Protocols



Christophe Lacomme *Editor*

# Plant Pathology

Techniques and Protocols

*Second Edition*

 Humana Press

# METHODS IN MOLECULAR BIOLOGY

*Series Editor*

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# **Plant Pathology**

**Techniques and Protocols**

**Second Edition**

Edited by

**Christophe Lacomme**

*Virology & Zoology, SASA, Edinburgh, UK*

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*Editor*

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ISSN 1064-3745  
Methods in Molecular Biology  
ISBN 978-1-4939-2619-0  
DOI 10.1007/978-1-4939-2620-6

ISSN 1940-6029 (electronic)  
ISBN 978-1-4939-2620-6 (eBook)

Library of Congress Control Number: 2015938158

Springer New York Heidelberg Dordrecht London  
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## Preface

Pathogens pose a threat to plants in natural communities (i.e., forests, grasslands), horticultural commodities, or cultivated crops. Risks of pathogen spread have increased with increased human mobility and the globalization of trade. In addition, factors such as environmental changes (local or global climate fluctuations) and changes to pesticide legislation impact on whether pathogens and their vectors establish in different habitats and the selective pressures that will give rise to new pathotypes and pesticide- or antibiotic-resistant variants. Damages caused worldwide by either emerging, re-emerging or endemic pathogens are significantly important. The International Plant Protection Convention, Regional and National Plant Protection Organizations, have developed phytosanitary measures to prevent the spread of regulated pathogens (particularly quarantine pathogens) between countries in order to protect agricultural and natural plant systems.

Safeguarding plant biosecurity relies heavily on the early detection and diagnosis of the pathogen. Other than diagnoses based on morphological characteristics, diagnostic methods can be separated into three main categories: bioassay, serological and molecular methods, and sometimes a combination of these methods will be used. Since the late 1970s, the serological method of ELISA, using polyclonal and especially monoclonal antibodies, has been the method of choice for most diagnostic laboratories, due to its cost effectiveness and capacity to provide reliable detection and diagnosis for a large number of samples. However, over the past decade an increasing number of DNA/RNA-based assays, particularly PCR-based assays, are routinely used in diagnostic laboratories because of their increased sensitivity and specificity, the relative ease with which tests can be developed, their adaption to detect multiple targets, their requirement for minimal quantities of target, and their capacity to be automated for high-throughput testing. Moreover sequencing has contributed considerably to the increased knowledge of plant and microbial genomes and is now widely used either as stand-alone methods or in addition to other methods for diagnosis. Techniques such as end-point (conventional) PCR, real-time PCR, and diagnostic microarrays are versatile and can be used as either a generic or species-specific detection/diagnostic method. One of their drawbacks, however, is their reliance on prior knowledge of the genome of the target pathogen or pathogens. The rapid evolution of bioinformatics and computing technology to analyze very high numbers of complex datasets will make next-generation, high-throughput parallel sequencing platforms (also known as deep sequencing) accessible as a detection and diagnostic method. The application of these metagenomic approaches to diseased material offers the possibility to identify pathogens that have yet to be fully characterized or described. Importantly, recent advances in plant pathogen diagnoses have delivered field deployable portable diagnostic systems that do not require thermal cycling equipment. This allows rapid on-site identification of pathogenic agents, thereby passing the need for laboratory-based analysis. The development of any diagnostic assay requires thorough validation to ensure for example sensitivity, specificity, repeatability, and reproducibility and that the assay is fit for purpose.

This second edition of *Plant Pathology Techniques and Protocols* covers diagnostic methods that are currently used in laboratories for a broad range of plant species and matrixes. These include serological and molecular methods that have one or more of the

following characteristics: suitability for high-throughput testing, detection of a group of pathogens or of sometimes uncharacterized pathogens, detection and identification of specific pathogens, and high sensitivity. Qualitative and quantitative tests are described, as well as recently developed cutting-edge diagnostic methods. These chapters target an audience of plant pathologists and molecular biologists who will find information on how to perform the tests in their laboratories. Also provided is background information on many pathogens, which are endemic, nonendemic, or emerging and with different lifecycles that cause diseases of significant importance in a wide variety of hosts. Finally I would like to thank all authors that have contributed to this second edition of *Plant Pathology Techniques and Protocols*.

*Edinburgh, UK*

*Christophe Lacomme*

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## Contents

Preface .....	v
Contributors .....	ix
1 Detection of the Bacterial Potato Pathogens <i>Pectobacterium</i> and <i>Dickeya</i> spp. Using Conventional and Real-Time PCR .....	1 <i>Sonia N. Humphris, Greig Cabill, John G. Elphinstone, Rachel Kelly, Neil M. Parkinson, Leighton Pritchard, Ian K. Toth, and Gerry S. Saddler</i>
2 Detection and Identification of <i>Phoma</i> Pathogens of Potato .....	17 <i>Denise A'Hara</i>
3 Diagnosis of <i>Ramularia collo-cygni</i> and <i>Rhynchosporium</i> spp. in Barley .....	29 <i>Neil Havis, James Fountaine, Kalina Gorniak, Linda Paterson, and Jeanette Taylor</i>
4 A Real-Time Multiplex PCR Assay Used in the Identification of Closely Related Fungal Pathogens at the Species Level .....	37 <i>Dominie G. Wright</i>
5 Diagnostics of Tree Diseases Caused by <i>Phytophthora austrocedri</i> Species .....	59 <i>Vincent Mulholland, Matthew Elliot, and Sarah Green</i>
6 Real-Time LAMP for <i>Chalara fraxinea</i> Diagnosis .....	75 <i>Jenny Tomlinson and Neil Boonham</i>
7 Loop-Mediated Isothermal Amplification Procedure (LAMP) for Detection of the Potato Zebra Chip Pathogen “ <i>Candidatus Liberibacter solanacearum</i> ” .....	85 <i>Aravind Ravindran, Julien Lévy, Elizabeth Pierson, and Dennis C. Gross</i>
8 Loop-Mediated Isothermal Amplification (LAMP) for Detection of Phytoplasmas in the Field .....	99 <i>Matt Dickinson</i>
9 Diagnosis of Phytoplasmas by Real-Time PCR Using Locked Nucleic Acid (LNA) Probes .....	113 <i>Sabrina Palmano, Vincent Mulholland, David Kenyon, Gerry Saddler, and Colin Jeffries</i>
10 Q-Bank Phytoplasma: A DNA Barcoding Tool for Phytoplasma Identification .....	123 <i>Nicoletta Contaldo, Samanta Paltrinieri, Olga Makarova, Assunta Bertaccini, and Mogens Nicolaisen</i>
11 High-Throughput Diagnosis of Potato Cyst Nematodes in Soil Samples .....	137 <i>Alex Reid, Fiona Evans, Vincent Mulholland, Yvonne Cole, and Jon Pickup</i>

12	Detection of Nepovirus Vector and Nonvector <i>Xiphinema</i> Species in Grapevine . . . . .	149
	<i>C. Van Ghelder, A. Reid, D. Kenyon, and D. Esmenjaud</i>	
13	Molecular and Serological Methods for the Diagnosis of Viruses in Potato Tubers . . . . .	161
	<i>Christophe Lacomme, Ross Holmes, and Fiona Evans</i>	
14	Immunocapture-Multiplex RT-PCR for the Simultaneous Detection and Identification of Plant Viruses and Their Strains: Study Case, Potato Virus Y (PVY) . . . . .	177
	<i>Mohamad Chikh-Ali and Alexander V. Karasev</i>	
15	SNaPshot and CE-SSCP: Two Simple and Cost-Effective Methods to Reveal Genetic Variability Within a Virus Species. . . . .	187
	<i>Agnès Delaunay, Sylvie Dallot, Denis Filloux, Virginie Dupuy, Philippe Roumagnac, and Emmanuel Jacquot</i>	
16	Detection and Characterization of Viral Species/Subspecies Using Isothermal Recombinase Polymerase Amplification (RPA) Assays . . . . .	207
	<i>Laurent Glaïs and Emmanuel Jacquot</i>	
17	Virus Testing by PCR and RT-PCR Amplification in Berry Fruit . . . . .	227
	<i>Stuart MacFarlane, Wendy McGavin, and Ioannis Tzanetakis</i>	
18	Metagenomics Approaches Based on Virion-Associated Nucleic Acids (VANA): An Innovative Tool for Assessing Without A Priori Viral Diversity of Plants. . . . .	249
	<i>Denis Filloux, Sylvie Dallot, Agnès Delaunay, Serge Galzi, Emmanuel Jacquot, and Philippe Roumagnac</i>	
19	Detection of <i>Potato spindle tuber viroid</i> and Other Related Viroids by a DIG Labelled RNA Probe . . . . .	259
	<i>Wendy A. Monger and Colin Jeffries</i>	
20	Microarray Platform for the Detection of a Range of Plant Viruses and Viroids . . . . .	273
	<i>Ian Adams, Catherine Harrison, Jenny Tomlinson, and Neil Boonham</i>	
21	Multiplex Detection of Plant Pathogens Through the Luminex Magplex Bead System . . . . .	283
	<i>René A.A. van der Vlugt, Henry van Raaij, Marjanne de Weerdt, and Jan H.W. Bergervoet</i>	
22	Next-Generation Sequencing of Elite Berry Germplasm and Data Analysis Using a Bioinformatics Pipeline for Virus Detection and Discovery . . . . .	301
	<i>Thien Ho, Robert R. Martin, and Ioannis E. Tzanetakis</i>	
23	Metagenomic Next-Generation Sequencing of Viruses Infecting Grapevines . . . . .	315
	<i>Johan T. Burger and Hans J. Maree</i>	
24	Droplet Digital PCR for Absolute Quantification of Pathogens . . . . .	331
	<i>Ion Gutiérrez-Aguirre, Nejc Rački, Tanja Dreö, and Maja Ravnikar</i>	
	Erratum . . . . .	E1
	Index . . . . .	349

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# Chapter 1

## Detection of the Bacterial Potato Pathogens *Pectobacterium* and *Dickeya* spp. Using Conventional and Real-Time PCR

Sonia N. Humphris, Greig Cahill, John G. Elphinstone, Rachel Kelly, Neil M. Parkinson, Leighton Pritchard, Ian K. Toth, and Gerry S. Saddler

### Abstract

Blackleg and soft rot of potato, caused by *Pectobacterium* and *Dickeya* spp., are major production constraints in many potato-growing regions of the world. Despite advances in our understanding of the causative organisms, disease epidemiology, and control, blackleg remains the principal cause of down-grading and rejection of potato seed in classification schemes across Northern Europe and many other parts of the world. Although symptom recognition is relatively straightforward and is applied universally in seed classification schemes, attributing disease to a specific organism is problematic and can only be achieved through the use of diagnostics. Similarly as disease spread is largely through the movement of asymptotically infected seed tubers and, possibly in the case of *Dickeya* spp., irrigation waters, accurate and sensitive diagnostics are a prerequisite for detection. This chapter describes the diagnostic pathway that can be applied to identify the principal potato pathogens within the genera *Pectobacterium* and *Dickeya*.

**Key words** *Pectobacterium*, *Dickeya*, Real-time PCR, Blackleg, Soft rot

---

### 1 Introduction

*Pectobacterium* and *Dickeya* species (spp.) are plant pathogenic bacteria belonging to the family *Enterobacteriaceae*. They mainly consist of broad host range pathogens that cause wilts, rots, and blackleg disease on a wide range of plants and crops worldwide [1]. The major pathogenicity determinant of these bacteria is their copious production of plant cell wall-degrading enzymes (PCWDE) including pectinases, cellulases, and proteases, which macerate host tissue [2]. The genera were previously known collectively as the “soft rot erwinias” [3]. However, in 1998 the genus *Erwinia* underwent a major revision resulting in the soft rot erwinias being reassigned to the genus *Pectobacterium* [4], a name originally proposed by Waldee in 1945 [5]. Subsequent study of these taxa

suggested further revision was required resulting in *Pectobacterium chrysanthemi* being reassigned to the newly established genus *Dickeya* [6]. *Dickeya* currently encompasses 7 species: *D. aquatica*, *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. paradisiaca*, *D. solani*, and *D. zeae* [6–9]. There are 6 species currently in the genus *Pectobacterium*, including *P. atrosepticum*, *P. betavasculorum*, *P. cacticidum*, *P. carotovorum*, *P. cypripedii* and *P. wasabiae* [10], with *P. carotovorum* being further subdivided into subsp. *carotovorum* and subsp. *odoriferum* [4]. A further subspecies “*P. carotovorum* subsp. *brasiliensis*” has recently been proposed [11] but has yet to be formally recognized.

A number of *Pectobacterium* and *Dickeya* species are known to cause blackleg, tuber soft rot, and stem wilt and rot in potato. These include *D. dadantii*, *D. dianthicola*, *D. solani*, *P. atrosepticum*, *P. wasabiae* and *P. carotovorum* subsp. *carotovorum* and *P. c. subsp. brasiliensis* [11–18]. World-wide, *Pectobacterium* is the main cause of blackleg and soft rot in potatoes, with *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* the most significant production constraints. *P. carotovorum* subsp. *carotovorum* has a broad host range and a world-wide distribution in contrast to *P. atrosepticum* which is largely restricted to potato and principally only found in temperate regions [19]. Although originally described as affecting potatoes grown in Brazil [11] and New Zealand [14], respectively, “*P. carotovorum* subsp. *brasiliensis*” and *P. wasabiae* can now both be found in many of the potato-growing regions of the world. With regard to *Dickeya*, *D. dianthicola* and *D. solani* predominate in Northern Europe with the earliest report of a *Dickeya* spp. (*E. chrysanthemi*) causing disease on potato dating back to the 1970s [20]. Until 2005, all European potato isolates were likely to have been *D. dianthicola* [13, 21], but, more recently, several studies on potato strains isolated from diseased plants and tubers from a wide range of European countries and Israel have identified a new pathogen, subsequently named *D. solani*, as the principal *Dickeya* species affecting potato across the region [8, 12, 13, 22]. In other parts of the world, *D. dadantii* is known to cause blackleg and soft rot in potatoes, with a recent report highlighting its importance in Zimbabwe [23].

Disease spread is thought to be largely due to movement of latently infected potato seed tubers, which can lead to the introduction of highly pathogenic *Pectobacterium* and *Dickeya* spp. into different countries resulting in new disease outbreaks [19, 22, 24, 25]. In addition, *Dickeya* spp. have been detected in irrigation water in a number of potato-growing regions of Northern Europe, but the significance of these findings in relation to disease epidemiology remains unclear [9, 12, 26].

Blackleg, caused by *Pectobacterium* and *Dickeya* spp., is characterized by the production of a slimy, wet, black rot lesion spreading from the rotting mother tuber up the stems, especially under wet



**Fig. 1** Symptoms of potato blackleg caused by *Pectobacterium atrosepticum* (a), *Dickeya solani* (b) and tuber soft rot caused by *Dickeya solani* (c)

conditions [19]. In dry conditions, symptoms tend to lead to stunting, yellowing, wilting, and desiccation of stems and leaves (Fig. 1). Under warm wet conditions, blackleg and soft rot symptoms in potato are similar whether caused by *Pectobacterium* or *Dickeya* spp., which makes it almost impossible to identify the causal agent by visual assessment alone. For this reason, it is important to be able to rapidly and reliably detect and identify the bacterial species or subspecies.

This chapter describes diagnostic methods for the isolation and differentiation of *Pectobacterium* and *Dickeya* spp. using dilution plating with conventional PCR or nucleic acid extraction with quantitative real-time PCR. The chapter provides methods for pathogen isolation from asymptomatic and symptomatic potato plants and tubers as well as irrigation waters and details diagnostic methods for the isolation and differentiation of *Pectobacterium* and *Dickeya* spp. using qualitative and quantitative PCR assays. The chapter covers pathogen isolation on selective crystal violet pectate (CVP) medium [27, 28] with conventional PCR and nucleic acid extraction with quantitative real-time PCR. While there are primers to identify most species and subspecies of *Pectobacterium* using conventional PCR, there is as yet only validated species-specific primers for *P. atrosepticum* and *P. wasabiae* using real-time PCR [29, 30]. The genera *Dickeya* can be detected as a group using conventional and real-time PCR. However, the only validated species-specific real-time PCR primers are currently available for *D. solani* and *D. dianthicola* [31]. It is expected that other *Dickeya* and *Pectobacterium* species-specific real-time PCR assays will be available in the near future.

## 2 Materials

### 2.1 Isolation of Bacteria from Plant Tissue

1. 70 % (aq., v/v) ethanol.
2. 10 % (aq., v/v) sodium hypochlorite.
3. Hand-held potato peeler or disposable scalpel.
4. Universal long Extraction bags (Bioreba).

5. 0.25 Strength Ringer's buffer: dissolve 1 tablet (Oxoid) in 500 mL of distilled water and sterilize by autoclaving.
6. Antioxidant: tetrasodium pyrophosphate or dithiothreitol.
7. Homex 6 (Bioreba) or mallet.

## 2.2 General and Selective Plating Media

1. Enrichment media: Double strength pectate enrichment medium (D-PEM; [32]):
  - Dissolve in order, the salts (0.64 g MgSO<sub>4</sub>; 2.16 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 2.16 g K<sub>2</sub>HPO<sub>4</sub>) in 300 mL distilled water.
  - Heat if required to dissolve salts then make up to 1,000 mL.
  - Suspend 3.4 g of sodium polypectate in 5 mL of absolute ethanol and add to the solution, mixing well with a magnetic stirrer.
  - Steam suspension until polypectate is completely dissolved, and adjust pH to 7.2 if necessary.
  - Dispense into small aliquots (50 mL) and sterilize by autoclaving at 120 °C for 15 min. Store at 4 °C until required.
2. Selective plating media:
  - Nutrient agar (NA) or Luria-Bertani agar (LBA).
  - Crystal violet pectate medium (CVP) can be successfully used as both a single or double layer media for the detection and isolation of *Pectobacterium* and *Dickeya* spp. [28]. Although the double layer is more time consuming and awkward to make, it can be more suited to samples with large numbers of bacteria due to its slower cavity development.
    - Single-layer CVP is prepared as two solutions, the crystal violet solution and the pectin solution. The two solutions should be prepared separately before being mixed together and autoclaved. Add the ingredients of both solutions sequentially in the order of the following recipes. Prepare the crystal violet solution in 500 mL distilled water and add 1.02 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 g tryptone, 5 g trisodium citrate, 2 g NaNO<sub>3</sub>, 4 g agar and 1.5 mL crystal violet (1 % aqueous solution). Each ingredient of the crystal violet mix should be dissolved by stirring the medium before adding the following one. The pectin solution contains 2 mL NaOH (5 M) and 18 g pectin in 500 mL distilled water, and the pectin should be dissolved by stirring with a magnetic stir bar using heat if required. Mix the pectin solution with the crystal violet solution while stirring, and adjust the pH to 7.0 if required, before autoclaving. After autoclaving, mix the medium using a magnetic stir bar to avoid bubble formation and pour while still hot into Petri dishes in a laminar flow cabinet. Allow the medium

to set overnight and then store at 4 °C until required. Before use, dry plates in a laminar flow or drying oven to remove excess surface moisture.

- Double-layer CVP media is prepared in two steps. The first step is to prepare the basal layer mix in 1 L distilled water by sequentially adding the ingredients in the order of the following recipe: 5.5 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 g tryptone, 1.5 mL crystal violet (0.1 % solution), 1.6 g NaNO<sub>3</sub> and 15 g agar. After autoclaving for 15 min at 120 °C, cool the mix to 45–50 °C and pour ~15 mL into Petri dishes. Allow the basal layer to set and dry in a laminar flow to remove excess surface moisture. The second step is to prepare the over layer by adding 2 mL of 5.5 % EDTA (pH 8.0), 2 mL NaOH (5 M) and 20 g pectin in 800 mL distilled water. Dissolve the pectin by stirring with a magnetic stir bar using heat if required. Adjust the pH to 7.0 before autoclaving. Pour 7 mL of the over layer onto the dried basal layer.

### **2.3 Conventional PCR**

#### 1. PCR amplification.

- Molecular grade water.
- Oligonucleotide primers (Table 1).
- Deoxyribonucleotides (dNTPs).
- 5× PCR Reaction Buffer containing 7.5 mM MgCl<sub>2</sub> and 5 U/μL Taq DNA polymerase (Promega)-positive reference sample (*see Note 1*).

#### 2. Gel electrophoresis.

- Agarose.
- SYBR safe (Life Technologies) or GelRed (10,000×; Thermo Scientific).
- Loading buffer if required (*see Note 2*).
- 100 bp or 1 kb ladder.
- Thermal cycler.
- Gel electrophoresis system.
- 10× TBE electrophoresis buffer: 1 M Tris, 1 M boric acid and 20 mM EDTA (pH 8.3). Dilute to 1× for use.
- UV gel documentation system.

### **2.4 Real-Time PCR**

#### 1. Nucleic acid extraction using kit.

- Promega Wizard Magnetic DNA purification for Food (Thermo Labsystems).
- Kingfisher 96 magnetic particle separator (Thermo Labsystems).

**Table 1**  
**Primers for conventional PCR**

Target organism	Gene target	Primer name	Primer sequence (5'-3')	Amplicon size (bp)	Reference
<i>Pectobacterium</i> and <i>Dickeya</i> spp.	16S rRNA	SR3F	GGT GCA AGC GTT AAT CGG AAT G	119	[33]
		SR1cR	AGA CTC TAG CCT GTC AGT TTT		
<i>Pectobacterium atrosepticum</i>	Genome	ECA1f ECA2r	CGG CAT CAT AAA AAC ACG GCA CAC TTC ATC CAG CGA	690	[34]
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Genome	EXPCCF	GAA CTT CGC ACC GCC GAC CTT CTA	550	[35]
		EXPCCR	GCC GTA ATT GCC TAC CTG CTT AAG		
<i>P. wasabiae</i>	YD protein gene	PW7011F	CTATGACGCTCGCGGGT TGCTGTT	140	[30]
		PW7011R	CGGCGGCGTCGTAGT GGAAAGTC		
“ <i>P. carotovorum</i> subsp. <i>brasiliensis</i> ”	16S-23S rRNA	BRI1f L1r	GCG TGC CGG GTT TAT GCA CT CAA GGC ATC CAC CGT	322	[11]
<i>Dickeya</i> spp.	pectate lyase gene	ADE1	GAT CAG AAA GCC CGC AGC CAG AT	420	[36]
		ADE2	CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC		

## 2. Nucleic acid extraction using lab protocol.

- Buffer B (Promega Cat No Z3201).
- 750 µL precipitation solution (Promega Cat No Z3191).
- Isopropanol.
- Sodium acetate (3 M).
- 0.25 Strength Ringer’s buffer.
- 70 % (aq., v/v) ethanol.
- TE buffer: 10 mM Tris (pH 8) and 1 mM EDTA.

## 3. Quantitative detection and differentiation.

- Molecular grade water.
- Oligonucleotide primers and TaqMan® probes (Table 4).
- TaqMan® Universal PCR MasterMix: the reaction mix is supplied at 2x concentration and contains AmpliTaq Gold® DNA Polymerase (Ultra-Pure), dNTPs and ROX™ as a passive reference.
- Real-time PCR machine.

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### 3 Methods

Bacteria can be extracted from the leaves and stem of potato plants and the peel and stolon end of tubers. The following methods are optimized for the detection from asymptomatic plants, but they can also be used for symptomatic plants (see Note 3). The methods describe (a) the isolation of *Pectobacterium* and *Dickeya* spp. from stem, tubers, and water samples, (b) the preparation of samples for molecular detection, (c) the qualitative detection and differentiation of *Pectobacterium* and *Dickeya* spp. using dilution plating on CVP and conventional PCR and (d) quantitative detection and differentiation using real-time PCR.

#### 3.1 Isolation from Stems, Tubers, and Water Samples

##### 3.1.1 Sampling Potato Tubers

Sampling and processing the peel and stolon end cores separately will determine whether the bacterial infection is systemic (found in the vascular tissue of stolon end) or can only be found externally as lenticel infection in tuber peel (see Note 4).

1. Wash tubers to remove excess soil.
2. Using a clean and disinfected hand-held potato peeler (see Note 5), remove one peel strip from each tuber in the sample, to include both the heel (stolon) and rose ends.
3. Rinse the tubers again, and then using a separate hand-held peeler or disposable scalpel, remove a small plug of tissue from the stolon end of each tuber in the sample (approximately 5–10 mm deep and wide) making sure not to take any peel.

##### 3.1.2 Sampling Potato Plants

1. Rinse plants to remove any soil or debris.
2. Using a disposable scalpel, remove a 5 cm section of each stem just above ground level and a selection of leaves from all stems in the sample.

##### 3.1.3 Processing of Plant and Tuber Samples

1. Place all plant and tuber samples into separate universal extraction bags (see Note 6) and weigh each bag.
2. Add 15 mL of 0.25 strength Ringer's buffer containing tetrasodium pyrophosphate (0.1 % final concentration) or dithiothreitol (final concentration 0.075 %) antioxidant (see Note 7) to each bag.
3. Pulverize the sample using a Homex 6 grinder or rubber mallet to give an oatmeal consistency.

##### 3.1.4 Sampling and Processing Irrigation Water

1. Collect water samples in sterile bottles (250 mL) and transport to the laboratory in a cool box. Process within 24 h of collection.
2. Subdivide into aliquots of 40 mL and clarify by centrifugation at a low speed ( $180 \times g$ ).

3. Remove 20 mL of supernatant and mix with an equal volume of D-PEM (*see Subheading 2.2*); incubate in an anaerobic chamber at 36 °C for 48 h.
4. Centrifuge at high speed (10,000  $\times g$ ) to concentrate the bacterial fraction. Resuspend the pellet prior to serial dilution and plating onto CVP medium.

**3.2 Qualitative Detection and Differentiation of *Pectobacterium* and *Dickeya* spp. Using Conventional PCR**

Isolating *Pectobacterium* and *Dickeya* spp. can be problematic when secondary saprophytic microorganisms are present as they can outgrow the bacteria being isolated. Plating on the semi-selective media CVP (*see Subheading 2.2*) preferentially increases the pectolytic populations. The selectivity of the media is based on the addition of crystal violet, which inhibits the growth of Gram-positive bacteria, and on the use of pectin as the main carbon source. *Pectobacterium* and *Dickeya* spp. form characteristic deep cavities in the medium, due to their ability to break down and metabolize pectin. Although *Pectobacterium* and *Dickeya* spp. are both plated onto CVP, they should be incubated at 27 and 37 °C, respectively, for colony formation (*see Note 8*).

**3.2.1 Selective Plating**

1. Pipette off the extract from the homogenized sample and prepare a dilution series from  $10^0$  to  $10^{-3}$  in 0.25 strength Ringer's buffer (*see Note 9*). This should ensure background saprophytes are diluted out and isolated *Pectobacterium* and *Dickeya* colonies are obtained.
2. Spread 100  $\mu$ L of each dilution for each sample on to duplicate CVP plates previously dried to remove excess surface moisture. Incubate one plate at 27 °C and one plate at 37 °C for 48 h.
3. For use as a back-up stock, a 1 mL aliquot of the homogenate can be removed and added to 200  $\mu$ L of 100 % sterile glycerol and stored at -20 °C or -80 °C for longer-term storage.
4. A dilution series of approximately  $10^{-1}$ – $10^{-4}$  CFU.mL $^{-1}$  of a positive control for *Pectobacterium* and *Dickeya* spp. should also be prepared.
5. After 48 h select all colonies showing characteristic deep cavities and prepare a suspension of each colony by resuspending in molecular grade water.
6. This suspension will serve as a template for conventional PCR. Prior to PCR an aliquot of the sample should be transferred to a screw-top vial and incubated in a heat block at 96 °C for 5 min. Boiled samples can be frozen at -20 °C until required.

If any colonies require to be kept for reference stocks, then select, if possible, at least two well-spaced isolated colonies/cavities per CVP plate and re-streak the bacteria onto a fresh CVP plate.

Incubate at 27 °C or 37 °C (depending on genera) for 48 h. Once there are clean colonies showing characteristic cavities, select a colony and streak on to an NA or LBA plate previously dried in a laminar flow cabinet long enough to remove excess surface moisture. Incubate at 27 °C or 37 °C for about 1 week to ensure that only *Pectobacterium* or *Dickeya* spp. colonies are present. These bacteria form round convex creamy-translucent colonies on NA or LBA.

### 3.2.2 Conventional PCR

Set up PCR reactions in a contamination-free environment using primers specific for the species of interest (Table 1). There are multiple primer combinations for *Pectobacterium* and *Dickeya* spp., reaction conditions and PCR product electrophoresis and visualization methods routinely used by the authors that are described below.

1. Prepare the master mix as described in Table 2 (*see Note 10*). Prepare enough master mix for the number of samples to be tested and aliquot 24 µL into each PCR tube or each well of a PCR plate.
2. Add 1 µL of boiled cell sample for each 25 µL reaction. Samples should be amplified using both undiluted and diluted (1:10) boiled cell suspensions.
3. Negative controls should include the reaction mix without any template (i.e., no boiled cell suspension) and the reaction mix with 1 µL of any buffers used for sample processing. A positive control should also be included (*see Note 1*).
4. Using a thermal cycler, run the specific amplification protocol for the appropriate primers as described in Table 3.
5. After PCR amplification, the amplicon can be resolved by agarose gel electrophoresis on a 1.5 % gel in TBE buffer containing 0.01 % SYBR safe or GelRed.

**Table 2**  
Master mix reagents for conventional PCR

Reagent	Working stock concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	–	16.38	–
Green GoTaq® reaction buffer with 7.5 mM MgCl <sub>2</sub>	5×	5.0	1×
dNTP mix (dATP, dTTP, dCTP, dGTP)	10 mM of each	0.5	0.2 mM of each
Forward primer	10 µM	1.0	0.4 µM
Reverse primer	10 µM	1.0	0.4 µM
Taq DNA polymerase	5 U/µL	0.12	1.5 U
Template	–	1.0	–

**Table 3**  
**Conventional PCR cycling conditions**

Target organism	Step 1	Step 2	Step 3
<i>Pectobacterium</i> and <i>Dickeya</i> spp.	94° for 5 min	40 cycles: 94 °C for 30 s, 68 °C for 45 s, 72 °C for 45 s	72 °C for 7 min
<i>Pectobacterium atrosepticum</i>	94° for 5 min	36 cycles: 94 °C for 30 s, 62 °C for 45 s, 72 °C for 45 s	72 °C for 7 min
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	94° for 5 min	30 cycles: 94 °C for 60 s, 60 °C for 1 min, 72 °C for 2 min	72 °C for 7 min
<i>P. wasabiae</i>	94° for 5 min	35 cycles: 94 °C for 60 s, 67 °C for 30 s, 72 °C for 60 s	72 °C for 7 min
“ <i>P. carotovorum</i> subsp. <i>brasiliensis</i> ”	94° for 5 min	25 cycles: 94 °C for 45 s, 62 °C for 45 s, 72 °C for 90 s	72 °C for 7 min
<i>Dickeya</i> spp.	94° for 5 min	25 cycles: 94 °C for 60 s, 72 °C for 2 min	72 °C for 7 min

6. Load 12 µL of the reaction if the PCR buffer has a loading dye added or 10 µL of the reaction mixed with 2 µL of loading dye if it doesn't.
7. Include a 100 bp or 1 kb DNA ladder.
8. The amplicon of interest can be visualized under UV light using a gel doc system, and the amplicon size can be established by comparing to a DNA ladder.

### **3.3 Quantitative Detection and Differentiation Using Real-Time PCR**

Real-time PCR analysis allows high-throughput detection, quantification, and identification of *Pectobacterium* and *Dickeya* populations. The following section describes the detection of *P. atrosepticum* and *Dickeya* spp. using real-time PCR with TaqMan® probes. Currently there are no validated TaqMan® real-time protocols for *P. wasabiae* or *P. carotovorum*. However, there is a SYBR Green qPCR assay for the detection of *P. wasabiae* [30]. TaqMan® chemistry uses a customized fluorogenic probe complementary to the target DNA sequence to enable the detection of a specific PCR product as it accumulates during PCR.

The following section describes (a) nucleic acid extraction from homogenized samples, (b) primers and probes for detection of *P. atrosepticum* and *Dickeya* spp., (c) preparation of a standard curve for quantification and (d) real-time PCR analysis.

### **3.4 Nucleic Acid Extraction**

1. Pipette off the extract from the homogenized sample (see Subheading 3.1.3) into a 15 mL centrifuge tube, and centrifuge at 4 °C for 10 min at 90×*g* to remove any remaining particulate matter.

2. Dispense two 5 mL aliquots of supernatant into separate 15 mL tubes and centrifuge at 4 °C and  $2,236 \times g$  for 15 min to pellet the bacteria.

One of the bacterial pellets can be stored at -20 °C (as a backup stock), and nucleic acid can be extracted from the other bacterial pellet using Promega Wizard Magnetic DNA purification for Food in combination with a Kingfisher 96 magnetic particle separator (Thermo Labsystems) following the manufacturers' instructions. Alternatively, nucleic acid can be extracted using the protocol below.

1. Resuspend the pellet in 1 mL of 0.25 strength Ringer's buffer.
2. To each tube, add 250 µL buffer B (Promega Cat No Z3201) and 750 µL precipitation solution (Promega Cat No Z3191).
3. Vortex tubes and incubate at room temperature for 5 min.
4. Centrifuge tubes at  $2,236 \times g$  for 15 min at room temperature.
5. Remove 750 µL of supernatant from the sample tubes while avoiding the pellet and add to an equal volume of ice cold isopropanol.
6. Add 75 µL sodium acetate (3 M) and gently invert the tubes to mix.
7. Incubate at room temperature for at least 1 h.
8. Centrifuge tubes at  $11,688 \times g$  for 4 min, after which pipette off supernatant while avoiding disturbing the pellet.
9. Wash the pellet with 150 µL of 70 % ethanol (aq., v/v) and vortex tube.
10. Centrifuge tubes at  $11,688 \times g$  for 2 min and pipette off the ethanol.
11. Allow the pellet to air-dry for 10 min.
12. Resuspend the pellet in 100 µL TE buffer. The neat NA can be stored at -20 °C until required.
13. The NA should be diluted 1:2 with molecular grade water before being used as a template for real-time PCR.

### **3.5 Preparation of a Standard Curve**

1. A known reference culture of *P. atrosepticum* and *Dickeya* spp. should be grown in LB broth at 27 °C or 37 °C with shaking for 16 h to give a bacterial density of ~ $10^9$  CFU/mL.
2. Prepare a tenfold dilution series of the bacterial suspension from  $10^9$  to  $10^0$  CFU/mL.
3. Determine total cell counts in each dilution by plating on CVP medium. All dilutions should be performed in triplicate and the average cell count of the three replicates determined.
4. Carry out nucleic acid extraction using 5 mL of the  $10^9$  CFU/mL bacterial suspension following the nucleic acid extraction method.

5. Determine the concentration of the extracted DNA using a NanoDrop or spectrophotometer.
6. Prepare a tenfold serial dilution of the extracted DNA.

Use the DNA dilution series as standards in the real-time PCR assays so that a standard curve can be produced and the amount of DNA in the unknown extracts determined. *P. atrosepticum* or *Dickeya* spp. infection can be expressed as log pg DNA.g<sup>-1</sup> fresh wet weight of leaf, stem, or tuber. The approximate CFU/g of fresh wet weight of leaf, stem, or tuber can also be calculated using the cell counts from the corresponding tenfold dilution series of the bacterial suspensions.

### **3.6 Real-Time PCR (TaqMan®) for the Detection and Quantification of *Pectobacterium atrosepticum* and *Dickeya solani***

Selected primers and probes for the detection and quantification of *Pectobacterium atrosepticum* (Table 4: Eca), total pectolytic bacteria (Table 4: PEC), *Dickeya* spp. (Table 4: Ech), *Dickeya solani* (Table 4: SolC or fusA) and the potato cytochrome oxidase (Table 4: COX) gene are listed below. The COX primer probe combination is an internal positive control that can be used to amplify a fragment of the potato cytochrome oxidase gene to determine reliable and uniform yields of pure NA from all extracts.

Real-time PCR reactions should be set up in a contamination-free environment using TaqMan® Universal PCR MasterMix. All TaqMan® reagents should be protected from light until ready for use as excessive exposure to light may affect the fluorescent probes. Real-time PCR should be performed in 25 µL reactions using 96 well optical plates and optical PCR seals or caps.

1. Prepare enough master mix on ice for the number of samples to be tested following the recipe in Table 5 (*see Notes 10 and 11*). All samples should be tested in duplicate.
2. Aliquot 23 µL of master mix into the wells of an optical plate.
3. For each reaction, add 2 µL NA which has been diluted 1:2 with molecular grade water.
4. A range of standards containing known amounts of DNA (*see Subheading 3.5*) should also be included in the real-time PCR along with no template controls (NTCs) for each assay on the plate.
5. Cover the plate using optical PCR seals or caps and centrifuge briefly.
6. Run the real-time PCR amplification using a Real-Time PCR Detection System and reaction conditions of an initial 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.
7. After the run is completed, examine the amplification plots of the standard, tested samples and controls using the real-time machine software. Record concentrations of standards and corresponding Ct values and check that averaged Ct values for tested samples are within the range of the standard.

**Table 4**  
Primers and probes for real-time PCR (see Note 11)

Target organism	Primer name	Primer and probe sequence (5'-3')	Reference
<i>Pectobacterium</i> and <i>Dickeya</i> spp.	PEC-1F PEC-1R PEC-P	GTG CAA GCG TTA ATC GGA ATG CTC TAC AAG ACT CTA GCC TGT CAG TTT T CTG GGC GTA AAG CGC ACG CA	[29]
<i>Pectobacterium atrosepticum</i>	ECA-CSL-1F ECA-CSL-89R ECA-CSL-36T-P	CGGCATCATAAAAACACGCC CCTGTGTAATATCCGAAAGGTGG ACATTCAAGGCTGATATTCCCCCTGCC	[29]
<i>Dickeya</i> spp.	ECH-1F ECH-1R ECH-P	GAG TCA AAA GCG TCT TGC GAA CCC TGT TAC CGC CGT GAA CTG ACA AGT GAT GTC CCC TTC GTC TAG AGG]	[29]
<i>Dickeya dianthicola</i>	DIA-A F DIA-A R DIA-A P	GGCCGCCTGAATACTACATT TGGTATCTCTACGCCCATCA ATTAACGGCGTCAACCCGGC	[31]
<i>Dickeya solani</i>	SOLC-F SOLC-R SOLC-P	GCCTACACCATTAGGGCTAT ACACTACAGCGCGCATAAAC CCAGGCCGTGCTCGAAATCC	[31]
<i>Dickeya solani</i>	fusA-F fusA-R fusA-P	GGTGTGTTGACCTGGTGAAA ATAGGTGAAGGTACACCCCTCATC TGAAAGCCATCAACTGGAATGATT	[37]
Potato (cytochrome oxidase gene)	COX-F COX-R COX-P	CGT CGC ATT CCA GAT TAT CCA CAA CTA CGG ATA TAT AAG AGC CAA AAC TG TGC TTA CGC TGG ATG GAA TGC CCT	[38]

**Table 5**  
Master mix reagents for real-time PCR

Reagent	Working stock concentration	Volume per singleplex reaction ( $\mu$ L)	Volume per multiplex reaction ( $\mu$ L)	Final concentration
Molecular grade water	–	7	3.5	–
TaqMan® Universal PCR Master Mix	2×	12.5	12.5	1×
Forward Primer	5 $\mu$ M	1.5	1.5 of each	0.3 $\mu$ M
Reverse Primer	5 $\mu$ M	1.5	1.5 of each	0.3 $\mu$ M
TaqMan probe	5 $\mu$ M	0.5	0.5 of each	0.1 $\mu$ M
Template (1:2 dilution)	–	2.0	2.0	–

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## 4 Notes

1. A positive control for PCR can either be 1 µL of gDNA from a known reference culture or 1 µL of a colony from a known culture re-suspended in molecular grade water.
2. The Promega GoTaq® DNA Polymerase has a 5× Green GoTaq® Reaction Buffer which contains two loading dyes (blue and yellow) that separate during electrophoresis to monitor migration progress. The sample can be loaded directly onto the gel after amplification as no additional loading dye is required for gel electrophoresis. A colorless buffer without loading dyes is also supplied, and this can be used when downstream applications involve absorbance or fluorescence.
3. Severely diseased plants and tubers can be tested by washing the sample under running water to remove excess soil but avoid breaking the skin. Excise a small amount of diseased tissue and suspend in 1 mL of 0.25 strength Ringer's Solution, vortex, and then allow to stand for 10 min. Use a sterile inoculating loop streak a loopful of the liquid onto CVP agar and proceed from **step 5** of Subheading [3.2.1](#).
4. If it is not important to differentiate between systemic or non-systemic infection, a core of tissue can be removed from the stolon end of the tuber to include the peel using a clean and disinfected hand-held potato peeler or disposable scalpel. The core (with the peel intact) can be processed as described in Subheading [3.1.3](#).
5. Ensure the peeler is cleaned and disinfected between each sample by rinsing with 0.2 M NaOH and then with 96 % EtOH and finally rinsing well with distilled (or tap) water. Allow to drain before peeling next sample.
6. It is advisable to use the extraction bags with the synthetic intermediate layer to separate most of the plant debris from the extract.
7. It is advisable to add an antioxidant to delay bacterial cell death by toxic substances present in extracts.
8. *P. atrosepticum* will form colonies up to 27 °C, *P. carotovorum* subsp. *carotovorum* up to 37 °C and *Dickeya* spp. up to and over 37 °C [[24](#)].
9. A 750–1,000 µL aliquot of the sample can at this point be enriched by mixing 1:1 (v/v) sample: D-PEM as described in Subheading [2.2](#). Proceed as described in Subheading [3.2.1](#) with the exception that the enriched samples should be diluted from 10<sup>0</sup> to 10<sup>-6</sup> in 0.25 strength Ringer's buffer.
10. Depending on the reaction mix used for PCR, the primer concentrations may require optimization with the final concentration in the range of 0.1–1.0 µM.

11. If carrying out multiplex reactions, the probes should be labelled with different reporter dyes and the primer and probe concentrations may require optimization for the most efficient amplification.

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# Chapter 2

## Detection and Identification of *Phoma* Pathogens of Potato

Denise A'Hara

### Abstract

*Phoma foveata*, *Phoma exigua* var. *exigua*, and *Phoma eupyrena* are fungal pathogens of potato, causing gangrene or pit rot symptoms in tubers, and they are responsible for significant crop losses. Various techniques are available to identify these pathogens in the laboratory. A multiplex Plexor® real-time PCR method which can detect and identify these pathogens in a single reaction will be presented.

**Key words** Multiplex, Fungal, *Phoma*, Gangrene, Potato, Bioassay, In vitro culture, Real-time PCR, Plexor®, PCR

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### 1 Introduction

Gangrene is a storage dry rot of potato (*Solanum tuberosum*) caused predominantly by the soil-borne fungus *Phoma foveata* (Foister) [1], which infects largely through wounds arising at harvest or grading. The less aggressive pathogen, *Phoma exigua* var. *exigua* [1], is also associated with gangrene-type symptoms, although these do not progress so extensively. Gangrene symptoms start with small sunken thumbprint-like lesions at wounds, lenticel or tuber eyes, which then enlarge, becoming irregular in shape, often with a characteristic wavy edge externally (Fig. 1a–e). Internally, a dark rot develops with a well-defined edge between the diseased and healthy tissue, and large cavities can be present (Fig. 1b, e). The size of the external lesion is rarely indicative of the dimensions of the internal lesion (Fig. 1c). Primarily a seed-borne disease, gangrene is responsible for crop losses in most potato-producing regions of the world including Europe, Northern Africa, New Zealand, Australia [2], and China [3].

*Phoma eupyrena* is a less aggressive pathogen responsible for causing pit rot symptoms in potato which can look like immature gangrene lesions. Cases of gangrene and pit rot have been increasing during the last few years (SASA, unpublished data), possibly due to the loss of the fungicide 2-AB as a control measure and



**Fig. 1** (a) External and (b) internal symptoms of gangrene (*Phoma exigua* var. *foveata*). (c) On the right of this tuber, a large external lesion can be seen, but the internal rot is not proportional in size as it has not progressed deeply into the tuber. (d) Characteristic wavy edge on the left hand side of this gangrene lesion. (e) Internal gangrene symptoms showing characteristic jet black discolouration, irregular shape, large cavities filled with white-gray fungal growth, and rusty reddish-brown discolouration. (f) Yellow colony of *Phoma foveata*. On close inspection, small specks of anthraquinone pigment production can be seen

sulfuric acid as a haulm desiccant, impacting on the spread and development of the pathogens responsible for these diseases.

Morphologically, *Phoma foveata* and *Phoma exigua* var. *exigua* are very similar and cannot be distinguished based on their pycnidia or conidia [1]. Pycnidia are globoid (90–200 µM) and dark brown to black in color, and their cylindrical conidia are 4–5 µM × 2–3 µM [4]. *Phoma eupyrena* is more easily identified from the other two *Phoma* species but all three have similar colony morphology, so laboratory expertise is required to differentiate them. *Phoma foveata* produces pigments known as anthraquinones (Fig. 1f) which exhibit as small yellow-brown flecks on the underside of the culture plate after approximately 7–10 days of growth. *Phoma exigua* var. *exigua* does not produce anthraquinone pigments, and this can be used to distinguish it from *Phoma foveata*. However, pigment production can be variable or lost completely, so colony morphology requires skilled interpretation, can be unreliable, and takes 7–14 days until colonies are mature. Therefore, it is necessary to be able to differentiate between these two species in a more reliable way. DNA-based techniques can assist with the rapid identification of these fungal species: No expertise is required in fungal morphology; these techniques are rapid to undertake and results are accurate in terms of identification.

However, at the molecular level there is considerable similarity between these two closely related *Phoma* varieties. MacDonald et al. [5] developed a RAPD-generated PCR-RFLP marker to distinguish between the two varieties, but the use of restriction enzymes makes this assay time-consuming and relatively expensive. Cullen et al. [6] developed conventional and quantitative PCR assays for the detection of *P. exigua* var. *foveata*, but the primers also detected the closely related *P. exigua* var. *exigua*. Aveskamp et al. [7] developed specific primers which can differentiate the two varieties with conventional PCR, but this is more time-consuming and less sensitive than real-time PCR. In order to facilitate the rapid identification of these three potato pathogens, for both diagnostic and research purposes, we describe below a multiplex assay, based on a real-time qPCR method known as Plexor® technology, which can distinguish between three *Phoma* potato pathogens in one reaction, thus saving on time, labor, and reagents.

## 2 Materials

### 2.1 Incubation Test

- Sterile masonry nail, approximately 4 mm diameter, or other blunt utensil to wound the tuber flesh.
- Sterile distilled water to create a humid environment.
- Paper toweling or capillary matting to wet with sterile water.
- Plastic container with lid to incubate the tubers in.
- Refrigerator at 5 °C.
- Methylated spirits and Bunsen burner to sterilize equipment.

### 2.2 Plating Media for Culturing *Phoma* sp.

#### 2.2.1 Malt Extract Agar

Malt extract (Oxoid)	30.0 g/l
Mycological peptone	5.0 g/l
Agar	15.0 g/l
Adjust pH to 5.4	

Suspend 50 g in 1 l of distilled water and boil to dissolve. Sterilize by autoclaving at 115 °C for 10 min. Once cooled to approximately 55 °C, dispense into Petri dishes and allow to cool. Store at 4 °C until required.

#### 2.2.2 Potato Dextrose Agar (PDA)

Potato extract (Oxoid)	4.0 g/l
Glucose	20.0 g/l
Agar	15.0 g/l
Adjust pH to 5.6	

Suspend 39 g in 1 l of water (purified as requested). Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 min. Mix well before pouring. Once cooled to approximately 55 °C, dispense into Petri dishes and allow to cool. Store at 4 °C until required.

### 2.2.3 Aseptic Techniques

Methylated spirit, a Bunsen burner, and scalpels/knife are required in order to sterilize the equipment.

## 2.3 Real-Time PCR

### 2.3.1 Nucleic Acid

#### Extraction Using Kit

- DNeasy plant mini kit (Qiagen).

### 2.3.2 Plexor® Detection

#### and Differentiation

- MOPS/EDTA buffer (Promega).
- Plexor® primers (Table 2).
- Plexor® Master Mix (Promega): the reaction mix is supplied at 2× concentration and the reaction mix includes deoxynucleotides and iso-dGTP modified with the quencher dabcyll.
- Real-time PCR machine (such as Stratagene MX3005p).

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## 3 Methods

### 3.1 Visual Inspection

If mature rots are present, a visual examination may be sufficient to identify gangrene symptoms and this provides the most rapid diagnosis. However, care has to be taken as the appearance of gangrene symptoms in tubers can be mistaken for other dry rot diseases, such as those caused by *Fusarium* species. Certain characteristic symptoms of gangrene infection can aid diagnosis:

1. Firstly, the presence of a wavy edge on the external lesion (Fig. 1a, d).
2. Secondly, when the affected tuber is cut in half through the center of the external lesion, if the size of the external lesion is not proportional to the size of the internal lesion, i.e., if the external lesion is large, but there is little internal rotting or vice versa, this is a good indication that the disease is gangrene (Fig. 1b, c, e).
3. Thirdly, if the internal symptoms are very irregular in shape, this can indicate gangrene. The internal lesion will be dry (see Note 1) and dark in color, with an extremely well-defined edge between the diseased and healthy tissue. Cavities will be present, often filled with fluffy fungal growth, which can vary in color; however, if there is black tissue present, this is likely to be due to pycnidia formation, which indicates gangrene infection (Fig. 1b, e). Likewise, if there is rusty reddish-brown tissue present, this is also indicative of gangrene.

The combined presence of these symptoms can be used to rule out *Fusarium* dry rot infections. Less well-developed infections would need to be subcultured in order to identify the fungal pathogen responsible.

### 3.2 Incubation Test

To detect for latent (no symptoms) contamination of tubers with *Phoma* species, a 100-tuber sample is tested

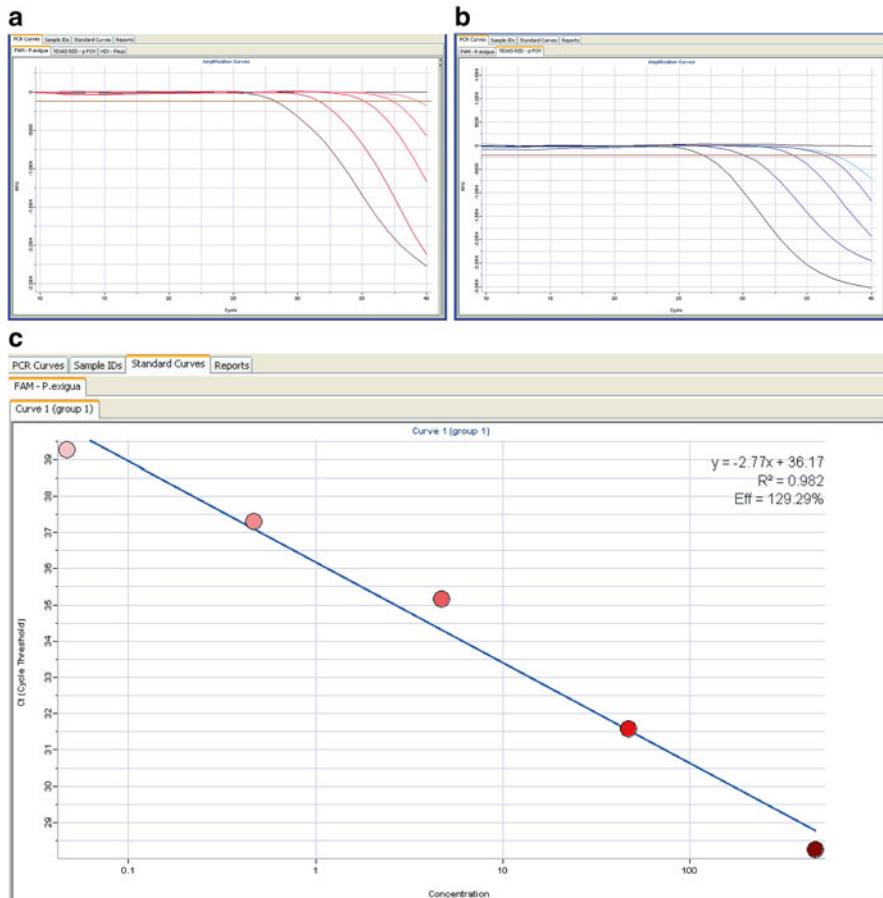
1. Utensils should be dipped in methylated spirit and flamed to sterilize before and between samples.
2. Wound tubers to a depth of 5 mm using a modified masonry nail (*see Note 2*). Tubers are wounded ten times, five wounds in an "X" formation on each side of the tuber.
3. Incubate tubers at 5 °C for a period of 4–6 weeks.
4. Once any rots develop, they will need to be subcultured to identify the causal organism, as described below.

### 3.3 Plating Medium

1. Using aseptic techniques, a knife should be used to cut into the lesion perpendicular to the center of the external lesion.
2. Once the full extent of the internal lesion is known, at the boundary between the diseased and healthy tissue, a sterile scalpel should be used to cut away a thin layer of tissue from the surface of the cut tissue, as this will remove contaminants that may have been dragged from the external skin of the tuber as the initial cut was made with the knife.
3. A final sterile scalpel is then used to scrape away a small amount of diseased tissue at the leading edge of infection (the boundary between diseased and healthy tissue) as this is where the fungus will be actively multiplying. Contaminants are more likely to be present near the initial starting point for the rot, particularly if there is an open wound, so this area should be avoided where possible.
4. Place the extracted tissue in the center of a Petri dish containing malt extract agar (Oxoid). On malt agar medium, *Phoma foveata* will display pigment production after approximately 7 days.
5. A second scraping of diseased tissue should be placed in the center of a potato dextrose agar (Oxoid) (PDA) plate. The use of PDA assists with the identification of other non-*Phoma* potato pathogenic fungi.
6. Incubate the plates for 7–14 days at ambient temperature.

### 3.4 Plexor® Assay

Based on real-time PCR technology, Plexor® (Promega) involves labeling one species-specific primer with a fluorescent dye and modified it with a methylisocytosine (iso-dC) residue at the 5'-end. The corresponding species-specific primer is not modified. Each set of species-specific primers is labeled with a different fluorescent



**Fig. 2** (a) Plexor results showing a decrease in fluorescence as cycling continues. Higher concentrations of template DNA show a decrease in fluorescence earlier in the cycling process than lower template DNA concentrations. Negative control sample shows no decrease in fluorescence. These results are for *Phoma exigua* var. *exigua*, using a tenfold dilution series starting with 2 ng DNA (amplified with *Phoma* complex primers). (b) Amplification curves obtained, in multiplex reactions, for *Phoma foveata* on a tenfold dilution series of target DNA starting with 2 ng DNA. Higher concentrations of template DNA show a decrease in fluorescence earlier in the cycling process than lower template DNA concentrations. Negative control sample shows no decrease in fluorescence. (c) Standard curve obtained on a tenfold dilution series of DNA from *Phoma exigua* var. *exigua* in a multiplex reaction. Concentration of DNA shown is in picograms

label to create a multiplex reaction. The qPCR reaction buffer includes dabcyl-iso-dGTP (iso-dG); during thermocycling this becomes incorporated at the position complimentary to the iso-dC label, effectively quenching the fluorescence over time [8], as shown in Fig. 2.

#### 3.4.1 Primer Design

To design primers to detect and differentiate *Phoma foveata*, *Phoma exigua* var. *exigua*, and *Phoma eupyrrena*, DNA sequences were generated from the respective fungal isolates held in SASA's culture collection by comparative sequence analyses using primers

**Table 1****Primers used to amplify *Phoma* DNA for sequencing purposes**

Primer name	Specific target	Sequence 5' to 3'	Amplicon size (bp)
Phoma 2	RAPD-PCR primer	GGACCCCTGTACTGACGTC	474
Phoma 7	<i>Phoma</i> sp. marker	AGCGGCTAGGATAGACAGGCG	
EF1-1Fa	EF-1 $\alpha$ gene	GCTGGTATCTCCAAGGATG	~870
EF1-1Ra	EF-1 $\alpha$ gene	TCRGTGAARGCCTCAAC	

**Table 2****Plexor® primers generated to detect *Phoma foveata*, *Phoma eupyrena*, and *Phoma* species**

Primer name	Target organism	Sequence 5' to 3'	Product size (bp)
PFoveataF	<i>P. foveata</i>	GGTGAACCTCTGTGCTCGATATGC <sup>a</sup>	80
PFoveataR		ATGACAGGAGTGAGACGATGATAGT	
PhomaF	<i>P. exigua</i>	GCCC GTT GGT CTCC ACTT GTA <sup>b</sup>	96
PhomaR	var. <i>exigua</i> and <i>P. foveata</i>	AGAAAGCCCGAAATCTAGAGCAAC	
PeupyrenaF PeupyrenaR	<i>P. eupyrena</i>	CAAGTGCCCACGAATGTACTGAG <sup>c</sup> TGATCTGACCTGTAAAACAGCATCG	121

<sup>a</sup>Modified at the 5'-end with iso-dC and Texas Red<sup>b</sup>Modified at the 5'-end with iso-dC and FAM<sup>c</sup>Modified at the 5'-end with iso-dC and Hex

derived from RAPD-PCR fragments (Phoma 2 and Phoma 7) [5] and translation elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) gene-specific primers (Schoch, unpublished data) (Table 1). In addition to the sequences generated above, the *Phoma* sequences published on GenBank as accession numbers EU880838 (*P. exigua* var. *exigua*) and EU880839 (*P. exigua* var. *foveata*) [7] were also used during the design of diagnostic primers. The Plexor® primer design software (Promega) was used to select suitable primer pairs.

The first primer pair, PhomaF and PhomaR, detects *Phoma exigua* var. *exigua* and *Phoma foveata* DNA sequences which are highly homologous. The forward primer was modified at the 5'-end with a fluorescein phosphoramidite (FAM; peak emission at 516 nm and peak excitation at 492 nm) label and an iso-dC residue. The second primer pair (PfoveataF and PfoveataR) detects *Phoma foveata* DNA sequences, and the third primer pair detects *Phoma eupyrena* (PeupyrenaF and PeupyrenaR) DNA sequences (Table 2). These were labeled at the 5'-end of the forward primer with Texas Red (peak emission at 620 nm and peak excitation at

584 nm) and Hex (peak emission at 556 nm and peak excitation at 535 nm), respectively. Additionally, both forward primers were modified at the 5'-end with an iso-dC residue. Reverse primers PFoveataR and PeupyrenaR were not labeled or modified.

### 3.4.2 DNA Extraction

A portion of diseased tuber flesh is scraped and homogenized using a reusable pestle (*see Note 3*) in a 1.5 ml microfuge tube. 400 µl AP1 buffer (Qiagen) is then added and the DNA extraction is performed according to the DNeasy plant mini kit's user instructions (Qiagen).

### 3.4.3 Real-Time Plexor<sup>TM</sup> Assay

Quantitative PCR amplifications are performed in 25 µl reactions in a Stratagene MP3005P thermocycler (*see Note 4*) using a master mix recipe shown in Table 3, with all three primer sets at a final concentration of 200nM (*see Note 5*) and <100 ng purified template DNA. Reactions are carried out using 2× Plexor<sup>®</sup> qPCR system master mix (Promega). The following amplification protocol is used: initial denaturation of 2 min at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 35 s, then 1 cycle of 60 °C for 15 s and 95 °C for 5 s. Following amplification, results are analyzed using Plexor<sup>®</sup> Analysis Software (Promega) which is available from their website ([www.promega.com](http://www.promega.com)).

Procedure:

1. Thaw Plexor<sup>®</sup> Master Mix and primers on ice.
2. Briefly vortex master mix and primers, and store on ice.
3. Prepare master mix as indicated below (Table 3).
4. Samples are tested in duplicate/triplicate.
5. Vortex reaction mixture briefly.
6. Add 20 µl of reaction mix to an optical-grade PCR plate for each sample.

**Table 3**  
**Plexor<sup>®</sup> reaction setup**

	Volume per reaction (µl)
Plexor <sup>®</sup> Master Mix, 2×	12.5
5 µM primer pair <i>P. foveata</i> ( <i>see Note 6</i> )	1.0
5 µM primer pair <i>Phoma</i> species	1.0
5 µM primer pair <i>P. eupyrena</i>	1.0
MOPS/EDTA buffer	4.5
Template DNA	5.0
Total volume	25

7. Add 5 µl of template (for non-template control, add 5 µl MOPS/EDTA buffer).
8. Spin plate briefly and place in Stratagene MX3005p machine.
9. Set up program according to Promega technical manual.
10. Select “SYBR Green (with dissociation curve),” then select “OK.”
11. Select “plate set up.”
12. Highlight wells to be used.
13. Select “unknown” as the well type.
14. Select “none” for the reference dye.
15. Click dyes to be used (Hex, FAM, Texas Red).
16. Select “thermal profile setup.”
17. Change plateau in segment 1–95 °C for 2 min.
18. Change plateau 1 in segment 2–95 °C for 5 s.
19. Change plateau 2 in segment 2–60 °C for 35 s.
20. Delete 3rd plateau (highlight, then click “delete”).
21. Ensure cycle number for segment 2 is 40.
22. Save the file.
23. Start the run.
24. After the run is complete, extract the data and analyze on Plexor® software (according to the Promega user instructions).

#### *3.4.4 Dilution of Standard Reference Template*

Inclusion of a dilution series of a standard reference in the Plexor® assay will provide an indication on the assay’s performance (Fig. 2a–c) and, when required, the limit of detection of the assay (Fig. 2b). This is provided when purchasing the 2× Plexor® Master Mix buffer.

1. Thaw and vortex a standard reference template.
2. Quantify DNA concentration.
3. Prepare serial dilutions of the standard reference template by adding 10 µl DNA to 90 µl MOPS/EDTA buffer. Change tips between dilutions.
4. Run Plexor® assay as above.
5. Extract data and analyze on Plexor® software.

#### *3.4.5 Interpretation of Results*

The assay is conclusive if all of the controls give the expected result: It is recommended to include a non-template control (no amplification expected; used to monitor potential contamination), a positive extraction control (amplification of each *Phoma* species target should occur in extraction control samples), and a positive amplification control (*Phoma* target DNA to monitor the performance of

*Phoma* real-time amplification). All tested samples are run in triplicate. The multiplex assay achieves the same level of sensitivity as the individual singleplex reactions. The multiplex detection limit is 80 fg for *P. foveata* (Fig. 2b), 160 fg for *Phoma* species, and 80 fg for *P. eupyrena*. The standard curves produced for each species show high correlation coefficient ( $R^2$ ) values of 0.99, 0.98, and 0.98 for *P. foveata*, *Phoma* complex, and *P. eupyrena*, respectively, indicating linear responses in detection related to the increasing DNA concentration, which can be used to estimate DNA concentration of unknown samples. Figure 2c shows the standard curve obtained for *Phoma* complex amplification of *P. exigua* var. *exigua* DNA.

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#### 4 Notes

1. Gangrene symptoms produce a dry rot, but the presence of secondary bacterial activity may cause wet rots to develop.
2. A 4 mm-wide masonry nail with the sharp end removed provides an ideal tool with which to puncture the skin of tubers when assessing for latent infection. The nails are robust and can be flame sterilized easily.
3. A plastic pestle designed for use with a 1.5 ml microfuge tube is used to homogenize the tuber tissue prior to DNA extraction. The pestles are used once only for each sample and then are washed in soapy liquid, rinsed in tap water, and then left to soak in 0.2 M sodium hydroxide overnight. A thorough rinse in tap water and then autoclaving at 115 °C for 15 min is performed before they can be reused.
4. If using a Stratagene machine, ensure that the lamp is warmed up prior to loading the samples. Switching the lamp on prior to setting up the master mix solution is usually sufficient time.
5. Prepare a primer stock solution by diluting individual primers to 100 µM and store in the dark (primers are light sensitive) at -20 °C. It is recommended to prepare a working dilution of primers from the 100 µM stock solutions to minimize freeze-thawing cycles from stock solution. Prepare a working dilution of all three primer pairs together at 5 µM. This is a 20-fold dilution, therefore 1 µl each primer in 17 µl of MOPS/EDTA buffer or 10 µl each primer in 170 µl MOPS/EDTA buffer. Diluted primers are stored in the dark at -20 °C.
6. Dilute primers and templates in MOPS/EDTA buffer (Promega), which is provided at pH 7.4. It is critical that this MOPS/EDTA buffer be used with the iso-dC-containing primers used in the Plexor® assay, as these primers are sensitive to pH below 7.0.

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# Chapter 3

## Diagnosis of *Ramularia collo-cygni* and *Rhynchosporium* spp. in Barley

**Neil Havis, James Fountaine, Kalina Gorniak, Linda Paterson, and Jeanette Taylor**

### Abstract

Ramularia leaf spot and Rhynchosporium leaf scald are two of the major diseases of barley crops in cooler temperate countries. The methods below are aimed at the identification and quantification of fungal DNA in leaf samples but can also be used for pathogen detection from seed or DNA extracted from environmental samplers. The methods describe in detail two individual quantitative PCR tests. The successful multiplexing of assays will lead to faster throughput of samples.

**Key words** Fungus, qPCR, DNA, Barley

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### 1 Introduction

Barley (*Hordeum vulgare*) is one of the major world crops and constitutes a major component of UK arable production. In 2013, over 800,000 ha of spring barley was estimated to have been sown in England alone, the highest for over a decade [1]. UK production is estimated to be 7.102 million tones [2].

Ramularia leaf spot (RLS, caused by the fungus *Ramularia collo-cygni*) has been shown to reduce yield by up to 1.0 t/ha in susceptible varieties [3]. Symptoms appear post-flowering in the crop. Small rectangular pepper spots appear on upper leaves. The spots often have a chlorotic halo and are bound by the leaf veins. Over time the symptoms coalesce to form large areas of necrotic tissue (Fig. 1). The fungus has been shown to be seed borne and can move through the crop asymptotically [4, 5]. Pure cultures of the fungus can be obtained by careful excision of conidiophores from the leaf surface of a barley plant showing fungal sporulation. The spores are small and a 40 $\times$  stereomicroscope is required. Potato dextrose agar amended with antibiotics is a suitable growth medium for the fungus.



**Fig. 1** Ramularia leaf spot (RLS) on spring barley



**Fig. 2** Rhynchosporium leaf scald in spring barley

The fungal pathogen *Rhynchosporium commune* is the major biotic factor involved in the formation of *Rhynchosporium* lesions on barley crops [5]. Lesions are initially grey/green but eventually darken with a distinct black edge (Fig. 2). *Rhynchosporium* is now considered to be the major economic disease to affect UK winter and spring barley crops with yield losses as high as 30–40 % being recorded [6]. These losses are accompanied by reductions in grain quality. The primary inoculum for *R. commune* is considered to arise from crop debris and seed-borne infection with secondary infection due to the release of rain splash spores from

infected lesions [7, 8]. The polycyclic nature of *R. commune* and the recent discovery of asymptomatic colonization of barley by the fungus suggest that the pathogen can undergo many generations *in planta* before symptoms appear in the crop.

Pure cultures of the fungus can be obtained from symptomatic leaves by surface sterilizing leaf tissue and placing on antibiotic amended malt yeast glucose agar. Pink colonies grow from the lesions and can be transferred to fresh media.

Classic symptoms are fairly easily distinguished to a trained pathologist, but earlier symptoms may still cause confusion. Although ELISA-based diagnostics were produced for both pathogens, most diagnosis is now done by quantitative PCR.

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## 2 Materials

1. Mortar and pestle.
2. T.E.N. Buffer: 31.52 g Trizma hydrochloride (400 nM), 14.1 g NaCl and 11.9 g of ethylenediaminetetraacetic acid disodium salt (50 nM); adjust to pH 8.0 with sodium hydroxide (NaOH). Add sterile distilled water to 500 ml and autoclave.
3. 2 % Sodium dodecyl sulphate solution: 10 g in 500 ml in sterile distilled water.
4. 1 %  $\beta$ -mercaptoethanol, aliquot 0.5 ml in 49.5 ml in sterile distilled water (*see Note 1*).
5. Extraction Buffer: 475 ml of T.E.N. Buffer, 475 ml of 2 % sodium dodecyl sulphate solution (lauryl sulphate), 0.8 g phenanthroline (5 mM), 20 g polyvinylpyrrolidone. Heat gently to dissolve. Allow buffer to cool and add 47.5 ml of 1 %  $\beta$ -mercaptoethanol. Add sterile distilled water to 1 l. Store for a maximum of 7 days at room temperature (preferably in the dark) (*see Note 2*).
6. 7.5 M ammonium acetate: Dissolve 57.81 g of ammonium acetate in approximately 50 ml of sterile distilled water, make up to a final volume of 100 ml with sterile distilled water.
7. Microcentrifuge.
8. Water bath.
9. Real-time PCR reagents (Eurogentec Ltd.).
10. Oligonucleotide primers and custom dye-labelled probes (Table 1 for details).
11. Spectrophotometer NanoDrop<sup>®</sup> ND-1000 (NanoDrop Technologies).
12. PCR plates with optical caps.
13. Real-time PCR machine Mx3000P and MxPro QPCR Software(Agilent Technologies) or equivalent.

**Table 1**  
**Primers and probes used in the detection of *Ramularia collo-cygni* and *Rhynchosporium* spp. (see Note 3)**

Primer/probe name	Sequence
<i>R. collo-cygni</i>	
RamF6	CGT CAT TTC ACC ACT CAA G
RamR6	CCT CTG CGA ATA GTT GCC
Ram6 molecular beacon probe	FAM-GCG ATT CCG GCT GAG CGG TTC GTC ATC GCG-BHQ-1
<i>Rhynchosporium</i> spp.	
Rsrtpcrlf	ATG TGC TTC CTT ATG GAC AGA TGT
Rsrtpcrlr	ATT ATT AAC AGA AAA ACC CCC TCAGAT
Rsrtpcrlp LNA probe	FAM-TATG*AG*GTGCC*AC*AGT-BHQ-1

\*Asterisk symbols in front of bases indicate which bases have 2-O, 4-C methylene linkages

### 3 Methods

#### 3.1 DNA Extraction

- Placed around 10 leaves in a mortar and pestle (see Note 4).
- Add liquid nitrogen into the mortar and carefully crush leaves down into very fine powder.
- To each mortar add Extraction Buffer (in fume hood) till the crushed leaves absorb all of the Extraction Buffer (see Note 5).
- Pour mixture into 15 ml tubes. These may placed in a -20 °C freezer for storage if required.
- Fill new 2 ml tubes to the top with leaf extract and place in a hot water bath at 70 °C for 20 min
- Spin down samples for 10 min at 10,000×*g*.
- Prepare 2 ml tubes with 900 µl 7.5 M ice cold ammonium acetate.
- Fill tube to the top with extract and vortex, then place on ice for 20 min (see Note 6).
- Centrifuge for 15 min at maximum speed (14,000×*g*).
- Prepare 2 ml tube with 800 µl ice cold isopropanol and pour supernatant into prepared tube (see Note 7).
- Stand at room temperature (22 °C) for 15 min before centrifuging at 14,000×*g* for 5 min.
- Pour off supernatant carefully and wash the pellet with 400 µl 70 % ethanol (see Note 8).
- Centrifuge at maximum speed (14,000 × *g*) for 5 min.

14. Carefully remove the supernatant and air-dry tube for 15 min.
15. Resuspend pellet in 50 µl sterile distilled water and leave sample over night in 4 °C.
16. Heat the sample by placing it in a heat block at 50 °C for 40 min to dissolve pellet, and pipette gently to ensure complete resuspension.
17. Samples are ready for nanodroping and can be stored in the fridge at 4 °C for 1–3 days.
18. Store in freezer –20 °C until ready for testing.

### 3.2 Real-Time PCRs

The next step in the process involves the setting up and running of real-time PCRs. Details of the assays are given below in Table 2. Care should be taken to avoid cross-contamination in the procedures. Assays should be set up in a separate sterile cabinet.

#### 3.2.1 Standard Curve Preparation

For the master mix and qPCR conditions for *R. collo-cygni* standard curve and for *Rhynchosporium* spp., see Subheading 3.2.2.

Using DNA extracted from pure cultures of *R. collo-cygni* and *Rhynchosporium* spp., prepare a fivefold dilution series in 25 µl reaction volume (see Note 9). Concentration ranges from 10 ng

**Table 2**  
**Standard reaction conditions for real-time PCR assays used for detection of *R. collo-cygni* and *Rhynchosporium* spp.**

Reaction component	Stock concentration	Volume in 25 µl reaction	Final concentration
<i>R. collo-cygni</i>			
PCR-grade water		5.125 µl	–
Master mix	2×	12.5 µl	1×
F primer	10 µM	1 µl	0.4 µM
R primer	10 µM	1 µl	0.4 µM
Molecular beacon probe	10 µM	0.375 µl	0.15 µM
Template	20 ng	5 µl	100 ng
<i>Rhynchosporium</i> sp.			
PCR-grade water	–	5.25 µl	–
Master mix	2×	12.5 µl	1×
F primer	10 µM	1 µl	0.4 µM
R primer	10 µM	1 µl	0.4 µM
LNA probe	10 µM	0.25 µl	0.1 µM
Template	20 ng	5 µl	100 ng

to 0.128 pg for *R. collo-cygni* and 50 ng to 0.0256 pg for *Rhynchosporium* spp. (see Notes 10 and 11).

The diluted *R. collo-cygni* and *Rhynchosporium* spp. DNA standards are run in triplicate on the plate containing the samples to be tested.

### 3.2.2 Real-Time PCR of *R. collo-cygni* and *Rhynchosporium* spp.

A standard curve was developed by plotting the logarithm of known concentrations of genomic DNA against the threshold cycle (Ct) values. Ct is defined as the cycle number at which the fluorescence of the PCR product is significantly different from the background. Ct value is inversely related to log of initial concentration; therefore, the lower the Ct value, the higher the initial DNA concentration.

Threshold cycles were calculated automatically by the MxPro QPCR Software (Agilent, UK). Detection threshold for *R. collo-cygni* was 0.128 pg. Detection threshold for *Rhynchosporium* spp. was 0.0256 pg.

#### *R. collo-cygni*

##### 1. Master Mix for *R. collo-cygni*.

The reaction mixture contained the following components (final concentration): 1× qPCR MasterMix Plus QGS (Eurogentec, Belgium), 0.4 µM forward and reverse primer, 0.15 µM Ramularia probe, 5 µl DNA template (20 ng/µl) and finally, PCR-grade water to make up the final volume. Real-time PCR assays were performed in a total volume of 25 µl (Table 2). Three technical replicates were carried out for each sample to confirm the reproducibility of the results. For negative (no template) control reactions, nuclease-free water was substituted for the DNA.

##### 2. Reaction conditions for *R. collo-cygni*.

The qPCR was carried out in a real-time Mx3000P (Agilent Technologies, UK) according to the following programme: An initial hot start of 10 min at 95 °C was followed by 50 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s. A final extension step of 95 °C for 1 min was added. Amplification produced a 115-bp PCR product.

#### *Rhynchosporium* spp.

##### 1. Master mix for *Rhynchosporium* spp.

For the cytochrome *b* LNA probe assay, the reaction mixture is as presented in Table 2. Reaction mix consists of 12.5 µl of PCR master mix FAST qPCR MasterMix Low Rox (Eurogentec, Belgium), 0.4 µM forward primer Rsrtpcr1f, 0.4 µM reverse primer Rsrtpcr1r, 0.1 µM LNA fluorogenic probe Rsrtpcr1p (Table 1) and sterile distilled water.

##### 2. Reaction conditions for *Rhynchosporium* spp.

Amplification of the 125-bp PCR product was carried out in an Mx3000P machine under the following conditions: 1 cycle at 94 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min.

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## 4 Notes

1. This should be made up in fume hood as toxic chemical.
2. Solutions containing mercaptoethanol should be made fresh, as mercaptoethanol is not stable in solution. The extraction buffer should be used within 7 days.
3. *R. collo-cygni* assay is specific to the individual species. *Rhynchosporium* primers were designed to the pathogen isolated from barley. This fungus was reported as *Rhynchosporium secalis* and had a wide host range. The barley-infecting pathogen has been renamed *Rhynchosporium commune*. Recent taxonomic studies have shown that *Rhynchosporium commune* is a complex of four closely related *Rhynchosporium* species [9]. The assay has not yet been tested against the other *Rhynchosporium* species on other hosts.
4. Symptomatic material can be sampled for *Rhynchosporium* spp. throughout the growing season. Leaf samples can be taken from the field and dried overnight at room temperature before storing at -20 °C prior to DNA extraction. Ramularia leaf spot symptoms are generally observed post-flowering in barley crops, but asymptomatic leaves can be sampled all year round and processed in a similar way to the *Rhynchosporium* spp. samples.
5. Ensure sample is not too thick (i.e., pourable) and the buffer and sample are mixed properly.
6. This step can be extended to a few hours.
7. This step can be extended to overnight if the samples are stored at -20 °C.
8. Wash can be repeated two to three times if the pellet remains dirty.
9. Phytopure kit was used for extracting DNA from pure cultures of *R. collo-cygni* and *Rhynchosporium* spp.
10. A stock solution of 50 ng of DNA was produced following DNA extraction and stored in aliquots at -20 °C.
11. Multiple aliquots of each dilution were prepared from the stock DNA. Each aliquot contained 20 µl, sufficient for the triplicate reactions, with one aliquot of each dilution used per run. This prevents freezing and thawing of the standard curve dilutions.

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## Acknowledgements

The authors would like to acknowledge funding from the Scottish Government, Home-Grown Cereals Authority, BBSRC and Syngenta Crop Protection.

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# Chapter 4

## A Real-Time Multiplex PCR Assay Used in the Identification of Closely Related Fungal Pathogens at the Species Level

Dominie G. Wright

### Abstract

The advent of real-time PCR and new chemistries such as TaqMan™ and SYBR™green has been used in plant pathology to aid in the identification of fungal, bacterial and viral pathogens. These chemistries have provided another tool to be used in the identification of fungal pathogens that are hard to differentiate on the basis of morphology. This work describes an assay that was developed to identify five different species of the pathogen *Tilletia* that causes smuts and bunts in cereals.

**Key words** Real-time multiplex PCR assay, TaqMan chemistry, Fluorescent probes, *Tilletia* species

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### 1 Introduction

This chapter is using a case study on how a real-time multiplex PCR using a fluorescent assay can help in the identification of a number of plant pathogens in one sample. For this particular assay, the chemistry used is TaqMan™ and not SYBR™green. The assay was developed to help with the identification of *Tilletia indica* and to distinguish it from other *Tilletia* species that are commonly mistaken for this pathogen.

#### 1.1 Pathogen Information

*Tilletia indica* Mitra causes the disease Karnal bunt, or Partial bunt, of wheat (*Triticum* spp.). Karnal bunt was first described in Karnal, India, in 1931. The pathogen is widespread in parts of South Asia and Southwest Asia [1, 2]. It has also been detected in restricted areas of the United States and Mexico and also in South Africa [3, 4].

Hosts include *Triticum aestivum*, *Triticum durum*, and *Triticum aestivum* × *Secale cereale*. Records on *Triticum aestivum* × *Secale cereale* are rare; however, *Secale* spp. have been shown to have the potential to be a host [5]. *Tilletia indica* has been shown to infect other grass species under glasshouse conditions but has never been detected in the field in these alternative hosts [6].



**Fig. 1** An infected grain of wheat showing the symptoms of Karnal bunt. Photographs are the courtesy of Department of Agriculture and Food, Western Australia

*T. indica* is a floret-infecting smut pathogen. Seeds are infected through the germinal end of the kernel, and the fungus develops within the pericarp where it produces a powdery, brownish-black mass of teliospores (Fig. 1). When fresh, the spore masses produce a foetid, decaying fish-like smell (trimethylamine). Unlike systemic smuts, it is not usual for all the seeds on an ear to be infected with *T. indica*, and heads with infected seeds do not differ in appearance from healthy heads. Seeds are usually only partially colonized, showing various degrees of infestation. Therefore, it is very difficult to detect the disease in the field. The symptoms are not usually seen until after harvest, unless infestation levels are high.

*T. indica* reduces grain quality by discoloring and imparting an objectionable odor to the grain and products made from it. It also causes a small reduction in yield. Generally *Triticum aestivum* containing more than 3 % bunted kernels is considered unsatisfactory for human consumption [3].

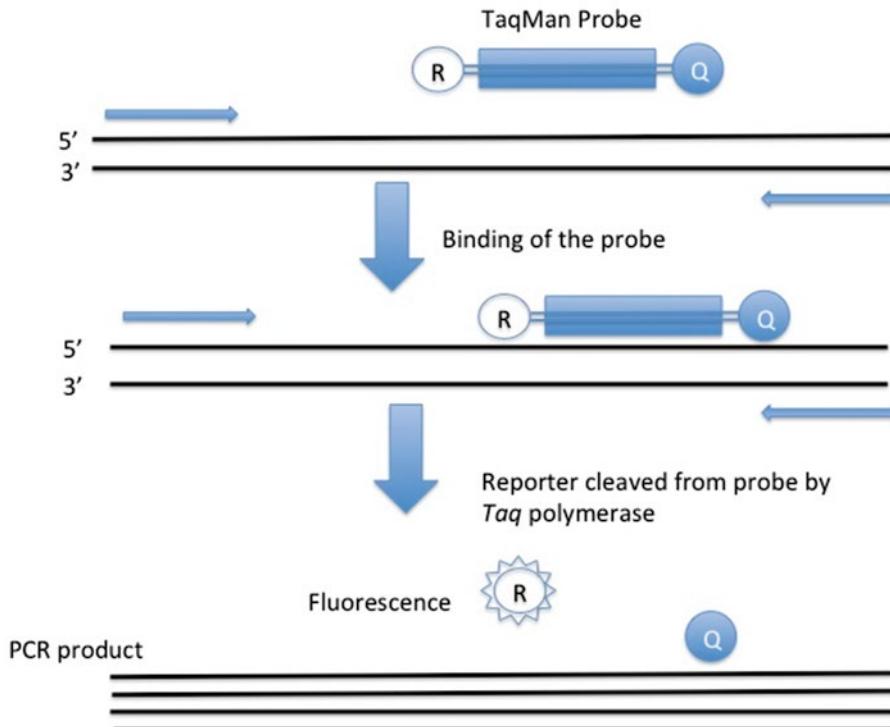
There are other *Tilletia* species that can be confused with *T. indica* and are commonly found in harvested grain or seeds. These include *T. walkeri* (a pathogen of *Lolium perenne* and *L. multiflorum*), *T. horrida* (a pathogen of *Oryza* spp.) and *T. ehrhartae* (a pathogen of *Ehrhartia calycina*). In Australia, *T. walkeri* and *T. ehrhartae* are found to contaminate harvested seed of *Triticum aestivum*. *T. walkeri* and *T. horrida* are present in the United States and are detected in harvested seed of *Triticum aestivum* especially where *Oryza* spp. and *Lolium* spp. are grown in rotation with *Triticum aestivum* [7–9]. Because of the morphological similarity of these pathogens, accurate identification is important.

## 1.2 Multiplex PCR

In conventional (end-point) PCR, the amplified product or amplicon is detected at the end of amplification by running the DNA on an agarose gel. In real-time PCR, the amplified product is detected and measured after each cycle of amplification. This is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA detected. The fluorescent molecule is bound either directly or indirectly [10]. For example, the use of SBYR™green I relies upon the binding of the fluorescent molecule to the minor groove of the double-stranded DNA [10]. Whereas in TaqMan™ chemistry, fluorescence results when the reporter molecule is cleaved from the probe by *Taq* polymerase [10]. The increase in DNA detected is proportional to the intensity of the fluorescent signal [11, 12]. The results from real-time PCR can either be qualitative (presence or absence) or quantitative (number of) and is entirely dependent upon the type of test developed and the results required. Real-time PCR and real-time quantitative PCR can be used for one genomic DNA target or multiple DNA targets to be detected or quantified simultaneously in a sample.

Multiplex PCR is made possible by the use of sequence-specific probes that are labelled with a distinct fluorescent dye and an appropriate quencher molecule individually. The fluorescent sequence-specific probe does not fluoresce in the absence of the specific target sequence, because it has not been hybridized, and the fluorescence of the reporter is transferred to the quencher [10]. However, when a specific target sequence has been detected during the extension step, the reporter molecule is cleaved from the quencher by polymerase nick translation and released; thus, fluorescence occurs (Fig. 2). The advantage of using this chemistry is it allows for multiple sequence-specific probes to be labelled with different reporter dyes, in a single reaction [11, 13]. It is most important that the emission spectrum for each fluorescent probes and quencher combination do not overlap each other, or this may lead to the signals being read on more than one channel, thereby giving confusing or conflicting results. Most companies such as QIAGEN and Bio-Rad provide tables with names of the fluorescent dyes and quenchers with their corresponding emission spectrum that can be used on their instruments. The fluorescent dye is usually at the 5' end of the oligonucleotide with the quencher placed at the 3' end. Optimally the quencher dye should only be 7–15 base pairs from the fluorescent reporter dye end. This may mean that the quencher needs to be placed at an internal thymine ("T") residue within the primer [14].

In this example, for the *Tilletia* species, a single-tube five-plex fluorescent assay was developed. The different probes were labelled with dyes that have non-overlapping, specific spectra for simultaneous detection [15].



**Fig. 2** Diagram of TaqMan™ probe chemistries showing the fluorescent reporter dye being cleaved from the probe by the *Taq* polymerase. *R* reporter dye, *Q* quencher

### 1.3 Design of the Genus and Species-Specific Primers

In most situations, the target pathogen may have very low levels of DNA present, which would then need amplification before being exposed to a multiplex real-time PCR reaction. In this case study, the protocol was done on a single teliospore and the DNA concentration was found to be as low as 0.1 pg. Therefore, a genus-specific primer was developed and used to amplify the *Tilletia* species DNA before proceeding to the multiplex real-time PCR. A species-specific primer-probe combination was used in the multiplex real-time PCR assay.

It is important when designing the primers to be used in real-time PCR that they are specific and will not detect or cross-react with other species that have similar genetic sequences. For a set of primers that are targeting a particular genus, you must ensure you pick a region with sufficient diversity that the genus is distinguished from other genera. However, you must also ensure the selected region is conserved enough so that all species within the genus can be detected. For specific species primers, you should ensure you pick a highly conserved region for that species that has high diversity when compared with other species within the same genus. For example, Tan et al. [15] targeted the internal transcribed

spacer 1(ITS1) region. *Tilletia* species have two variable regions (ITS1 and ITS2) separated by the conserved 5.8S rRNA gene. Work by Levy et al. [16] showed there is a >98 % similarity between *T. indica* and *T. walkeri* sequences; however, in the ITS 1 region, *T. walkeri* has a diagnostically important restriction enzyme site (*Scal*1) that is not present with *T. indica* and *T. horrida* [16, 17]. The works done by Levy et al. [18] and Tan and Murray [19] have shown that by targeting this region, the phylogeny of *T. walkeri*, *T. indica* and *T. horrida* could be resolved.

The size of the primer is very important as well. For real-time PCR, the optimum size of primers is between 15 and 30 base pairs [12, 20]. The primers should give you an amplicon size between 70 and 300 bp, because the larger the amplicon is, the greater the variation in reproducibility. There are now many software packages to help with the design of the primers and the probes to be used such as Primer3 (Whitehead Institute, Cambridge, MA) [10]. For example, in this case study [15], the software from Biosearch Technologies (<http://www.biosearchtech.com>) was used, along with Sigma-Proligo (St Louis, MO). Regardless of which software package is used, it is really important that the ITS sequences (or the targeted regions) are examined using GenBank (or similar) and then aligned using software such as TreeBASE S2240 or Clustal W [21] to ensure that there are no overlap and possible cross-reactions when designing your primers and probes.

The probes and primers for *T. indica* and *T. walkeri* were designed in this example by anchoring the sequences of the two probes for these two species on the ITS1 fragment where the nucleotide base polymorphism (nt. 117 of AF398434, Table 1) is located. The probe for *T. walkeri* was synthesized with Sigma-Proligo (St Louis, MO).

The probes and primers for *T. ehrhartae*, *T. caries*, and *T. horrida* were designed with the aid of the qPCR assay design software from Biosearch Technologies Inc. (<http://www.biosearchtech.com>) (verified 28 November 2008), which also supplied the custom primers and probes used in this study.

The ITS sequences of the following *Tilletia* species were examined in GenBank (Table 5) and then aligned using TreeBASE S2240 for the design of four pairs of primers and five dual-labelled probes (Fig. 3; Table 1).

#### 1.4 Determining Probe Specificities

The testing of the probe specificities against the target pathogens is an important component of the developmental process in the assay. Therefore, when designing a multiplex assay, all combinations of the target pathogens need to be tested, at equal concentrations. It is best to do this in a sequential logical pattern, for example, if you were targeting three pathogens (P1, P2, and P3), you would test the three pathogens individually, then in combination of two

**Table 1**

***Tilletia* species, their host and origin (geographical) used in the development of the genus-specific primers and the species-specific primers used in the PCR assays**

Species	Collection No	Host	Origin/year
<i>T. indica</i>	Ti 1	<i>Triticum aestivum</i>	Sonora, Mexico
<i>T. indica</i>	Ti 2	<i>T. aestivum</i>	Sonora, Mexico
<i>T. indica</i>	Ti 3	<i>T. aestivum</i>	Sonora, Mexico
<i>T. indica</i>	Ti 6	<i>T. aestivum</i>	Pakistan
<i>T. indica</i>	Ti 7	<i>T. aestivum</i>	Dakka, India
<i>T. indica</i>	Ti 8	<i>T. aestivum</i>	Ropar, India
<i>T. indica</i>	Ti 9	<i>T. aestivum</i>	Guerdersmir, India
<i>T. indica</i>	Ti 10	<i>T. aestivum</i>	California, USA
<i>T. indica</i>	WL1562	<i>T. aestivum</i>	India
<i>T. indica</i>	P2	<i>T. aestivum</i>	Amritsar, Punjab
<i>T. indica</i>	P3	<i>T. aestivum</i>	Ferozepur, Punjab
<i>T. indica</i>	P4	<i>T. aestivum</i>	Bathinda, Punjab
<i>T. indica</i>	P5	<i>T. aestivum</i>	Nawanshahar, Punjab
<i>T. indica</i>	P6	<i>T. aestivum</i>	Faridkot, Punjab
<i>T. indica</i>	P7	<i>T. aestivum</i>	Sangrur, Punjab
<i>T. indica</i>	P8	<i>T. aestivum</i>	Mansa, Punjab
<i>T. indica</i>	P9	<i>T. aestivum</i>	Gurdaspur, Punjab
<i>T. indica</i>	P10	<i>T. aestivum</i>	Hoshiarpur, India
<i>T. indica</i>	P11	<i>T. aestivum</i>	Ludhiana, India
<i>T. indica</i>	P12	<i>T. aestivum</i>	Ropar, India
<i>T. indica</i>	P13	<i>T. aestivum</i>	Pantnagar, India
<i>T. indica</i>	P14	<i>T. aestivum</i>	Haryana, India
<i>T. indica</i>	P15	<i>T. aestivum</i>	Pradesh, India
<i>T. indica</i>	P16	<i>T. aestivum</i>	Uttar Pradesh, India
<i>T. indica</i>	Ps2	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	Ps6	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	Ps7	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	Ps9	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	Ps12	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	Ps14	<i>T. aestivum</i>	Gurdaspur, India

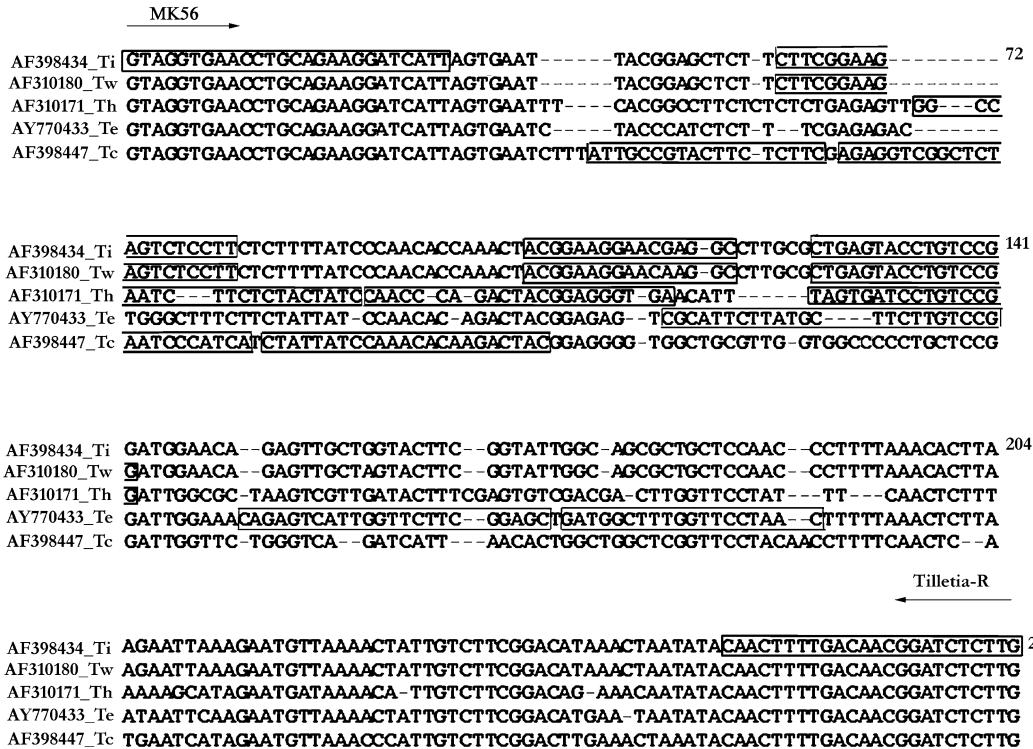
(continued)

**Table 1**  
**(continued)**

Species	Collection No	Host	Origin/year
<i>T. indica</i>	Ps17	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	Ps21	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	Ps23	<i>T. aestivum</i>	Gurdaspur, India
<i>T. indica</i>	M8602	<i>T. aestivum</i>	Mexico
<i>T. indica</i>	Jy01	<i>T. aestivum</i>	India
<i>T. walkeri</i>	210G	SW <sup>a</sup>	Oregon, USA
<i>T. walkeri</i>	Tw4	<i>Lolium multiflorum</i>	Georgia, USA
<i>T. walkeri</i>	DAR16720	<i>L. perenne</i>	NSW, Australia
<i>T. walkeri</i>	DAR16802	<i>L. perenne</i>	NSW, Australia
<i>T. horrida</i>	Th2	<i>Oryza sativa</i>	California, USA
<i>T. ehrhartae</i>	VPRI32078	<i>Ehrharta calycina</i>	SA, Australia
<i>T. ehrhartae</i>	BRIP45365	<i>Ehrharta calycina</i>	SA, Australia
<i>T. ehrhartae</i>	BRIP26818	<i>Ehrharta calycina</i>	WA, Australia
<i>T. ehrhartae</i>	BRIP28392	<i>Ehrharta calycina</i>	SA, Australia
<i>T. ehrhartae</i>	BRIP39762	<i>Ehrharta calycina</i>	SA, Australia
<i>T. ehrhartae</i>	BRIP45363	<i>Ehrharta calycina</i>	SA, Australia
<i>T. caries</i>	S4	<i>T. aestivum</i>	Sejet, Denmark
<i>T. caries</i>	S6	<i>T. aestivum</i>	Sejet, Denmark
<i>T. controversa</i> <sup>b</sup>	756	<i>Triticum</i> sp.	Idaho, USA
<i>T. controversa</i>	M973111	<i>Triticum</i> sp.	Ontario, Canada
<i>T. controversa</i>	177	<i>Triticum</i> sp.	Utah, USA
<i>T. bromi</i>	64	<i>Bromus japonicus</i>	Idaho, USA
<i>T. bromi</i>	120	<i>B. japonicus</i>	Idaho, USA
<i>T. fusca</i>	314A	<i>Vulpia microstachys</i>	Washington, USA
<i>T. fusca</i>	344	<i>V. microstachys</i>	Washington, USA
<i>T. caries</i>	DAR40492	<i>T. aestivum</i>	NSW, Australia
<i>T. caries</i>	DAR34387	<i>T. aestivum</i>	VIC, Australia
<i>T. laevis</i>	DAR73302	<i>T. aestivum</i>	NSW, Australia
<i>T. laevis</i>	WW05/0037	<i>T. aestivum</i>	NSW, Australia

Cited from [15] with permission

<sup>a</sup>SW isolate derived from seed wash<sup>b</sup>*T. controversa* is the name used by CAB International and most North American workers, whereas *T. contraversa* is the name advocated by Vánky (1994)



**Fig. 3** Sequences and positions of primers and probes used in the diagnosis of *Tilletia indica* (Ti), *T. walkeri* (Tw), *T. horrida* (Th), *T. ehrhartae* (Te), and *T. caries* (Tc) used in the real-time multiplex assay. The two arrows indicate the forward (MK56) and reverse (Tilletia-R) primers used in the amplification of Tilletia specific DNA. The relative positions of the pair of forward and reverse primers and the intervening probe sequence (Table 1) required for the real-time assay of each species are indicated. Numbers on the right refer to nucleotide positions in the GenBank accession AF398434 (cited from [15] with permission)

pathogens together (P1 + P2; P1 + P3; P2 + P3) and then all three together (P1 + P2 + P3).

So in relation to the case study [15], the following was done. DNA templates comprising equal concentrations of a mixture of two Tilletia species (*T. indica* + *T. ehrhartae*), (*T. indica* + *T. horrida*), (*T. indica* + *T. caries*) and (*T. indica* + *T. walkeri*) were tested. For three species *T. indica* + *T. walkeri* + *T. ehrhartae*, *T. indica* + *T. walkeri* + *T. caries*, *T. indica* + *T. ehrhartae* + *T. caries* and *T. indica* + *T. walkeri* + *T. horrida* were tested, and this was then done for four species and for all five species in the five-plex assay to assess the probe specificities in a mixture of template DNA. The DNA samples of the five target species used were P2, Tw4, Th2, S6, and BRIP45363 (Table 2).

The templates of mixed DNA were prepared from DNA samples of concentration 10 ng/µL. Thus, the DNA concentration of each species in a mixture of two, three, four, and five species

**Table 2**  
Primer and probe sequences and modifications used in the multiplex diagnostic assay for *T. indica* and other related *Tilletia* spp.

Primer pairs (sequence 5'-3')	Probes (modifications 5', 3')	Channel	Target
KB-DL-For: CTTCGGAAAGAGTCTCCCTT (nt. 1–21 <sup>a</sup> )	ACGGAAAGGAACGAGGC (nt. 67–82) (6-FAM, BHQ1)	Green	<i>T. indica</i>
KB-DL-Rev: CCGGACAGGTACTCAG (nt. 89–104)	ACGGAAAGGAACAAGGC (nt. 67–82 <sup>b</sup> ) (JOE, BHQ1)	Yellow	<i>T. walkeri</i>
Hor-DL-For: GGCCAATCTCTCTAC TATC (nt. 40–59 <sup>c</sup> )	CAACCCAGACTACGGAGGGTGA (nt. 60–81)	Orange	<i>T. horrida</i>
Hor-DL-Rev: CGGGACAGGATCACTA (nt. 87–102)	(CAL Fluor Red 610, BHQ2)		
Tri-DL-For: ATTGCCGTACTTCCTTC (nt. 56–73 <sup>d</sup> )	AGAGGTCCGGCTCTAATCCCATCA (nt. 75–97)	Red	Broad range <sup>e</sup>
Tri-DL-Rev: GTAGTCTTGTGTTGGA TAATAG (nt. 99–112)	(Quasar 670, BHQ2)		
Ehr-DL-For: CGCATTCCTATGCTTC TTG (nt. 72–90 <sup>f</sup> )	CAGAGTCATTGGTCTTCGGAGC (nt. 104–126)	Crimson	<i>T. ehrhartae</i>
Ehr-DL-Rev: GTTAGGAACCAAAGC CATC (nt. 128–146)	(Quasar 705, BHQ2)		

Cited from [15] with permission

<sup>a</sup>GenBank No. AF310174  
<sup>b</sup>AF310180

<sup>c</sup>AF310171  
<sup>d</sup>AF398447

<sup>e</sup>Includes *T. caries*, *T. laevis*, *T. controversa*, *T. fusca*, *T. bromi* and *T. gloeosporioides*  
<sup>f</sup>AY770433

would be 5, 3.3, 2.5, and 2 ng/ $\mu$ L, respectively, to end up with a total concentration of 10 ng/ $\mu$ L.

The specificity of the probe for *T. indica* was investigated in a DNA mixture of *T. indica*: *T. walkeri*, *T. indica*: *T. ehrhartae* or *T. indica*: *T. caries* in ratios of 1: 0.1 pg and 0.1: 1 pg to ensure there was no cross-reaction between the primers and probes detecting *T. indica* when the concentration was equivalent to 1 spore or 10 spores being present in the sample.

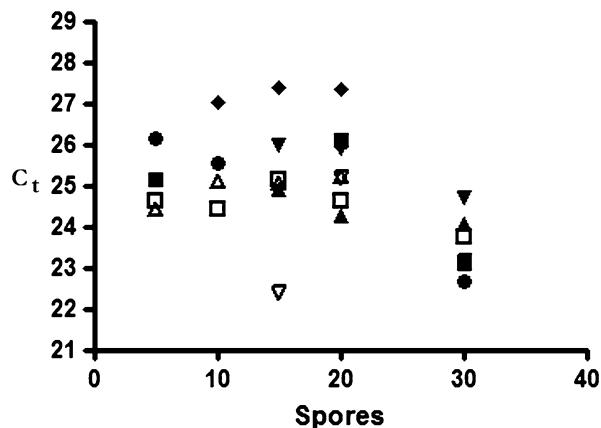
### **1.5 Extraction of DNA from *Tilletia* Species**

One of the major limiting steps in identifying *Tilletia* species by molecular methods was the extraction of DNA. In the past, the majority of methods relied upon germinating the teliospores of the fungi and then doing a DNA extraction from the mycelial mats. This can take 3 weeks or more to occur. Peterson et al. [22] found that the average teliospore germination rate was 55 %, which severely reduces the chances of identifying the teliospores by these molecular methods. The method by Tan et al. [15] removed this step by breaking a single teliospore open on a slide and then performing a DNA extraction step.

There are two important controls to include in the DNA extraction: (a) A known positive sample is used at the same time, to show that the DNA extraction step has worked, and (b) a known negative sample of the same material you are using at the time of extraction. The positive control confirms that your extraction is good, and the negative will confirm that you have not contaminated any of your samples at any stage of the process. The quantity of DNA extracted will also determine if an extra amplification step is required before proceeding to the multiplex PCR. In this case study on *Tilletia* species [15], the protocol was done on a single teliospore, and the DNA concentration was found to be as low as 0.1 pg. In this situation, a genus-specific primer was developed and used for the amplification step before the proceeding to the real-time multiplex PCR.

### **1.6 Determination of Relationship Between Target Pathogen Numbers and $C_T$ Values**

It is necessary to determine  $C_T$  values of the target pathogens both within the medium that you want to detect the pathogen within and if possible in pure culture. In this case study, grain (50 g) was spiked with known quantities of *Tilletia* spores (from the same species) before undergoing the DNA extraction method that had been developed. Single-spore analysis was also done on *T. indica*. Single species and mixtures of species are used when determining if there is a relationship. Determining this relationship will indicate if the multiplex PCR can be quantitative or qualitative (detected or not detected). In this case study, there was no significant difference in the mean  $C_T$  values obtained between the different numbers of spores in the grain samples (Fig. 4).



**Fig. 4** Correlation of  $C_t$  values with the number (5, 10, 15, 20, and 30) of *Tilletia ehrhartae* spores in grain samples. The mean  $C_t$  values (s.d.) obtained were as follows: 5 spores, 25.09 (0.77); 10 spores, 25.52 (1.10); 15 spores, 25.14 (1.64); 20 spores, 25.58 (0.98); and 30 spores, 23.58 (0.75). Cited from [15] with permission

The sensitivity of the test for single spores was 10–40 % (only 10–40 % of the known *T. indica* spores gave positive PCR results) [23]. This was because the reference material (which was exotic to Australia) had to be autoclaved twice for the work to be done, causing a loss in genetic material.

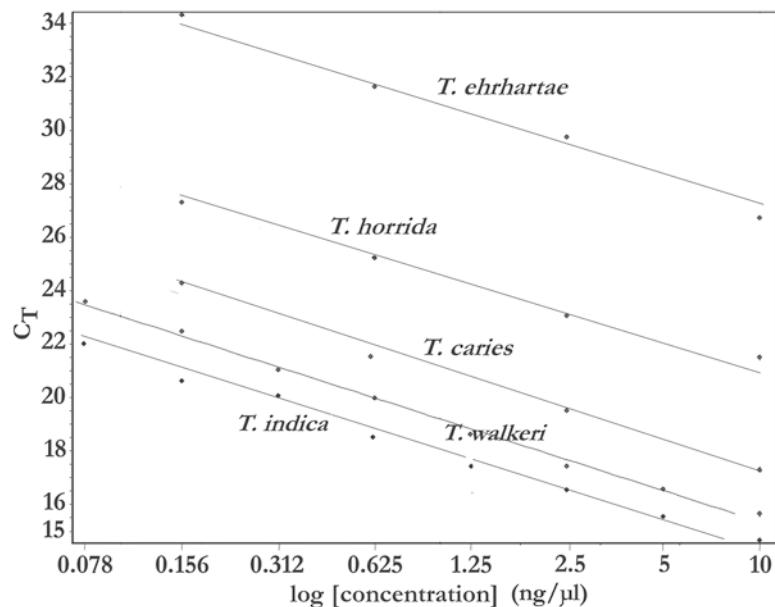
When doing this work, it is important to ensure that the number of replicates used is statistically valid to ensure that significant differences can be detected. A minimum needed is a triplicate measurement of each sample along with statistical analysis to determine whether differences in  $C_t$  values are significant.

### 1.7 Standard Curve

A standard curve is required to ensure that the efficiency of the amplification is consistent at different concentrations of the template and can be used to examine the specificity of the primer-probes produced. The standard curve is developed from a serial dilution of the template at a known concentration. These samples are then processed as per the protocol developed. The standard curve is the log of the initial template concentration against the  $C_t$  for each dilution. Most multiplex PCR machines have the software inbuilt to plot and develop this curve for you. An example of this is shown in Fig. 5. However, the equation to use for this work is

$$\text{Exponential amplification} = 10^{(-1/\text{slope})}$$

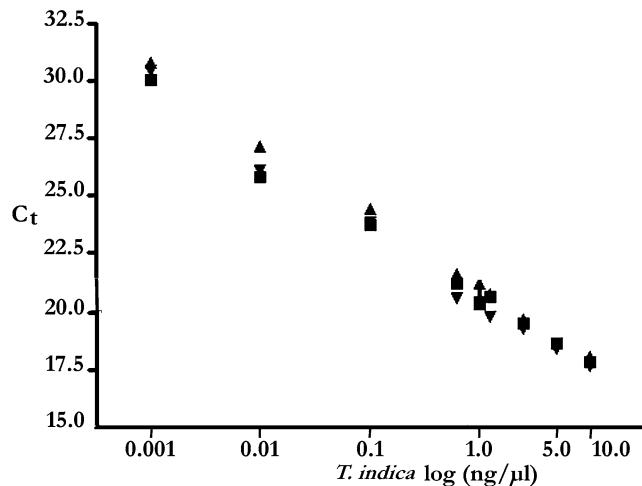
$$\text{Efficiency} = \left[ 10^{(-1/\text{slope})} \right] - 1 \times 100 \quad (\text{cited from [24]})$$



**Fig. 5** Overlay of the five standard curves correlating the  $C_t$  values (the value of the cycle where the amplification curve crosses the threshold line) with the  $\log(\text{concentration})$  for the quantification of the five target pathogens in the multiplex assay in Fig. 7. The standard curve was generated using the software by Rotor-Gene 1.7.75 (Corbett Research, Mortlake, New South Wales) (cited from [15] with permission)

The efficiency is relatively stable in the early exponential phase and gradually declines to zero as the PCR components deplete, along with the polymerase activity [24]. The authors state that the calculation of the amplification efficiency from the standard curve may overestimate the efficiencies because it does not take into account the change of efficiency over time. However, if the efficiency is calculated from raw data, a more accurate representation is obtained [24]. The efficiency value should be between 90 and 100 % [20]. This suggests that within the amplification process, the amount of PCR product doubles within each cycle.

Regardless, the standard curve should generate a linear regression line. It is important that a minimum of five points should be on this line and that it is done in triplicate [10, 20]. The range of concentrations used to develop the standard curve should be representative of the concentrations of the target to be detected in the assay. The square of the correlation coefficient ( $R^2$ ) indicates how well the line fits, and the closer the value is to 1, the better the fit [12, 20]. In this case study [15], the  $R^2$  values for the different species were as follows: *T. indica* 0.971, *T. walkeri* 0.996, *T. caries* 0.990, *T. horrida* 0.995 and *T. ehrhartae* 0.993. These results indicate a high correlation and a good fit of the standard curve (Fig. 6).



**Fig. 6** Correlation of  $C_t$  values with the log(concentration) for three replicate real-time assays of *Tilletia indica*. The range of standard deviations for  $C_t$  values and the concentrations were 0.08–0.68  $C_t$  and 0.000028–3.46 ng. The error values (which are the mean squared error of the single data points fit to the regression line) for each of the replicate assays were 0.0832, 0.146, and 0.175. Values below 0.2 are acceptable criteria for the accuracy of the quantification (cited from [15] with permission)

The standard curve can also be used to determine the sensitivity of the assay for the target pathogen. The results from the single-spore analysis on *T. indica* (Fig. 6) showed that the range of detection was from 0.1 to 10 pg.

## 2 Materials

### 2.1 DNA Extraction

1. Sterilized microscope coverslips, and cut into 1 × 1 mm pieces.
2. Microscope slides.
3. Tris-EDTA buffer (1 M Tris pH 8.0, 0.5 M EDTA, distilled water).
4. Sterilized needles (either dissecting needle, sewing needle, or surgical needle).
5. Fine needle nose forceps.
6. Dissecting microscope (50×).
7. Tilletia species spores on a microscope slide.
8. QIAGEN DNeasy Plant Mini Kit (QIAGEN, Australia).
9. Eppendorf tubes (1.5 and 2 mL).
10. Disposable mini-grinding pestles (Sigma, USA).

## 2.2 Amplification of Extracted DNA

1. Amplification Buffer L: 1× (50 mM Tris, pH 9.0, 20 mM NaCl, 1 % Triton X-100, 0.1 % gelatin).
2. *Taq* DNA polymerase (Invitrogen).
3. dNTP mix.
4. 50 mM MgCl<sub>2</sub>.
5. RNase-free water.
6. Thermocycler.
7. Eppendorf tubes.
8. 0.2 mL PCR tubes.
9. Pipettes and pipette tips.
10. Ice for keeping reagents cold or frozen ice bricks with wells to hold the Eppendorf tubes/PCR tubes.
11. Primers: MK56 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') [19].
12. Tilletia-R (5'-CAA GAG ATC CGT TGT CAA AAG TTG-3') [19].

## 2.3 Real-Time Multiplex PCR

1. Real-time PCR instrument (e.g., Rotor-Gene™ 6000, or LightCycler 480).
2. dNTP mix.
3. Immolase DNA Polymerase (Bioline).
4. RNase-free water.
5. MgCl<sub>2</sub> (Bioline).
6. PCR Amp. Buffer (Bioline).
7. Primer and Probes—refer to Table 4.
8. Eppendorf tubes.
9. 0.2 mL PCR tubes or 96 well plates.
10. Pipettes and pipette tips.
11. Ice for keeping reagents cold or frozen ice bricks with wells to hold the Eppendorf tubes/PCR tubes.

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## 3 Methods

### 3.1 Extraction of DNA from a Single Spore

1. Place a piece of the coverslip onto the microscope slide using the forceps.
2. Place 1 µL Tris-EDTA buffer onto the coverslip.
3. Place the slide with the Tilletia species spores under the microscope. Pick up a single spore using the sterilized needle (the spores are attracted by static charge).
4. Move the other slide (with the piece of the coverslip) underneath the microscope and dip the needle into the Tris-EDTA buffer. You should see a single spore in the droplet.

5. Using the forceps, pick up another piece of a coverslip and place over the top of the spore.
6. Now gently but firmly press onto the coverslip to squash the spore.
7. Pick up the coverslip sandwich and place into an Eppendorf tube. Seal the tube until you are ready to perform the amplification step. This can be stored at 4 °C.

### **3.2 Extraction of DNA from a Pellet**

1. A pellet obtained from the differential sieve-wash technique for the detection of *Tilletia* spores in grain [25] is placed into an Eppendorf tube with 50 µL of extraction buffer from the Qiagen DNeasy kit.
2. Grind this pellet to a smooth paste using a disposable mini-grinding pestle.
3. Add another 350 µL of extraction buffer from the Qiagen DNeasy kit to the pellet.
4. Then follow the manufacturer's instructions to complete the extraction procedure.
5. Store the extracted DNA at either 4 °C (if only for a day) or in the freezer (-20 to -80 °C) until being used.

### **3.3 Making PCR Master Mixes for Amplification**

1. Thaw the components of the master and primer mixes.
2. Make up stock solutions of 5× PCR master mix, 10× Primer mix (Tables 3 and 4).
3. Mix the reagents thoroughly before dispensing the stock solutions into smaller quantities that become your work solutions.
4. After dispensing into smaller quantities, and Eppendorf tubes are labelled correctly, then these working solutions can be stored in the freezer (see Note 1).

**Table 3**  
**5× PCR master mix: 50 lots of 20 µL reactions**

Component	Stock	Vol (µL)
Amp. Buffer L	10×	100
MgCl <sub>2</sub>	50 mM	30
dNTP mix	10 mM	20
<i>Taq</i> DNA polymerase (Invitrogen)	5 U/µL	10
RNase-free water		40
Total volume		200

**Table 4**  
**10× primer mix: 50 lots of 20 μL reactions**

Component	Stock (μM)	Vol (μL)
Forward primer MK56	100	5
Reverse primer Tilletia-R	100	5
Molecular water		90
Total volume		100

Forward: MK56 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3')  
 Reverse: Tilletia-R (5'-CAA GAG ATC CGT TGT CAA AAG TTG-3')

**Table 5**  
**PCR master mix composition**

Component	Stock	Vol (μL)
PCR master mix	5×	4
Primer mix	10×	2
RNase-free water		13
Template DNA (add at step 4)		1
Total volume		20

### **3.4 Amplification of DNA Before Proceeding to Real-Time PCR**

1. Design genus-specific primers to amplify the pathogen of interest. In this case study, the following primers were designed and used to amplify Tilletia species.  
 Forward: MK56 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3').  
 Reverse: Tilletia-R (5'-CAA GAG ATC CGT TGT CAA AAG TTG-3').
2. Thaw the working solutions from the stock solutions and make up the reaction mix (as shown below in Table 5) for the appropriate number of reactions.
3. Mix the reaction mix thoroughly and dispense 19 μL into 0.2 mL PCR tubes.
4. Add the template DNA to the individual PCR tubes containing the reaction mix.  
 NB: If using the single spore from the previous step (DNA extraction step), add 19 μL of the reaction mix directly to this Eppendorf tube and crush the coverslip sandwich using the pipette tip. Be careful to not break the Eppendorf tube in the process.

5. Perform the amplification step on a thermocycler using the following conditions. These temperature profiles relate to an Eppendorf Mastercycler.

PCR conditions: An initial cycle of 95 °C for 3 min, followed by 20 cycles of 94 °C for 20 s, 63 °C for 30 s, 72 °C for 30 s, with the annealing temperature decreased by 1 °C per cycle for five cycles to 59 °C, and finally followed by a 10 min and 1 min incubation at 72 and 4 °C, respectively. The products may be stored at 4 °C.

6. If visualizing on a gel, 10 µL of reaction products is loaded with a suitable marker and run on a 2 % gel. The expected fragment size is 260 bp. However, this fragment will not be visible if the PCR is done on a single teliospore, as there will not be enough DNA present.

### **3.5 Real-Time Five-Plex Fluorescent PCR Assay**

1. This step utilizes the DNA from the amplification step.
2. Thaw the components of the PCR master mix and the primer-probe mix (*see Note 2*).
3. Make up the stock solutions of the 4× PCR master mix and the 10× primer-probe mix (Tables 6 and 7).
4. Mix the reagents thoroughly before dispensing the stock solutions into smaller quantities that become your work solutions.
5. After dispensing into smaller quantities, and Eppendorf tubes are labelled correctly, then these working solutions can be stored in the freezer.
6. The multiplex reaction mix is made up as shown below (Table 8) just before use.
7. Mix thoroughly, and dispense 19 µL into 0.2 mL tubes to be used in a Rotor-Gene, or into multiwell plate that is used with other instruments.

**Table 6**  
**4× PCR master mix (multiplex): 50 lots of 20 µL reactions**

Component	Stock	Vol (µL)
Amp. Buffer (Bioline)	10×	100
MgCl <sub>2</sub> (Bioline)	50 mM	100
dNTP mix	10 mM	20
Immolase DNA polymerase (Bioline)	5 U/µL	10
RNase-free water		20
Total volume		250

**Table 7**  
**10× primer-probe mix: 50 lots of 20 µL reactions**

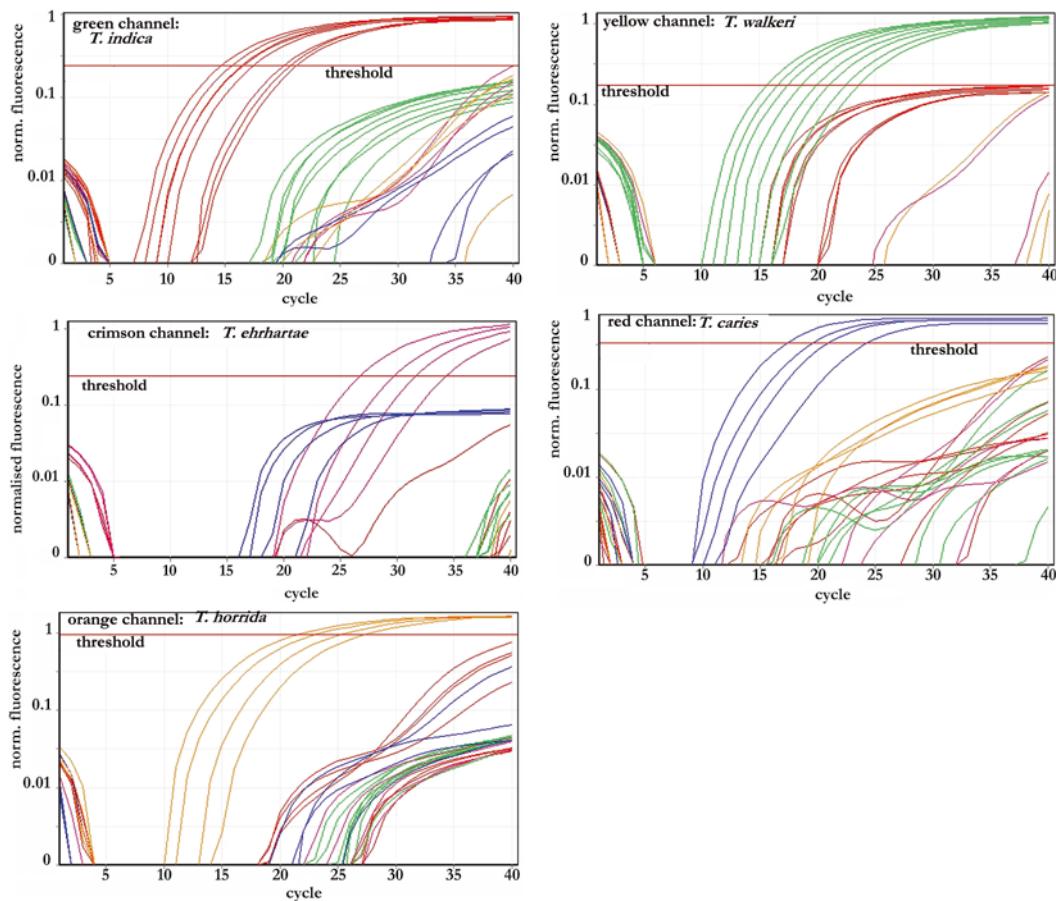
Component	Stock (µM)	Vol (µL)
KB-DL-For1	100	4
KB-DL-Rev1	100	9
Ehr-DL-For	100	4
Ehr-DL-Rev	100	9
Tri-DL-For	100	4
Tri-DL-Rev	100	9
Hor-DL-For	100	4
Hor-DL-Rev	100	9
<i>T. indica</i> probe 5' FAM, 3'BHQ1	100	2
<i>T. walkeri</i> probe 5' JOE, 3'BHQ1	100	2
<i>T. ehrhartae</i> probe 5' Q705, 3'BHQ2	100	2
<i>T. caries</i> probe 5' Q670, 3'BHQ2	100	2
<i>T. horrida</i> probe 5' CFR, 3'BHQ2	100	2
RNase-free water		38
Total volume		100

**Table 8**  
**Multiplex reaction mix composition**

Reagents	Vol (µL)
4× PCR master mix	5
10× primer-probe mix	2
RNase-free water	12
Template DNA	1
Total volume	20

8. Add the template DNA into the individual tubes or wells containing the reaction mix, and proceed to run the reactions according to the instrumentation specifications.

Multiplex PCR conditions: An initial cycle of 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 65 °C for 60 s with the annealing temperature decreased by 1 °C per cycle for 6 cycles to 60 °C. Dynamic tube normalization option was used to determine the average background of each individual sample



**Fig. 7** Fluorescence data acquired simultaneously by five channels in Rotor-Gene 6000 (Corbett Research, Mortlake, New South Wales) and normalized to a log scale for *Tilletia indica*, *T. walkeri*, *T. ehrhartae*, *T. horrida*, and *T. caries* in a multiplex real-time PCR assay (cited from [15] with permission)

before amplification commences. Fluorescence data was recorded to five channels: green, yellow, orange, red, and crimson. An example of the results is shown in Fig. 7.

9. Refer to **Notes 3–5** when using this assay with unknown samples.

### 3.6 Standard Curve

1. Dilute each of the *Tilletia* species standards from 10 to 0.0625 ng/ $\mu$ L using a twofold dilution series. This will give you the five points that are needed on the curve (*see Notes 6 and 7*).
2. Run these standard curves in triplicate following the instructions for the multiplex PCR (Subheading 3.5). An example of the results is shown in Fig. 6.

## 4 Notes

1. It is important to dispense stock solutions into working solution sizes, as freeze-thawing process affects the quality of the reagents and then affects the results. Therefore, work out what quantities (number of reactions) you will be doing on a regular basis.
2. The primer-probe mix is sensitive to light. So it is important to keep the exposure of these reagents to light at a minimum. Reduce the amount of natural light in the room by closing blinds.
3. Standard curve must always be included in a sample of unknowns, as you cannot compare different runs as conditions may vary between runs. Samples must always be in duplicate as the minimum, triplicate is preferred. Remember to include controls of known samples and no template controls in your run. Some papers also suggest that you include a negative sample as well which has had a DNA extraction done on a molecular water sample [12, 20]. However, if you use a negative water sample from the amplification step in the real-time multiplex, be aware that primer dimers can occur and create confusion with the results.
4. If the PCR efficiency is significantly less, this suggests that there are inhibitors within the reaction, that the primers and probes developed are not optimal or that the reaction conditions are not optimal. Poor pipetting techniques and the wrong serial dilution were used. If the PCR efficiency is greater than 100 %, this indicates the formation of primer dimers, pipetting error (or incorrectly calibrated) and other formation of non-specific products [12, 20].
5. The threshold cycle ( $C_T$ ) value (the value of the cycle where the amplification curve crosses the threshold line) obtained is used to set the threshold for that *Tilletia* species being tested. In general, a  $C_T$  value of greater than that set in this step is considered negative.
6. Ideally the standards should be made from single-spore isolates of the *Tilletia* species.
7. The use of a nano-drop to determine the concentration of DNA is very useful. However, be aware that it can give incorrect readings if it has not been calibrated correctly and the wrong solution has been used during the calibration process. Ensure you use the same solution that the DNA has been extracted with.

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# Chapter 5

## Diagnostics of Tree Diseases Caused by *Phytophthora austrocedri* Species

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### Abstract

We present methods for the detection and quantification of four *Phytophthora* species which are pathogenic on trees; *Phytophthora ramorum*, *Phytophthora kernoviae*, *Phytophthora lateralis*, and *Phytophthora austrocedri*. Nucleic acid extraction methods are presented for phloem tissue from trees, soil, and pure cultures on agar plates. Real-time PCR methods are presented and include primer and probe sets for each species, general advice on real-time PCR setup and data analysis. A method for sequence-based identification, useful for pure cultures, is also included.

**Key words** *Phytophthora ramorum*, *Phytophthora kernoviae*, *Phytophthora lateralis*, *Phytophthora austrocedri*, Real-time PCR, TaqMan, Quantification, Calibration curve, PCR inhibition, Internal positive control, ITS amplification, Identification

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### 1 Introduction

Species of *Phytophthora* (from Greek *phytón*, “plant” and *phthorá*, “destruction”) are among the world’s most destructive plant pathogens, causing damage not only to forest and amenity trees but also to shrubs and important food and fiber crops. Until fairly recently about six species of *Phytophthora* were recognized as causes of woody plant diseases in Britain, attacking the roots and root collar leading to symptoms of lower stem bleeding and crown dieback in affected trees. Over the past 10 years, however, at least four more tree-infecting species of *Phytophthora* have been detected in Britain: *P. ramorum*, *P. kernoviae*, *P. lateralis*, and *P. austrocedri* [1] (Fig. 1a–d).

*Phytophthora ramorum*, a foliar and canker pathogen, first emerged in the 1990s in both Europe and North America and has since transferred rapidly and repeatedly to various hosts and ecosystems in the two continents [1]. In the UK *P. ramorum* is the causal agent of a recent landscape-scale dieback of larch, resulting



**Fig. 1** Typical symptoms on plants infected with *Phytophthora* sp. (a) The red/brown discolouration of *Chamaecyparis lawsoniana* foliage typical of *Phytophthora lateralis* infection. (b) Necrosis of needles of *Larix kaempferi* caused by *Phytophthora ramorum* infection. (c) Extensive mortality of *Juniperus communis* due to an outbreak of *Phytophthora austrocedri*. (d) Foliar dieback on *Rhododendron ponticum* typical of *Phytophthora kernoviae* infection

in the felling of millions of trees. *Phytophthora kernoviae*, which was first found in Cornwall in 2003 [2], is an aerially transmitted pathogen like *P. ramorum* and in Britain is known to infect Rhododendron, beech and Vaccinium; the latter an ecologically important understory component of seminatural ancient woodland. *Phytophthora lateralis*, a devastating pathogen of Lawson cypress in its native range of southwest Oregon and northern California [3] has since 2010 been reported killing Lawson cypress via both root and aerial infections at several locations throughout the UK [4]. *Phytophthora austrocedri* is a root pathogen first described in 2007 associated with widespread dieback and mortality of *Austrocedrus chilensis* in Argentina [5, 6]. *Phytophthora austrocedri* was not known to occur anywhere else in the world until

2011 when it was reported causing extensive mortality of juniper trees in northern England [7]. Since then, surveys have revealed many more geographically disparate *P. austrocedri*-infected juniper woodlands in northern England and Scotland with more symptomatic sites under investigation [8]. This is raising serious concerns since juniper is a priority species in the UK Biodiversity Action Plan due to a decline in its distribution, population viability, and regeneration.

This upsurge in *Phytophthora* diseases of trees in Britain is likely due to two factors; firstly that some of these pathogens have been introduced from other geographical locations by the burgeoning international trade in live plants, and secondly, that their local establishment and spread within the new area may be linked to warmer, wetter, and windier site conditions over the last decade. This is because climate and the soil environment are integral to the spread and establishment of *Phytophthora* pathogens, which disseminate via free swimming zoospores; thus, wind-driven rain and waterlogged soils are frequent precursors of disease.

*Phytophthora ramorum* and *P. kernoviae* are quarantine regulated pathogens meaning that outbreaks within the UK are subject to statutory control measures to prevent further spread. *Phytophthora lateralis* and *P. austrocedri* are not listed organisms, but action is being taken either to remove infected plants or contain outbreaks by controlling access, correcting drainage problems, and implementing hygiene protocols to reduce the risk of further spread [1]. Essential to effective control or containment measures is an ability to detect these pathogens rapidly in the environment. However, these Phytophtoras, particularly the slow growing *P. lateralis* and *P. austrocedri*, can be difficult to isolate from plant material or soil onto selective media. For more reliable detection, TaqMan real-time PCR assays are now available for all four organisms, enabling rapid diagnosis from a range of environmental samples.

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## 2 Materials

### 2.1 Sampling, DNA Extraction, Real-Time PCR, and Sequencing

1. MACHEREY-NAGEL Plant II DNA Kit.
2. Saw and knife for sampling wood.
3. Microcentrifuge.
4. Sterile sand.
5. Micropesles.
6. 3 mm stainless steel bead.
7. 10 mm stainless steel bead.
8. Liquid nitrogen.
9. Mixer Mill (Retsch MM300 or QIAGEN Tissuelyzer) with 2 × 24 tube adaptors.

10. Planetary Ball Mill (Retsch PM400) with 4 × 250 ml mortar bowls.
11. Promega Wizard Magnetic DNA purification system for food kit.
12. Microplate compatible centrifuge.
13. Water bath.
14. UV spectroscope (e.g., NanoDrop).
15. Qiagen Plant Mini Kit.
16. 3 mm tungsten beads.
17. Vortex mixer.
18. Environmental mastermix 2.0 (Life Technologies, Paisley).
19. TaqMan internal positive control.
20. Micropipettors (air-displacement or positive-displacement, electronic or manual).
21. Eppendorf Comfort Thermomixer.
22. Real-time PCR machine (e.g., 7900HT or 7500 real-time PCR instrument (Applied Biosystems). Other instruments are also usable, e.g., Agilent Mx3005p).
23. Real-time PCR plasticware and seals (optical grade).
24. ITS primers and reagents for conventional PCR.
25. Thermal cycler.
26. Gel apparatus and imaging equipment.
27. KingFisher mL robotic workstation and BindIt 3.3 software.
28. Exonuclease I and shrimp alkaline phosphatase (Affymetrix-USB).
29. DNA sequencing reagents (e.g., BigDye Terminator version 3.1 Cycle Sequencing Kit) and apparatus, e.g., ABI Prism 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) (or an external provider of sequencing services).

## **2.2 Media Preparation for Fungi Cultivation**

1. SMA medium (*see Table 1*).
2. Trace element solution composition (*see Table 2*).
3. SMA + MRP medium.

SMA + MRP medium is prepared as for the Phytophthora minimal medium (SMA medium) of Elliott et al. [9] and then supplemented before autoclaving with 0.5 ml of 4 % MBC (benomyl hydrochloride) solution. The pH is adjusted to 6.5 with 1 M NaOH. After autoclaving at 121 °C for 15 min the agar is cooled and then further supplemented with 0.4 ml of a 2.5 % suspension of Pimaricin and 3 ml of a 1 % w/v solution of Rifamycin SV.

**Table 1**  
**SMA medium**

Ingredient	Amount/liter
Sucrose	10 g
L-asparagine	1.0 g
KH <sub>2</sub> PO <sub>4</sub>	0.5 g
MgSO <sub>4</sub> •7H <sub>2</sub> O	0.25 g
Trace element solution ( <i>see</i> Table 2)	1.0 ml
Thiamine hydrochloride	1.0 mg
Difco Bacto-Agar	10.0 g

**Table 2**  
**Trace element solution composition**

Ingredient	Amount/liter
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> •10H <sub>2</sub> O	88 mg
CuSO <sub>4</sub> •5H <sub>2</sub> O	393 mg
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> •6H <sub>2</sub> O	910 mg
MnCl <sub>2</sub> •4H <sub>2</sub> O	72 mg
Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O	50 mg
ZnSO <sub>4</sub> •7H <sub>2</sub> O	4,403 mg
EDTA	5 g

4. V8 broth medium.

V8 broth is adapted from Johnston and Booth [10]; 20 % Campbell's V-8 juice and 0.5 % CaCO<sub>3</sub> are combined and then autoclaved at 121 °C for 15 min.

5. Carrot broth.

Carrot broth is adapted from Drenth and Sendall [11]. 200 g of carrots are cleaned and thickly sliced and then macerated in a blender with 500 ml of distilled water. Filter through muslin, squeezing to extract all of the juice. Make up to 1 l and autoclave at 121 °C for 15 min.

### 3 Methods

#### 3.1 DNA Extraction from Phloem Tissue Using the MACHEREY-NAGEL Plant II Kit

This method below uses a 96-well plate-compatible centrifuge for column processing (see Note 1). DNA extractions are usually performed in multiples of eight (see Note 2).

1. Phloem tissue is collected from just behind the leading margin of tree stem or branch lesions.
2. 65–99 mg of tissue is added to each extraction tube (supplied with kit). A 3 mm stainless steel bead is placed in each tube and the tubes are sealed using supplied cap strips.
3. The tubes are frozen in liquid nitrogen and tissue is disrupted in a Retch MM300 mixer mill for 60–90 s at 30 Hz.
4. Centrifuge tubes at  $5,600 \times g$  for 2 min in a microplate centrifuge.
5. Remove cap strips and add 500  $\mu$ l PL1 buffer and 10  $\mu$ l RNaseA (12 mg/ml).
6. Mix by shaking vigorously, then centrifuge briefly ( $1,500 \times g$  for 30 s) to move solids and liquids from the caps, to the bottom of the tubes.
7. Incubate samples in a water bath at 65 °C for 30 min. Complete the extraction procedure using the MACHEREY-NAGEL Plant II Kit as described by the kit supplier.
8. Elute DNA with 100  $\mu$ l of preheated elution Buffer PE (65 °C) added directly to the membrane of the binding strips. Centrifuge for 2 min at  $5,600$ – $6,000 \times g$ .
9. Measure concentration of eluted DNA using spectroscopy.

#### 3.2 DNA Extraction of *Phytophthora* from Soil Using the Promega Wizard Magnetic DNA Purification System for Food and KingFisher mL Robotic Workstation [12]

1. Soil samples (approximately 500 g) are air-dried for 5 days or until they are completely dry. Once dry, the samples are placed into a large plastic bag (300 mm × 450 mm) and thoroughly mixed to reduce the soil to fine particles.
2. Twelve 10 mm stainless steel ball-bearings are placed into a 250 ml mortar bowl from a Retsch PM400 Planetary Ball Mill. The mixed soil (approx. 150 cm<sup>3</sup>) is then added to the bowl to within 1 cm of the rim and 120 ml of CTAB buffer is added (20 g CTAB, 21.4 g Sodium Phosphate, 87.7 g NaCl in 1 l sterile distilled water). The mortar bowl is securely fastened into the Retsch PM400 and milled at 300 rpm for 5 min to mix the soil to a liquid paste.
3. After milling, two 2 ml aliquots are taken from each sample and placed into 2 ml microcentrifuge tubes. These 2 ml aliquots are centrifuged for 2 min at  $5,000 \times g$ .
4. 500  $\mu$ l of supernatant is then removed from each of the two tubes and transferred to a new 2 ml centrifuge tube resulting in a total 1,000  $\mu$ l of supernatant. 250  $\mu$ l of Buffer B and 750  $\mu$ l

of precipitation fluid from the Promega Wizard Magnetic DNA purification system for food kit (Promega Cat. No. FF3750) are added before the tubes are vortexed and centrifuged at  $11,000 \times g$  for 10 min.

5. 750  $\mu$ l of the resulting supernatant is then transferred to a new 2 ml tube. 50  $\mu$ l of the magnetic beads and 600  $\mu$ l of isopropanol are added and then vortexed before the tubes are incubated for 5 min at room temperature with occasional mixing by inverting.
6. The prepared sample is added to the first tube in the KingFisher m BindIt L robotic workstation rack. The second well contains 1,000  $\mu$ l buffer B (from the Promega kit), the third and forth wells contain 1,000  $\mu$ l of 70 % ethanol and the fifth well contains 200  $\mu$ l of molecular grade water for elution.
7. Thermo Fisher BindIt 3.3 software is used to create the program for the KingFisher mL (see Note 3).

### **3.3 DNA Extraction of *Phytophthora* from Pure Culture Using the QIAGEN Plant Mini Kit**

1. Isolates are grown in either V8 broth or carrot broth for 1–2 weeks (i.e., for *P. austrocedri*, incubation times are 14 days at 17 °C, in the dark). 1 ml of culture is placed in a 2 ml microcentrifuge tube.
2. Mycelia are pelleted by centrifugation in a microcentrifuge for 5 min at  $14,000 \times g$ . The supernatant is discarded and a 3 mm tungsten carbide bead is added to each tube.
3. The tubes are frozen in liquid nitrogen for 30 s, and subsequently placed into the 2×24 adaptor set. The adaptors are fixed into the clamps of the Retsch MM300 mixer mill and immediately shaken for 1 min at 30 Hz.
4. The samples are removed from the adaptors, refrozen in liquid nitrogen and placed back into the adaptor, but reversing the original positions. Replace in the clamps of the mixer mill and shake for 1 min at 30 Hz.
5. Retrieve the tubes and add 400  $\mu$ l Buffer AP1 and 4  $\mu$ l RNaseA solution (100 mg/ml). Vortex vigorously and then incubate at 65 °C for 10 min. Mix two or three times during this incubation. Complete the extraction procedure using the MACHEREY-NAGEL Plant II Kit as described by the kit supplier.
6. Elute DNA using 100  $\mu$ l of the AE elution buffer pipetted directly onto the membrane of the spin column. Incubate for 5 min at room temperature and then centrifuge for 1 min at  $\geq 6,000 \times g$ .

### **3.4 Real-Time PCR**

Despite the DNA purification method, some preparations of DNA obtained from the woody tissue and soil contain inhibitors which can prevent amplification using standard master mixes. The methods described use a specific mastermix to alleviate the inhibitors

encountered in this assay (Environmental Mastermix 2.0, Life Technologies) (e.g., [13]) (*see Note 4*). In addition to inhibitor resistance, the *P. austrocedri* assay is designed to include an internal positive control which should alert the user to the presence of a sample which may overcome the inhibition resistance of the mastermix

#### 3.4.1 Making Dilutions for Calibration Curve

After obtaining the DNA preparation to be used in construction of the calibration curve (S0), dilute a portion to the most concentrated amount needed (S1, the first standard dilution). An S1 dilution of 5,000 pg/ $\mu$ l is used, adding 2  $\mu$ l per PCR reaction to give 10 ng/PCR. After making the S1 dilution, incubate at 4 °C with gentle shaking for 16–24 h (e.g., using an Eppendorf Comfort mixer). Subsequent tenfold dilutions (S2 to S5) are made and incubated at 4 °C with gentle shaking for 1 h after each dilution (*see Note 5*). Store the calibration standards at 4 °C. The standards should be monitored for performance degradation by comparison to previous data, including loss of reliability in detection of the highest dilutions.

#### 3.4.2 Setting Up Real-Time PCR Reactions

In general, reactions should be set up as triplicates. For each sample (or control, or standard), add 3.5 $\times$  the volume of each reagent needed for a single reaction to one microcentrifuge tube. Vortex each tube for 30 s, and then briefly pulse-spin the tube to collect the reaction mix. Dispense 20  $\mu$ l into each of three wells (*see Note 6*).

In addition to samples and standards, one should ensure that negative controls are included. There are two main types of negative controls: extraction blanks and water blanks. The first is an extraction performed at the same time as the samples, where the mycelia or phloem tissue is replaced by an equivalent amount of water. The extraction blank is then processed like any other sample and tested alongside the others. This will show if the extraction process has caused cross-contamination between samples. The second control, the water blank, substitutes the template DNA for water. This will control for contamination in the PCR reaction constituents. Real-time PCR plates are run on a real-time PCR instrument (7900HT or 7500 (Applied Biosystems)).

1. *P. austrocedri* real-time PCR detection [13] (Tables 3, 4, and 5).
2. *P. ramorum* and *P. kernoviae* real-time PCR detection [14] (Tables 6, 7, 8, and 9).
3. *P. lateralis* real-time PCR detection [15] (Tables 10, 11, and 12).

#### 3.4.3 Analysis of Results

Each real-time PCR reaction can be characterized in terms of a value, on the X-axis, at which an amplification curve intersects the “threshold” line, positioned to pass through the early exponential part of the amplification curves. The X-axis value at the point that the threshold line intersects the amplification curve is called the

**Table 3**  
**Primers and probe sequence for *P. austrocedri* diagnosis**

Primer name	Sequence <sup>a</sup>
Paus-481-F	TGG TGA ACC GTA GCT GTA TTT AAG C
Paus-554-R	GGA ACA ACC GCC ACT CTA CTT C
Paus-507-TM <sup>b</sup>	TGG CAT TTG AAC CGR CGA TGT G

<sup>a</sup>Primers shown 5' to 3'

<sup>b</sup>A combination of 5'-FAM fluorochrome and 3'-BHQ-1 dark quencher is used for the TaqMan probe, but other combinations should work, and adaptations can be used to fit in with local circumstances

**Table 4**  
**Real-time PCR mix for *P. austrocedri* diagnosis**

Ingredient	Volume (1×) (μl)	Volume (3.5×) (μl)	Supplier
Environmental mastermix 2.0 (2×)	10	35	Life Technologies
Paus-481-F (5 pmol/μl)	1	3.5	
Paus-554-R (5 pmol/μl)	1	3.5	
Paus-507-TM (5 pmol/μl)	1	3.5	
IPC mastermix VIC probe (10×)	2	7	Life Technologies
IPC DNA (50×)	0.4	1.4	Life Technologies
H <sub>2</sub> O	2.6	9.1	
Template DNA	2	7	

**Table 5**  
**Cycling conditions**

No. of cycles	Temperature (°C)	Time
1×	95	10 min
	95	30 s
40×	60	30 s
	72	60 s

**Table 6**  
**Primers and probe sequence for *P. ramorum* diagnosis**

Primer name	Sequence <sup>a</sup>
Pram 114-Fc	TCA TGG CGA GCG CTG GA
Pram 190-R	AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT
Pram 1527-134-T <sup>b</sup>	TTC GGG TCT GAG CTA GTAG

<sup>a</sup>Primers shown 5' to 3'

<sup>b</sup>A combination of FAM and TAMRA is used for the TaqMan probe

**Table 7**  
**Primers and probe sequence for *P. kernoviae* diagnosis**

Primer name	Sequence <sup>a</sup>
Pkern 615F	CCG AAC AAT CTG CTT ATT GTG TCT
Pkern 722R	GTT CAA AAG CCA AGC TAC ACA CTA
Pkern 606T <sup>b</sup>	TGC TTT GGC GTT TGC GAA GTT GGT

<sup>a</sup>Primers shown 5' to 3'

<sup>b</sup>A combination of 5'-TET fluorochrome and 3'-TAMRA quencher is used for the TaqMan probe

**Table 8**  
**Real-time PCR mix for *P. ramorum* diagnosis**

Ingredient	Volume (1x) (μl)	Volume (3.5x) (μl)	Supplier
Environmental mastermix 2.0 (2x)	12.5	43.75	Life Technologies
Pram 114-Fc (5 pmol/μl)	1.5	5.25	
Pram 190-R (5 pmol/μl)	1.5	5.25	
Pram 1527-134-T (5 pmol/μl)	0.5	1.75	
H <sub>2</sub> O	7	24.5	
Template DNA	2	7	

The ingredients for *P. kernoviae* are the same, substituting the Pkern primers for the Pram

**Table 9**  
**Cycling conditions**

No. of cycles	Temperature (°C)	Time
1x	94	10 min
40x	94	15 s
	60	60 s

**Table 10**  
**Primers and probe sequence for *P. lateralis* diagnosis**

Primer name	Sequence <sup>a</sup>
qPlat-F	ACG GGA TCG TGT TCT AGC AG
qPlat-R	TAG CTG CAC GTC GTT GCT AC
qPlat-P <sup>b</sup>	TTT TCC CGC TTT CCT TGG GG

<sup>a</sup>Primers shown 5' to 3'

<sup>b</sup>A combination of FAM and BHQ1 is used for the TaqMan probe

**Table 11**  
**Real-time PCR mix for *P. lateralis* diagnosis**

Ingredient	Volume (1×) (μl)	Volume (3.5×) (μl)	Supplier
Environmental mastermix 2.0 (2×)	12.5	43.75	Life Technologies
qPlat-F (5 pmol/μl)	1.5	5.25	
qPlat-R (5 pmol/μl)	1.5	5.25	
qPlat-P (5 pmol/μl)	0.5	1.75	
H <sub>2</sub> O	7	24.5	
Template DNA	2	7	

**Table 12**  
**Cycling conditions**

No. of cycles	Temperature (°C)	Time
1×	95	10 min
40×	95	10 s
	60	45 s

cycle threshold ( $C_T$ ). Provided the amplification curve fulfils certain quality criteria, this figure will largely be determined by the initial amount of template added to the reaction mixture. In addition to setting the threshold to pass through the amplification curves, it is also important to set the baseline region correctly. The baseline is a region in the early cycles of amplification where the amount of product is too low to be detected. This baseline region

is used to set background fluorescence across all wells of a plate, and so permit its subtraction. The baseline is set as follows:

1. Set an appropriate threshold line.
2. Find the lowest  $C_T$  value for the experiment (i.e. the  $C_T$  value of the leftmost curve).
3. Round this  $C_T$  value down to an integer (e.g., if the  $C_T$  is 16.7, round to 16).
4. Set the upper baseline limit to three cycles less than the lowest rounded value ( $16 - 3 = 13$ ). Subtracting 3 from the lowest  $C_T$  value seems to be a commonly accepted method. The lower baseline limit is usually left unchanged. Apply the baseline adjustment.
5. Reset the threshold if necessary.
6. Reset the baseline if the lowest  $C_T$  value has changed (e.g., if the value was 16.7 and is now 16.2 the upper limit of baseline would remain the same; if the  $C_T$  has dropped to 15.6 the upper limit would need to be adjusted). This should now have correctly set baseline and threshold.

This process needs to be repeated for the internal positive controls (IPC) if used, and usually requires a different threshold level and a different baseline setting. This is because the amount of IPC template is low giving a concomitantly high  $C_T$  value, so as not to interfere with the multiplexed assay for pathogen detection. Once the threshold and baseline settings are satisfactory for the IPC one should check that the  $C_T$  value is similar for all PCRs. In general,  $\pm 1$   $C_T$  value appears to be the normal deviation for the IPC. Considerable variation from this expected deviation should be investigated. Any wells with unusual IPC results should be eliminated as a precaution.

The next task is to examine the standard curve produced. In general it is sufficient to use the analysis software provided with the real-time machine. If necessary, one can export the data to use in a spreadsheet for other analyses. The triplicate values should be treated as separate data points, and not averaged. This will allow variation, or obviously incorrect values, to be seen and eliminated if justified (i.e., obvious outliers). The standard curve usually shows numerical information, in addition to the graph. The  $R^2$  value (the square of the correlation coefficient) shows how close the data points lie to a predicted line of regression. For a standard curve,  $R^2$  values should be  $\geq 0.98$ . Finally, the slope of the graph should be between -3.6 and -3.1 corresponding to an amplification efficiency of 90–110 % (-3.32 being the 'ideal' slope, meaning that the reaction proceeds at 100 % efficiency). Efficiency can be calculated using the following equation:

$$E = \left( 10^{(-1/\text{Slope})} - 1 \right) \times 100$$

Once one is satisfied that the internal positive controls have worked (and the negative controls, of course), the data from the samples can be considered. Software estimates of amount of target in a well are usually automatic, the answer being in the units used for the standard curve (e.g., pg of target per tube). As all samples are performed in triplicate, an outlier should be obvious. However, there may occasionally be two outliers and the data point discarded could be closer to the real value than the data points retained.

### **3.5 Cultivation of Fungi from Tissue and Confirmatory Sequencing Using Standard PCR**

Phloem samples from lesion margins are plated on to SMA + MRP *Phytophthora* selective medium [2] and incubated at room temperature (15–24 °C) in the dark. Putative *Phytophthora* colonies are individually subcultured onto V8 or carrot agar to obtain a pure culture.

DNA is obtained from isolates as described above. To amplify the internal transcribed spacer (ITS) region, PCR is performed using the *Phytophthora*-specific forward primer Ph2 [16] and universal reverse primer ITS4 [17]. A simplified version of the PCR recipe is used, utilizing a 2× master mix (JumpStart REDTaq ReadyMix Sigma-Aldrich, catalogue number P 0982), using 0.25 µM final concentration of each primer and 1 µl of template DNA. Amplification is performed with initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 7 min. The PCR product is cleaned up using Exonuclease I and Shrimp Alkaline Phosphatase (SAP) Exonuclease I is used to degrade single-stranded DNA such as unused primers, and SAP is used to treat unincorporated dNTPs prior to sequencing (see Note 7). PCR products are sequenced in both direction, using primers Ph2 and ITS4, with the BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI Prism 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following recommendation to users (Table 13).

**Table 13**  
**Primers used for *Phytophthora* confirmatory sequencing**

<b>Primer name</b>	<b>Sequence<sup>a</sup></b>	<b>Reference</b>
Ph2	ATA CTG TGG GGA CGA AAG TC	[16]
ITS4	TCC TCC GCT TAT TGA TAT GC	[17]

<sup>a</sup>Primers shown 5' to 3'

## 4 Notes

1. A vacuum method is also available—see manufacturer's instructions for details.
2. This method is also useful for isolation of DNA from liquid cultures. Mycelia from pure fungal cultures (other than *P. austrocedri*) is harvested by centrifuging 1 ml samples of broth

**Table 14**  
**KingFisher mL program for soil extraction**

Bind DNA (Tube A)	Beginning of step—precollect no Release time 00:00:30, speed—bottom mix Mixing/pause: mixing time, speed 00:04:30, fast Pause for manual handling—no End of step postmix—no Collect count 3 Collect time [s] 1
Wash_1 (Tube B)	Beginning of step—precollect no Release time 00:00:30, speed—bottom mix Mixing/pause: mixing time, speed 00:01:00, fast Pause for manual handling—no End of step postmix—no Collect count 3 Collect time [s] 1
Wash_2 (Tube C)	Beginning of step—precollect no Release time 00:00:30, speed—bottom mix Mixing/pause: mixing time, speed 00:01:00, fast Pause for manual handling—no End of step postmix—no Collect count 3 Collect time [s] 1
Wash_3 (Tube D)	Beginning of step—precollect no Release beads—no Mixing/pause: mixing time, speed 00:00:30, slow Pause for manual handling—no End of step postmix—no Collect beads—no
Elution (Tube E)	Beginning of step—precollect no Release beads—yes Mixing/pause: mixing time 00:15:00, speed—bottom mix Pause for manual handling—yes Pause message—paused for heat (5 min at 65 °C) End of step postmix—no Collect count 3 Collect time [s] 30
ReleaseBeads1 (Tube D)	Release time 00:00:30, speed—bottom mix

cultures, resuspending the pellet in 500 µl buffer PL1 with 10 µl RNaseA solution and grinding the tissue with sterile sand using a micropestle and continuing the extraction method below from **step 6** onwards.

3. KingFisher mL program for soil extraction (Table 14).
4. Other master mixes have been tested and shown to be resistant to the inhibitors from phloem extractions (e.g., Takara PCR Mastermix, A. Schlenzig, SASA, Pers. Comm.).
5. The volumes used should not be so small as to minimize inaccuracy—a minimum of 10 µl of DNA into 90 µl of diluent (either molecular grade water or TE buffer) is recommended. Also, the use of 2.0 ml curved-bottomed microcentrifuge tubes is suggested, rather than the conical-bottomed tubes (to improve movement of the liquid during agitation). The dilution series used for real-time PCR may start at a higher concentration than given in this description, but depends on the expected range of results—qualification should always be performed by interpolation rather than extrapolation between DNA standards.
6. Accuracy seems to improve by the use of reverse pipetting to dispense the reaction mixture, presumably because of the characteristics of the liquid (e.g., viscosity, density, wetting capacity, surface tension) of the liquid. Use of a positive displacement pipette may also improve accuracy.
7. Equal volumes of exonuclease I (10 units/µl, Affymetrix-USB, catalogue number 70073) and SAP (1 unit/µl, Affymetrix-USE, catalogue number 78390) are combined and mixed gently by pipetting, and 1 µl of this mixture is added to each PCR reaction and the reactions are incubated at 37 °C for 45 min, and inactivated at 80 °C for 15 min.

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# Chapter 6

## Real-Time LAMP for *Chalara fraxinea* Diagnosis

Jenny Tomlinson and Neil Boonham

### Abstract

*Chalara fraxinea* is the causal agent of ash dieback, a disease affecting *Fraxinus excelsior* and *F. angustifolia* across Europe. Loop-mediated isothermal amplification (LAMP) is a rapid, DNA-based method which can be used for specific detection of plant pathogens in infected material. The combination of a rapid LAMP assay for *C. fraxinea* with a simple sample preparation method in a user-friendly kit format raises the potential for testing to be carried out away from conventional laboratory facilities, to expedite measure to manage this damaging disease.

**Key words** Detection, On-site testing, Loop-mediated isothermal amplification, *Chalara fraxinea*, Ash dieback

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### 1 Introduction

*Chalara fraxinea* T. Kowalski [1] is the causal agent of ash dieback in *Fraxinus excelsior* and *F. angustifolia*, first identified in Poland in 2006. Dieback symptoms are preceded by the development of necrotic lesions in the bark and xylem of *C. fraxinea*-infected trees. The lesions are often lenticular or diamond shaped and may extend from infected side shoots (Fig. 1). The teleomorph of *C. fraxinea* was initially identified as the common saprophyte *Hymenoscyphus albidus* until Queloz et al. [2] determined that the teleomorph is actually a distinct species, *H. fraxineus* (synonym: *H. pseudoalbidus*). Ash dieback has the potential to cause significant damage and has resulted, directly or indirectly, in the loss of large proportions of the ash population in countries where outbreaks have occurred. In order to respond appropriately to control the disease, rapid methods for detection of *C. fraxinea* in potentially infected trees are required.

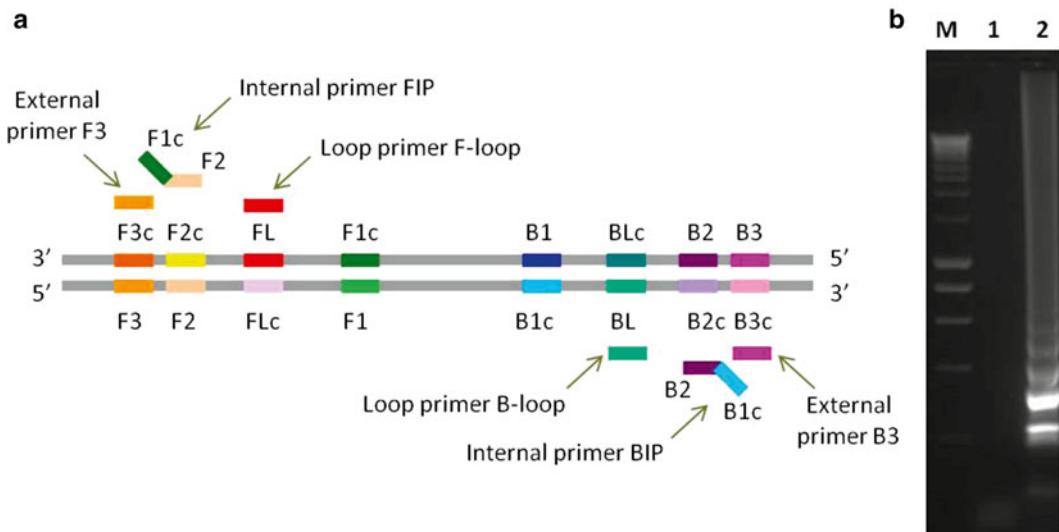
Isolation of *C. fraxinea* from infected plant material is relatively slow and likely to produce false-negative results [3]; for this reason real-time PCR for *C. fraxinea* is the preferred method for high-throughput testing in the laboratory [3, 4]. In general, real-time PCR is a very useful method for rapid testing of large sample



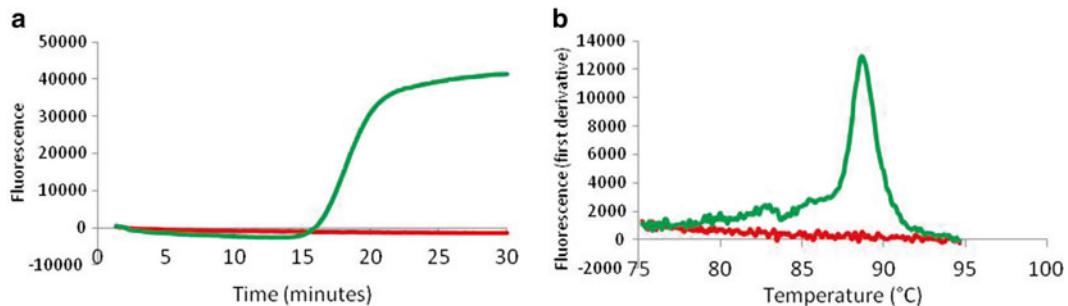
**Fig. 1** Symptoms of *Chalara fraxinea*

numbers and has the potential for automation. However, the use of PCR is limited to fairly well-equipped laboratories due to the complexity and expense of thermal cycling equipment. Methods which use isothermal amplification instead of PCR have an immediate advantage in this respect as they do not require thermal cycling equipment. Loop-mediated isothermal amplification (LAMP) is an amplification method which uses a novel arrangement of primers and DNA polymerase with strand displacement activity to generate amplification products which contain single-stranded loop regions (Fig. 2) [5–7]. These single-stranded regions can act as primer binding sites without the need for thermal denaturation and reannealing, so the amplification can occur at a single temperature (typically 65 °C). Amplification of nucleic acids by LAMP is extremely efficient; a large amount of product can be generated in a short period of time (Fig. 2) and the sensitivity of LAMP assays typically exceed that of conventional PCR.

Because LAMP generates a large amount of amplification product, greatly exceeding the amount typically produced by PCR, a wide range of methods can be used to detect positive reactions, including visual assessment of turbidity and various color change reactions. A major consideration when using LAMP is the need to avoid cross contamination due the very large amount of amplification products that are generated. For routine use there is therefore a significant advantage to the use of closed-tube detection methods, such as measurement of turbidity or fluorescence. Real-time



**Fig. 2** Primers used for loop-mediated isothermal amplification (LAMP) (a) and the typical ladder-like appearance of a LAMP product analyzed by agarose gel electrophoresis and examination of the gel under UV illumination (b). M, DNA size marker; 1, negative reaction; 2, positive reaction



**Fig. 3** Typical real-time LAMP results: (a) amplification plot showing increasing fluorescence with amplification of the target sequence; (b) anneal plot showing a specific peak at approx. 88 °C. Positive reaction shown in green; negative reaction shown in red

amplification plots can be informative in giving an indication of amplification efficiency and the amount of target in a sample, as the time to a positive result ( $T_p$  value) is inversely proportional to the amount of target. Real-time LAMP using a fluorescent intercalating dye, such as SYBR Green, also has the advantage of allowing additional interrogation of the amplified products without the need to open the tubes, by observation of melting or annealing temperatures (Fig. 3). LAMP products consist of alternately inverted repeats of the target sequence concatenated in products of different total length, which melt/anneal at a specific temperature which is characteristic for each assay. This allows specific products

to be distinguished from nonspecific amplification products, primer dimers, and other artifacts, increasing the robustness of the results obtained and also assisting in troubleshooting in the event of unexpected or atypical results. Real-time LAMP has been reported for the detection of a variety of plant pathogens [8–12].

Another characteristic feature of LAMP is the tolerance of the enzyme to substances which can inhibit amplification by PCR using Taq polymerase. In the context of plant pathogen detection, significant inhibitors include polysaccharides and polyphenolics from plant material and humic acid from soil. Tolerance of inhibitory substances permits the use of crude extraction methods which could not be used with PCR without additional purification. This is of particular significance for testing outside normal laboratory facilities, where the ability to test samples after minimal processing is a major advantage. In the context of the detection of *C. fraxinea*, wood can be a particularly challenging matrix for nucleic acid extraction. An approach which is often used for simplified extraction of nucleic acid from plant samples is to use alkaline lysis followed by neutralization [13–15]. Buffers with a pH of around 13–14 can be used to lyse cells with or without physical disruption of the material. Samples which have been lysed using NaOH or KOH can be neutralized before being added to LAMP reactions [14]. A useful variant of this method uses alkaline polyethylene glycol (PEG) as the lysis buffer; as this reagent can be neutralized by dilution, the addition of a neutralizing agent is not necessary [15]. Extraction of DNA from wood samples, including *C. fraxinea*-infected ash, can be efficiently achieved by shaking with ball bearings in alkaline PEG buffer followed by dilution in molecular-grade water.

The combination of simplified sample preparation and rapid detection using real-time LAMP is particularly well suited to use in non-laboratory conditions, such as at outbreak sites. End users in these scenarios are unlikely to be molecular biology specialists, so the availability of reagents in a kit format, requiring minimal handling, greatly increases the likely uptake of LAMP for this kind of application. The *C. fraxinea* Plant Assay kit from OptiGene uses a combination of alkaline PEG extraction and real-time LAMP for the detection of *C. fraxinea* in ash showing signs of ash dieback. Being able to perform testing at the site of sampling without needing to send samples to a laboratory can have significant advantages in enabling decisions related to disease management to be made more rapidly. LAMP can have particular advantages of sensitivity and specificity over other field testing methods such as lateral flow devices, which in any case are not currently available for *C. fraxinea*.

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## 2 Materials

1. Scalpels (or other suitable blades) for sampling.
2. Disposable gloves (*see Note 1*).
3. High-level disinfectant (*see Note 1*).
4. Alkaline PEG buffer: 60 % PEG 200 (Sigma) plus 20 mM KOH (or NaOH), pH 13.3–13.5 (*see Note 2*).
5. 5 ml screw cap tubes.
6. Ball bearings (7/16", stainless steel).
7. Nuclease-free water.
8. 1.5 ml or 2 ml microcentrifuge tubes.
9. Pipettors and tips or disposable plastic loops (*see Note 3*).
10. Genie assay strips: *C. fraxinea* assay and COX (plant control) assay (OptiGene) (*see Note 4*).
11. Genie II instrument (OptiGene).

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## 3 Methods

### 3.1 Sampling

1. A crucial part of pathogen detection in symptomatic plant material is the initial visual assessment of symptoms. Identify the suspected lesion(s) and remove the outer layer of bark using a scalpel or other suitable blade to locate the leading edge. Robustness is increased by taking samples from different locations around the lesion if possible. Bear in mind that symptoms may vary with factors such as age of the host tree, age of the lesion, and time of year. Also *see Note 5*.
2. Use a scalpel or similar blade to cut thin pieces, ideally from the leading edge, to total approximately 2 × 2 cm in area (e.g., 4 pieces of approx. 1 cm<sup>2</sup>). Use disposable blades or decontaminate between samples (*see Note 6*). It is important to avoid cross contamination between samples due to the sensitivity of LAMP (*see Note 1*).

### 3.2 Sample Preparation

1. Place the sample material into a 5 ml screw cap tube containing a 7/16" ball bearing and 1 ml alkaline PEG buffer and shake vigorously for 1 min (*see Note 7*).
2. Dilute the sample by transferring 3 µl into a fresh tube containing 72 µl nuclease-free water or Dilution Buffer supplied with the OptiGene kit. *See also Note 3*.
3. Test the samples within approx. 1 h of preparation (*see Note 8*). *See also Note 9*.

### 3.3 Running LAMP on the Genie II

1. To test up to six samples, prepare one *C. fraxinea* strip and one COX (plant control strip) (*see Notes 10 and 11*). Prepare each strip by resuspending the pellet in each well in 25 µl resuspension buffer (*see Notes 4, 12, 13*.)
2. Transfer 3 µl sample to the appropriate wells (*see Note 14*). Each strip should contain a negative control reaction and a positive control reaction.
3. Place the strips into Blocks A and B of the Genie II instrument. Run the reactions on a Genie II instrument with the following reaction conditions: no preheat; 30 min @ 65 °C; anneal from 75 to 95 °C at 0.05 °C/s (*see Note 15*.)
4. Positive reactions should display both a sigmoidal amplification curve and a single anneal peak at the correct temperature (Fig. 3). DNA or PEG extracts from *C. fraxinea*-infected ash samples should give annealing peaks at approx. 88.5 °C for the *C. fraxinea* assay and approx. 85 °C for the COX assay. *See also Note 16*.

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## 4 Notes

1. Due to the sensitivity of LAMP and the efficiency with which amplification products are generated, care must be taken to prevent cross contamination between samples and carry-over contamination of amplified products, which would lead to false-positive results. This can be achieved by taking simple precautions. Work areas and any equipment (racks, pipettors if using) should be wiped with high-level disinfectant or 5 % bleach solution before and after testing. If multiple samples are to be processed at one time, disposable gloves should be worn and changed between samples. When a closed-tube detection method such as real-time fluorescence detection is used, the tubes should be disposed of immediately after use without reopening.
2. To make approx. 100 ml alkaline PEG buffer, combine 60 g PEG 200, 0.93 ml 2 M KOH (or NaOH), and 39 ml water. Check the pH and adjust if necessary to 13.3–13.5.
3. The PEG sample preparation method and spiking of LAMP reactions can be carried out using a pipettor in the laboratory, but an alternative for simplified use outside the laboratory (or for nonspecialist operators) is to use disposable plastic inoculating loops for the transfer of small volumes of liquid. The method is sufficiently robust that the dilution of crude PEG extracts prior to testing, and the transferral of diluted samples into pre-prepared LAMP reactions, can be carried out using loops instead of pipettors.

4. LAMP requires the use of a thermostable DNA polymerase with strand displacement activity, such as *Bst* large fragment (New England Biolabs). OptiGene Isothermal Master Mix, as used in the *C. fraxinea* Plant Assay kit, contains an alternative polymerase which can be used with much shorter reaction times of 30 min or less. As this master mix contains an intercalating fluorescent dye it is convenient for use with real-time fluorescence detection.
5. The PEG extraction method as described is optimal for testing wood samples. Other matrices including foliage, rachis, and individual apothecia can also be tested but may require modification of the sample preparation method.
6. Non-disposable blades should be cleaned to prevent carry-over contamination. For example, spray with laboratory disinfectant and dry thoroughly between samples.
7. Larger pieces should be cut up if necessary to fit in the tube and move freely when shaken. The PEG buffer should take on the color of the sample but the material will not be completely homogenized.
8. PEG extracts are not stable for long-term storage and should be tested straight away or within a few hours.
9. Conventional DNA extraction methods can be used instead of the alkaline PEG method for testing in laboratory conditions. The limit of detection of the *C. fraxinea* LAMP assay is approx. 7 pg fungal DNA per reaction.
10. The robustness of testing for pathogens can be increased by parallel testing for DNA from the plant host, for example, using the LAMP assay for the detection of plant DNA targeting cytochrome oxidase (the COX assay). By testing for the host plant, false negatives caused by suboptimal samples or improper or inefficient extraction can be identified. A negative result for *C. fraxinea* which is accompanied by a negative result for COX may be a false negative so should be considered inconclusive, and testing should be repeated if possible.
11. Up to six samples may be tested with one pair of strips. Samples should be run in parallel with both positive and negative controls. As a minimum, a no-template control (NTC) should be run to help to identify issues with contamination or nonspecific amplification artifacts (e.g., due to improper handling or storage of reagents). An extraction blank may also be tested to check for contamination in sample preparation (e.g., of the PEG buffer or other extraction reagents, if using). Positive control reactions should be run to confirm that the LAMP reagents in a particular run are functional, as improper handling or storage of reagents may result in delayed amplification or atypical amplification plots or annealing peaks.

DNA extracted from *C. fraxinea* in culture or from infected ash samples using conventional extraction methods can be used as a positive control. Note that if the positive control on a strip gives a negative result, it is not possible to assign any negative results for the samples on the same strips as these may be false negatives.

12. Lyophilized reagents are ideal for testing in non-laboratory conditions as they can be stored at ambient temperature and simply resuspended before use. Reactions made up from non-lyophilized reagents (or lyophilized reagents once resuspended) should be stored at 4 °C or on ice and used on the same day to avoid the generation of nonspecific amplification artifacts which can occur after prolonged storage.
13. After resuspending the lyophilized reagents, leave the strips for a few minutes to allow any foaming to subside. The strips can also be briefly centrifuged or tapped on a flat surface to ensure that the reagents are at the bottom of the wells before the caps are opened.
14. When testing PEG extracts using LAMP, an overall dilution of 1 in 250 generally gives very good results (a greater dilution factor can reduce sensitivity; at lower dilution factors, the PEG or the sample itself may interfere with amplification and/or fluorescence detection). As described here, the neat PEG extracts are diluted 1 in 25 in nuclease-free water/Dilution Buffer, and then approx 3 µl diluted sample is added to a 25 µl LAMP reaction; however, different dilution factors and sample volumes may also be used.
15. The Genie II instrument is portable and battery powered, making it suitable for use in a range of testing scenarios. Other closed-tube approaches include the use of the metal ion indicator hydroxynaphthol blue and the measurement (or direct observation) of turbidity. Other detection methods, including gel electrophoresis (Fig. 2b), can be used in the laboratory, but only if pre- and post-amplification handling can be meticulously separated at all times.
16. Amplification plots should be sigmoidal, as shown in Fig. 3. Linear amplification plots or plots with a shallow gradient may indicate inefficient amplification due to a problem with the reagents or suboptimal processing of the sample. In order to confirm that amplification was specific, the annealing plots should also be examined. Nonspecific amplification can result in normal amplification plots but can be discriminated from true positives if the annealing plot shows no peak, if there are multiple peaks and/or if there are peaks at incorrect temperatures.

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# Chapter 7

## Loop-Mediated Isothermal Amplification Procedure (LAMP) for Detection of the Potato Zebra Chip Pathogen “*Candidatus Liberibacter solanacearum*”

Aravind Ravindran, Julien Lévy, Elizabeth Pierson,  
and Dennis C. Gross

### Abstract

An efficient loop-mediated isothermal amplification procedure (LAMP) for the detection of “*Candidatus Liberibacter solanacearum*” (Lso), the bacterial causal agent of potato zebra chip (ZC) disease, is described in this chapter. Similar to the polymerase chain reaction (PCR), the LAMP employs a bacterial polymerase to amplify specific DNA sequences. However, the method differs from conventional PCR in that it uses six primers specific to the target region to generate a loop structure and autocycling strand displacement rather than thermocycling for sequence amplification. Moreover, unlike PCR that requires agarose gel electrophoresis for resolution, the positive LAMP results can be visualized directly as a precipitate within the reaction tubes. The 16S rDNA gene of “*Ca. Liberibacter solanacearum*” was used as the target for the design of the six LAMP primers. The LAMP technique is a reliable, rapid, and cost-effective method of detecting the “*Ca. Liberibacter solanacearum*” pathogen in the potato/tomato psyllid, *Bactericera cockerelli*, and in field-grown potato plants and tubers.

**Key words** Bacteria, DNA extraction, LAMP, Liberibacter, Potato, Psyllid, Zebra chip

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### 1 Introduction

Zebra chip disease has had a significant impact on potato production in North America and New Zealand [1]. The disease is caused by the nonculturable bacterial species “*Candidatus Liberibacter solanacearum*” (Lso), which is transmitted by the potato/tomato psyllid, *Bactericera cockerelli* (Sulc.) [2] (Fig. 1). Symptom development in potato plants infected by Lso begins with the development of purple on mature leaves, leaf curling, and chlorosis, which is typically most pronounced on the youngest leaves. As the disease progresses, symptoms include shortened and swollen internodes, aerial tubers, and eventually wilting and premature death [1] (Fig. 1). Potato tubers affected by zebra chip disease develop darkened stripes when



**Fig. 1** Zebra chip disease vector and symptoms. (a) Adult potato psyllid (*Bactericera cockerelli*) and (b) nymph feeding on the leaf surface (4 $\times$  magnifications). (c) Early symptoms of ZC on potato include the yellowing of the younger leaves and leaf rolling and (d) development of purple leaf coloration. (e) Later symptoms include the formation of aerial tubers and (f) severe wilting leading to death. Notice the plant infected

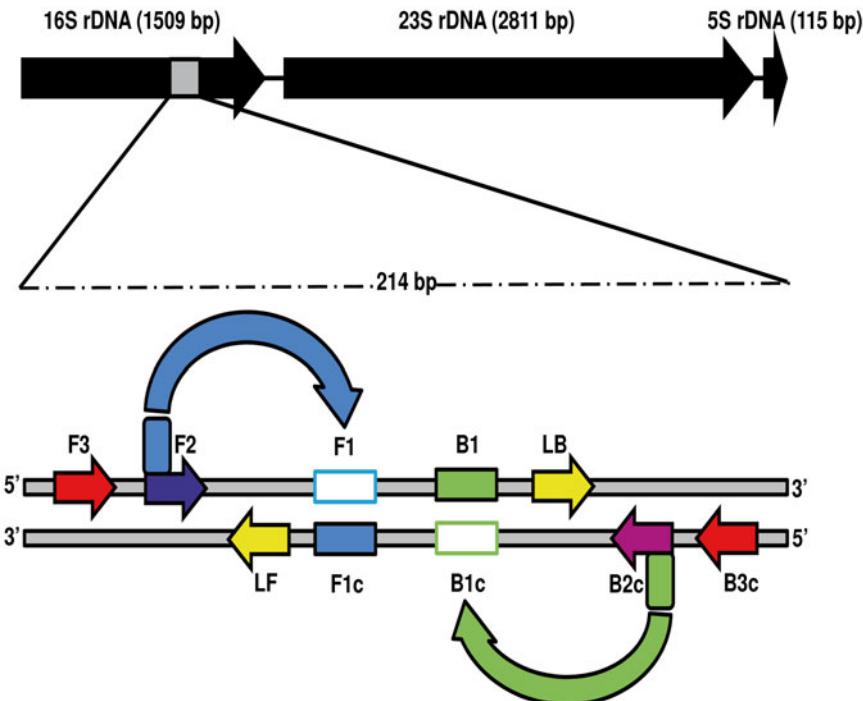
fried that render tubers unsuitable for potato chip or French fry production (Fig. 1). Resistance to zebra chip disease has not been identified in commercially available potato cultivars. Consequently, disease management along with efforts to breed for disease resistance depends on reliable detection of the pathogen both within the insect vector and infected plant tissues. Moreover, dependable early detection of Lso in plants immediately following infection has been problematic because bacterial titers typically remain too low for reliable detection via PCR until several weeks after infection [3].

Loop-mediated isothermal amplification (LAMP) as developed by Notomi et al. [4] is being used increasingly for the detection of a variety of pathogens [5, 6]. In previous work [7], we demonstrated that the LAMP method presented here reliably detected “*Ca. Liberibacter solanacearum*” the bacterial causal agent of potato zebra chip disease. Although it was not as sensitive as quantitative real-time PCR, LAMP detection was equivalent to conventional PCR in tests of ZC-infected potato plants from the field. The LAMP method is based on autocycling strand displacement DNA synthesis facilitated by a *Bacillus stearothermophilus* (*Bst*) DNA polymerase. As described previously [7], the method is centered on the generation of a dumbbell-shaped loop structure resulting from the use of six primers: two inner primers, forward inner primer (FIP) and backward inner primer (BIP); two outer primers, forward outer primer (F3) and backward outer primer (B3c); and two loop primers, loop forward (LF) and loop backward (LB) that are specific to eight independent regions of the target sequence (Table 1, Fig. 2). *Bst* DNA polymerase has autocycling strand displacement activity and is used in a one-step process

**Table 1**  
The Lso 16S rDNA primer sequence for each of the six LAMP primers

Lamp primer name	Sequences (5'-3')
LsoTX16SLamp-F3	CTGCATGGCTGTCGTCA
LsoTX16SLamp-B3c	GTAACCACCATTGTAGC
LsoTX16SLamp-FIP (F1c-F2)	TAGAGGTAGGGTTGC-GTGTGAGATGTTGGG
LsoTX16SLamp-BIP (B1-B2c)	GGGTACTTATAGGGA-CCATGAGGACTTGACG
LsoTX16SLamp-LF	GCTCGTTGCGGGACTTA
LsoTX16SLamp-LB	GGAGGAAGGTGGGGATG

◀  
**Fig. 1** (continued) by zebra chip disease (center) relative to the healthy field potatoes. (g) Darkening of the medullary rays renders fried tubers unsuitable for chip or French fry production relative to healthy tubers (h). Photos A and B courtesy of Cecilia Tamborindeguy; C, D, G, and H courtesy of J. Levy; and E and F courtesy of D. C. Gross



**Fig. 2** Schematic diagram showing the eight annealing locations of the six LAMP primers. The primers (shown in Table 1) were designed to target unique sequences within a 214 bp region of the 16S rDNA gene of *Lso*. To initiate the first-strand synthesis of the target sequence, the inner primers FIP and BIP anneal to the F2 and B2c regions of the “sense” and “complementary” strands of the double-stranded DNA template (shown in *violet* and *magenta*, respectively). The outer primers F3 and B3c then hybridize to the F3 and B3c regions (shown in *red*) and displace the synthesized first strand; these primers are added in a lower concentration because they are only required for first-strand synthesis during the first 5 min of the amplification process. Unlike the template, the newly synthesized “sense first strand” includes additional sequences corresponding to the FIP primer at the 5' end, i.e., a sequence complementary to the F1 region of the sense strand (shown in *blue*). This sequence facilitates the formation of a loop structure at the 5' end. Similarly the newly synthesized “complementary first strand” contains additional sequences derived from the BIP primer that are complementary to the B1c region (shown in *green*) and facilitate the formation of a loop structure at the 5' end. Subsequent amplification of the newly synthesized “sense first strand” using the BIP primer or of the “complementary first strand” by FIP primer produces the requisite dumbbell-shaped template with loop structures at either end. Cycling amplification using this dumbbell-shaped template produces concatemeric amplicons of the target sequence. To accelerate the LAMP reaction, two additional primers LF and LB anneal to the LF and LB regions shown in *yellow*

that amplifies a target DNA sequence at a single temperature rather than thermocycling as used in conventional PCR and quantitative real-time PCR (qPCR) for DNA polymerase amplification. Furthermore, positive LAMP results can be visualized directly as a precipitate within the reaction tubes, unlike PCR that requires agarose gel electrophoresis for resolution. The end result is a highly specific method of pathogen detection in significantly less time and with fewer equipment requirements.

As described previously [7], for Lso detection the basic LAMP primers (F3, B3c, FIP, and BIP) were designed to specifically target six independent regions of the 16S rDNA sequence (Fig. 2). In addition, two loop primers (LF and LB) were developed to the same gene, and these provide enhanced specificity and reaction efficiency for an accelerated LAMP reaction [8]. The LAMP produces substantial amounts of large concatemeric DNA strands composed of repeating units of the target sequence. A by-product of the LAMP is the formation of an insoluble white precipitate of magnesium pyrophosphate, which becomes increasingly visible with time in the reaction tube. The results (no product vs. visible turbidity) make it simple to distinguish between negative and positive reactions within the reaction tube. LAMP amplification requires less than 60 min (as compared to up to 2 h for conventional PCR amplification), and no post-PCR analysis via gel electrophoresis is needed to visualize the outcome.

### 1.1 Primer Design for LAMP

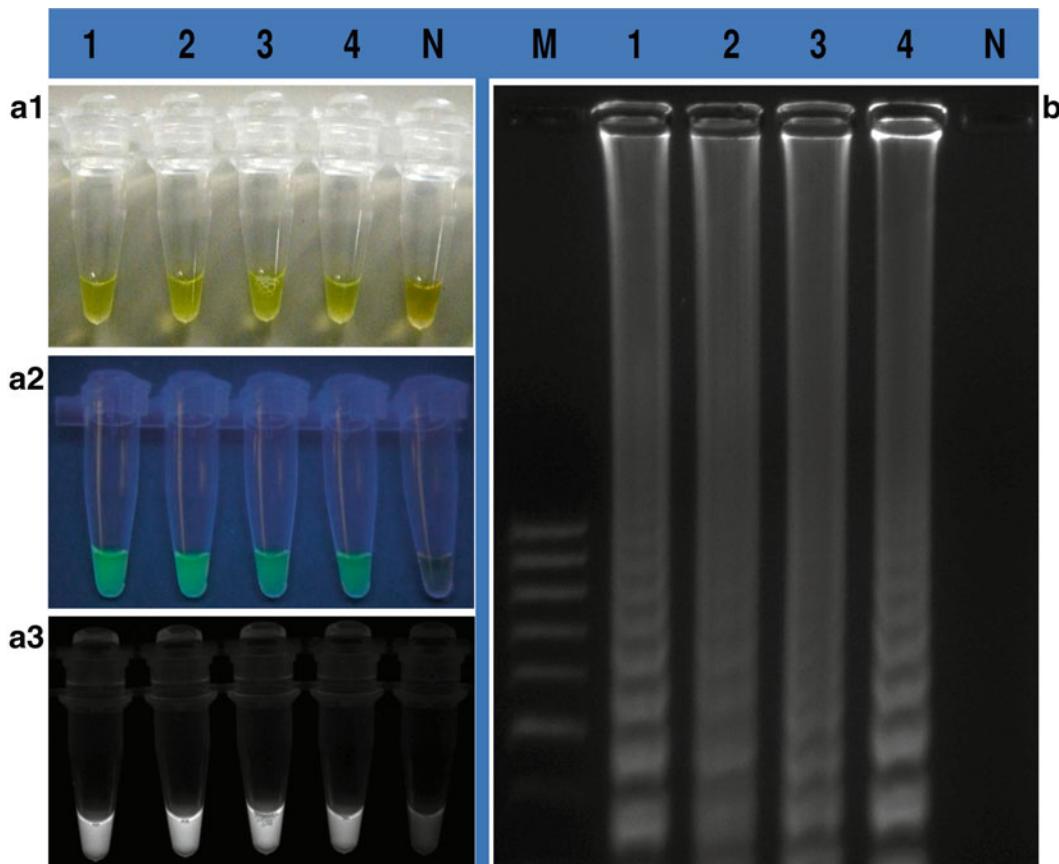
LAMP primer design was performed with the aid of Primer Explorer V4 software (Eiken Co., Ltd., Japan: <http://primerexplorer.jp/e/>). Sets of six LAMP primers were designed to several housekeeping genes, the 16S rDNA gene, and the 16S/23S rDNA intergenic region. Those primer sets were tested for amplification of the targeted genes. Initially LAMP was somewhat unreliable for all of these primer sets, resulting in either no reproducible amplification or false positives, suggesting that further improvement in primer design was necessary. To enhance reliability, it was necessary to verify the primer folding predictions, and so the free energy of the most stable DNA secondary structure was calculated using the UNAFold software program [9]. Optimization of the turn-back primers (FIP and BIP) was required for stable loop formation, and this was carried out using the least absolute shrinkage and selection operator (LASSO) regression analysis method ([http://gerg.gsc.riken.jp/TP\\_optimization/](http://gerg.gsc.riken.jp/TP_optimization/)) [10]. Ultimately, the LAMP primer set (LsoTX16SLAMP) targeting the 16S rDNA of “*Ca. Liberibacter solanacearum*” (GenBank accession NC\_014774) was selected based on it yielding the most reproducible results (see Note 1) (Table 1). Standard desalting primers were obtained from either Invitrogen (Grand Island, NY) or IDT (Coralville, IA) (see Notes 2 and 3).

### 1.2 Optimization of LAMP

Once the primer set and concentration was determined, it was important to perform assays to optimize the concentrations of other reagents and the reaction conditions, e.g., betaine (see Note 4), MgSO<sub>4</sub> (see Note 5), *Bst* DNA polymerase enzyme (see Note 6), and the amplification temperature and time (see Notes 7 and 8).

### 1.3 Visual Detection of LAMP Products

LAMP amplification products can be visualized directly in the reaction tubes due to the formation of a magnesium pyrophosphate precipitate during the synthesis of DNA. Positive and negative



**Fig. 3** Results of loop-mediated isothermal amplification (LAMP) using the LsoTX16SLAMP primer set for detection of “*Candidatus Liberibacter solanacearum*” (Lso) in infected plant and insect samples. **(a)** Visualization of results in tubes with 25  $\mu$ L reaction volumes: *a1*, under normal light (positive, bright fluorescent yellow; negative, nonfluorescent yellow); *a2*, under UV light (color image, positive, bright fluorescent green; negative, no fluorescence); *a3*, under UV light (*black and white* image, positive, bright fluorescent white; negative, no fluorescence). *Lanes 1* and *2*, samples from infected potato plants; *lanes 3* and *4*, samples from psyllids vector; and *lane N*, negative control (no DNA). **(b)** Ethidium bromide stained agarose gel showing ladderlike DNA banding pattern from LAMP. *Lane M*, molecular size markers (a 100-bp DNA ladder ranging from 100 to 1,000 bp); *lanes 1–4* are as described above; and *lane N*, negative control (no DNA)

reactions easily can be distinguished by the presence or absence of visible turbidity, respectively (Fig. 3). Several methods were evaluated for enhancement of visual detection of LAMP products. The best involved the addition of MnCl<sub>2</sub> and calcein to the reaction mix, which results in the development of green fluorescence for positive reactions. This fluorescence becomes more pronounced under UV light (Fig. 3). After adding these reagents, the calcein binds with the manganese to produce an yellow coloration in the reaction mix. During DNA synthesis, one of the by-products of the LAMP reaction, pyrophosphate, scavenges the manganese ion from calcein. The other by-product of the LAMP reaction, a magnesium ion,

combines with the manganese-free calcein to produce a bright green fluorescence [11]. The positive reaction can be unmistakably differentiated from the negative reaction under UV light by the presence or absence of bright green fluorescence, respectively. When resolved via gel electrophoresis, LAMP products produce a regular ladderlike DNA banding pattern resulting from the formation of different-sized DNA concatemers of the target DNA sequences (i.e., different numbers of linked target sequences) (Fig. 3).

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## 2 Materials

### 2.1 Specialized Equipment and Supplies

1. Sterile 1.5-, 0.5-, and 0.2-mL Eppendorf microfuge tubes and racks.
2. RNase/DNase-free sterile filter tips (P2, P10, P20, P200, P1000).
3. Benchtop microcentrifuge.
4. Equipment for grinding psyllids (Micro-Vial Homogenizer System, Wilmad-LabGlass).
5. Heating block (MyBlock™ Mini Digital Dry Bath, Benchmark Scientific) with temperature uniformity of  $\pm 0.2\%$ .
6. Microvolume spectrophotometer (NanoDrop Technologies).

### 2.2 DNA Extraction

1. Bacterial DNA extraction buffer for potato plants (autoclaved): 100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), and 1.4 M NaCl.
2. Bacterial DNA extraction buffer for potato/tomato psyllids (autoclaved): 100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), and 1.4 M NaCl.
3. 0.2 mg/mL proteinase K.
4. 10 % cetyl trimethyl ammonium bromide (CTAB) stored at 37 °C.
5. 20 % sodium dodecyl sulfate (SDS) warmed to dissolve the precipitate.
6. Chloroform/isoamyl alcohol (24:1).
7. 3 M sodium acetate.
8. 5 M ammonium acetate.
9. Ice-cold isopropanol.
10. 70 % ethanol.

### 2.3 Reagents for LAMP Reaction

1. Ultrapure and RNase-free sterile distilled water.
2. ThermoPol Reaction Buffer (New England BioLabs).
3. dNTPs.

4. Betaine (Sigma-Aldrich).
5. Calcein, MgSO<sub>4</sub>, and MnCl<sub>2</sub>.
6. Primers (Invitrogen/Integrated DNA Technologies (IDT)) (F3, B3c, FIP, BIP, LF, and LB). HPLC grade FIP and BIP primers are optional but recommended (Invitrogen).
7. *Bst* (*Bacillus stearothermophilus*) DNA polymerase (8 units/μL).

## 2.4 Reagents for Electrophoresis

1. 10× TBE buffer: Dissolve 108 g of Tris and 55 g of boric acid in 40 mL of EDTA solution, and bring volume to 1 L with deionized water.
2. Electrophoresis running buffer (1× TBE): 10× TBE diluted tenfold with deionized water.
3. 5× Loading buffer: 0.5 M EDTA, 0.25 % w/v bromophenol blue, 0.25 % w/v xylene cyanol, 30 % glycerol, in deionized water.
4. Ethidium bromide (EB) solution 10 g/L: Dissolve 1 g of EB in 100 mL of double-distilled water using a magnetic stir for several hours to ensure complete dissolving. Store in a brown glass bottle at 4 °C.

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## 3 Methods

### 3.1 Extraction of DNA from Potato Plant Tissue (Tuber, Stem, Petiole, or Leaves)

1. Grind the 0.5 g of tissue (tuber, petiole, stem, or leaves) in 1 mL of extraction buffer and homogenize sample with mortar and pestle [12, 13].
2. Collect the solution in the microfuge tube and add 100 μL of CTAB (10 %) and 75 μL of SDS (20 %) per tube; this is incubated at 65 °C for 1 h.
3. Spin the solution at 9,224×*g* for 10 min in a microfuge at room temperature.
4. Collect the supernatant (clear solution) in new 1.5 mL tubes and add an equal volume/500 μL of chloroform/isoamyl alcohol (24:1).
5. Spin the solution at 9,224×*g* at 4 °C for 10 min.
6. Collect the aqueous phase in the fresh 1.5 mL tubes and add 0.6 volume of isopropanol and 60 μL of 3 M sodium acetate (pH 5.2) and incubate for 30 min at -20 °C.
7. Spin the solution at 9,224×*g* at 4 °C for 10 min.
8. Discard the supernatant (do not disturb the invisible DNA pellet).
9. Wash the pellet with 500 μL of cold 70 % ethanol.

10. Spin the solution at  $9,224 \times g$  at 4 °C for 10 min and discard the alcohol phase (solution).
11. Dry the pellet under a vacuum for 30–60 min (avoid any remainder of ethanol).
12. Dissolve the pellet in 50 µL of ultrapure sterile distilled water.
13. Keep the tubes at 37 °C for 30 min.
14. Determine total DNA concentration and quality using a micro-volume spectrophotometer.
15. Store at –20 °C.

### **3.2 Extraction of DNA from Psyllids**

1. Place 3–5 psyllids in a 1.5 mL tube; add ~450 µL extraction buffer with 50 µL proteinase K [3, 14].
2. Grind with a Micro-Vial Homogenizer System (pestle autoclaved), try to avoid foaming.
3. Add 100 µL of CTAB (10 %) and 75 µL of SDS (20 %) to each tube and incubate at 65 °C for 30 min.
4. Spin the solution at  $9,224 \times g$  for 10 min at room temperature.
5. Collect the supernatant (clear solution) in new tubes and add an equal volume/500 µL of chloroform:/isoamyl alcohol (24:1).
6. Spin the solution at  $9,224 \times g$  at 4 °C for 10 min.
7. Collect the aqueous phase in the fresh tubes and add 250 µL of 5 M ammonium acetate and keep on ice for 30 min.
8. Centrifuge at  $9,224 \times g$  for 10 min at 4 °C.
9. Transfer supernatant (DNA solution) to a new 1.5 mL tube.
10. Add 0.6 volume of isopropanol, mix gently, and keep on ice for 30 min.
11. Discard the supernatant (do not disturb the invisible DNA pellet).
12. Wash the pellet with 500 µL of cold 70 % ethanol.
13. Spin the solution at  $9,224 \times g$  at 4 °C for 10 min and discard the alcohol phase (solution).
14. Dry the pellet in a vacuum for 30–60 min (avoid any remainder of ethanol).
15. Dissolve the pellet in 50 µL of ultrapure sterile distilled water.
16. Keep the tubes at 37 °C for 30 min.
17. Determine total DNA concentration and quality using a micro-volume spectrophotometer.
18. Store at –20 °C.

### 3.3 LAMP Assay

- Set up LAMP reaction mixture according to Table 2, adjusting the final volume to 25 µL (see Notes 9–11).
- Perform LAMP reactions at 60 °C for 60 min (and the reaction is terminated at 80 °C for 5 min).
- Direct detection: LAMP amplicons can be detected directly in the reaction tubes by formation of an insoluble magnesium pyrophosphate precipitate during amplification; turbidity indicates a positive reaction, whereas a negative result remains clear.
- UV detection: if desired, MnCl<sub>2</sub> and calcein can be added to the reaction mix. Detection of amplicons can be improved under UV light (wavelength 254/365 nm), which is used to illuminate the fluorescent product for color photography and black and white imaging. A positive reaction is indicated by green fluorescence and a negative reaction by the lack of fluorescence.

**Table 2**  
**Composition of the LAMP reaction mixture for Lso detection**

No.	Components	Stock conc.	Working conc.	Required conc.	Required vol./reaction (µL)	10× conc. (µL)
1.	Ultrapure sterile distilled water (RNase-free water)				6.20	62.0
2.	ThermoPol reaction buffer	10×	10×	2×	5.00	50.0
3.	Betaine	5 M	5 M	1.6 M	4.00	40.0
4.	MgSO <sub>4</sub>	100 mM	100 mM	12 mM	1.50	15.0
5.	dNTPs	25 mM	25 mM	2.8 mM	1.40	14.0
6.	F3 primer	100 pM	100 pM	10 pM	0.10	1.0
7.	B3c primer	100 pM	100 pM	10 pM	0.10	1.0
8.	FIP primer	100 pM	100 pM	40 pM	0.40	4.0
9.	BIP primer	100 pM	100 pM	40 pM	0.40	4.0
10.	LF primer	100 pM	100 pM	20 pM	0.20	2.0
11.	LB primer	100 pM	100 pM	20 pM	0.20	2.0
12.	MnCl <sub>2</sub> <sup>a</sup>	20 mM	20 mM	1 mM	1.25	12.5
13.	Calcein <sup>a</sup>	1 mM	1 mM	50 µM	1.25	12.5
14.	8 units of <i>Bst</i> DNA polymerase				1.00	
15.	Template DNA (50 ng/µL)				2.00	
Total reaction volume					25.00	

<sup>a</sup>These reagents are optional and added as desired for enhancement of visualization

5. Gel detection: Results may be further confirmed via gel electrophoresis on a 2 % agarose gel. The expected result is a uniform ladderlike pattern resulting from the resolution of different-sized concatemers of the target DNA sequence.

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## 4 Notes

1. LAMP primers require consideration of GC content, melting temperature, and the self-compatibility of the sequences. For organisms such as “*Ca. Liberibacter solanacearum*” with a low genomic GC content (33–41 %), the selection of primer sequences with adequate GC content is the most critical determinant of LAMP success. LAMP primers targeted to the Lso 16S rDNA gene had more than 50 % GC content. Furthermore, turn-back primers (FIP and BIP) were subjected to UNAFold and LASSO software analysis for optimization. The inclusion of the additional loop primers contributed to improved Lso detection and reliability.
2. The F3 and B3c primers, required only for the initial displacement reaction, were added at a lower (fourfold) concentration than the FIP and BIP primers. The LF and LB primers are not required for the generation of the template or subsequent amplification and theoretically can be omitted from the reaction [8]. However, when they were added at the recommended concentration (e.g., twofold lower than the FIP and BIP primers), they greatly increase the speed of the reaction by providing an increased number of starting points for DNA synthesis.
3. The LAMP turn-back primers, FIP and BIP, are required for stem loop formation and thus are the most crucial for reliable amplification. HPLC purified FIP and BIP primers provided more reliable and reproducible results.
4. Betaine was used in the LAMP reaction mixture to reduce base stacking [15] and increase overall rate of reaction and target specificity (e.g., by significantly reducing nonspecific amplification).
5. MgSO<sub>4</sub> concentration had the greatest effect on LAMP amplification, and thus, careful consideration of MgSO<sub>4</sub> concentration is required. This is likely due to the effects of Mg<sup>2+</sup> on DNA polymerase activity and primer annealing. The best concentration of Mg<sup>2+</sup> for the Lso assay was determined to be 12 mM.
6. *Bst* DNA polymerase concentration can be optimized at 2, 4, and 8 units. The best *Bst* DNA polymerase concentration was 8 units in the LAMP method for detection of Lso.
7. Bands for detection of Lso using LAMP are observed on amplifications between 60 and 63 °C. Therefore, 60 °C was

determined as the best temperature based on the production of the brightest bands.

8. Bands can be observed after 45 min of reaction, but much clearer bands are observed at 60 min. Therefore, 60 min was chosen as the best duration for the LAMP reaction.
9. To avoid contamination, the LAMP reaction mixture should be prepared using a different set of pipettes (wiped with disinfectant), using clean filter tips, and processed in a different room or laminar flow chamber.
10. The entire LAMP reaction setup should be conducted on ice, to avoid nonspecific room temperature effects on the activity of the *Bst* DNA polymerase.
11. Avoid violent mixing as it may inactivate the *Bst* DNA polymerase. Mix gently by pipetting and spinning down in a microcentrifuge at  $92 \times g$  for 20 s.

## Acknowledgments

This work was supported by Texas A&M AgriLife Research Project Number H-8832 (DCG) and Texas Department of Agriculture, Specialty Crop Research, and Product Development Grant Program award SCFB-1213-014. We thank Cecilia Tamborindeguy for providing images of insects and contributions to method development. We also thank J. Creighton Miller Jr. and the Texas Potato Breeding Program for contributions to method development.

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# Chapter 8

## Loop-Mediated Isothermal Amplification (LAMP) for Detection of Phytoplasmas in the Field

Matt Dickinson

### Abstract

Loop-mediated isothermal amplification (LAMP) is a nucleic acid-based detection method with many applications. This chapter details its use for detection of phytoplasma diseases, combining a rapid 2-min DNA extraction method with real-time LAMP product detection such that the entire procedure can be undertaken and completed in the field within 40 min. Furthermore, the assays include an anneal curve validation step to guard against false positives and an assay for host plant DNA to guard against false negatives.

**Key words** Detection, In-field testing, Loop-mediated isothermal amplification, Phytoplasma, Validation

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### 1 Introduction

Numerous nucleic acid-based systems have been developed for the detection of plant pathogens, including conventional and real-time PCR. Such methods are especially valuable for phytoplasma diseases because the causal organisms cannot be routinely grown in culture and their pleomorphic nature makes them difficult to identify by microscopic techniques. However, conventional PCR often requires nested steps to attain sufficient sensitivity [1], and this, combined with the use of gel electrophoresis for detection of amplification products, leaves the method prone to contamination by aerosols leading to the potential for false positives. As a closed-tube system, real-time PCR has the advantage of reduced contamination risk, and a number of real-time assays have been developed based on TaqMan probes and SYBR Green. Furthermore, some of these assays are generic to detect all phytoplasmas [2–4] while others are group specific, which enable identification of the 16Sr group or “*Candidatus* species” [5–8]. Real-time assays are also sensitive and can be used for quantification. However, the *Taq* polymerase enzyme is prone to inhibition by compounds in crude

plant and insect extracts, such that the DNA samples generally have to be purified to ensure that they support PCR. In addition, because of the need to rapidly heat and cool samples in the amplification programs, few real-time PCR machines are suitable for use outside of the laboratory, such as for in-field use or use in remote locations.

LAMP is an alternative method that uses a strand-displacing DNA polymerase, e.g., *Bst* polymerase along with external and internal primers, plus optional “loop” primers [9, 10] (see Chapter 6, Fig. 2) to generate amplification products containing loop regions at a single temperature, normally around 65 °C. Typically the amplicons are around 200–300 bp in length, and because repeat sequences are generated, the amplicons appear as ladders on agarose gels. One advantage of products being generated at a single temperature is that battery-operated machines can be developed, and the *Bst*-type polymerases have been found to be much less prone to inhibitors in DNA extracts than *Taq* polymerase, such that crude DNA preparations can be used for LAMP. In previously published work, LAMP primers and assays have been developed and validated for a number of different phytoplasma 16Sr (“*Candidatus* species”) groups [11–13]. In this protocol, we describe how these and other assays can be used in a closed-tube LAMP assay that involves detection in real time of a fluorescent double-stranded DNA intercalating dye. This means that the assays can be undertaken in the field and at remote locations with minimal equipment or risk of contamination of samples. Both generic and group-specific assays have been developed, along with a generic assay for plant DNA to guard against false negatives, and the assays also include an anneal curve validation step for the amplification products.

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## 2 Materials

1. Tubes filled with 2–3 stainless steel beads (5 mm diameter) and 10 mL ELISA buffer 236 mM Tris-HCl, 137 mM NaCl, 2 % PVP K-25, 2 mM PEG 6000, 0.05 % Tween 20, pH 8.2 or Na acetate buffer 50 mM Na acetate, pH 5.5, 50 mM NaCl, 30 mM ascorbic acid (see Note 1).
2. 0.5 g plant material (normally leaves, but flowers and roots from *Catharanthus roseus*, grapevine berries [13], and trunk borings for coconut palms have also been tested).
3. LAMP primers (see Note 2). Internal primers FIP and BIP should be HPLC purified. Primers should be stored at –20 °C as 100 µM stocks. For each assay, “working primer mixes” should be made from these stocks by combining 152 µL water with 4 µL F3 primer, 4 µL B3 primer, 20 µL FIP primer, 20 µL BIP primer,

20 µL FL primer, and 20 µL BL primer. These “working primer mixes” can also be stored at -20 °C until required.

4. Eight-well strips of 0.2-mL tubes for the Genie machines (OptiGene, UK) (*see Note 3*).
5. Rack for holding the 0.2-mL reaction tube strips.
6. 2× isothermal mastermix (OptiGene, UK) (*see Note 4*).
7. Molecular biology grade water.
8. Genie II or Genie III real-time LAMP detection machine (OptiGene, UK) (*see Note 5*).
9. 1.5-mL Eppendorf tubes (or similar).
10. Pipettors and filter tips (*see Note 6*).
11. Positive control DNA sample.

### 3 Methods

#### 3.1 DNA Extraction and LAMP Reactions in the Field

1. In the laboratory, and prior to going to the field, use dedicated pipettors and tips that have not been used for work with DNA to make up the LAMP reaction mixes for the required number of reactions (*see Table 1* and **Note 3**). For every eight reactions, make up a mix that contains 50 µL water, 25 µL “working primer mix,” and 200 µL 2× isothermal mastermix in a 1.5-mL Eppendorf. If the 16Sr group of the likely phytoplasma is not known, we recommend using the universal phytoplasma primers in the initial field assay to determine whether any phytoplasma is present. Once the presence of a phytoplasma in a sample has been determined, group-specific primers can be used to determine the phylogenetic group (or the DNA sample can be returned to the lab for subsequent analysis (*see Note 1*)). If it is a specific 16Sr group phytoplasma that the diagnostic test is being used for in the field, make up reaction mixes with the primers from Table 1 for this specific phytoplasma.
2. Mix briefly by pipetting, and aliquot 23 µL into each of the 8 × 0.2-mL tubes in a strip.
3. Repeat the above procedure with the plant *cox* gene primers (Table 1), aliquoting into a second set of 8 × 0.2-mL tubes in a strip. Cover the tubes with cling film, or close all the lids.
4. The reaction mixes are stable at ambient temperature so the prepared tubes can all be transported to the field site without the need for storage on ice.
5. Once in the field, take approx. 0.5 g of plant material from each sample plant and place in the DNA extraction tubes (*see Note 7*). We suggest doing sampling in batches of six samples at a time. Shake the plant extracts vigorously for 2 min to disrupt the plant material.

**Table 1**  
**LAMP primer sequences for a generic phytoplasma assay and for a range of 16Sr group-specific assays and for the generic plant *cox* gene assay**

Target for assay	Primer name	Sequence (5'-3')
16SrI phytoplasmas	SrIF3	TAATATTAAAGGGCCCTATAAGCTCAGTGG
	SrIB3	CACGGATCTTCACTTATTACAGCTT
	SrIFIP	ATTTTGCTTATTGTGTTATGGTTATAGAGCACACGCCATAATAAGCGTGAAGG
	SrIBIP	GAAGGTTAAAAATCAAAGGAACATAAGGGTTAATTGCGTCCTTCATCGG
	SrIFL	CTAAATGGACTTGAACCACCGA
	SrIBL	ACAGTGGATGCCTTGGCACT
16SrII phytoplasmas	SrIIF3	CCGAATGGGGCAACCTAC
	SrIB3	CTCGTGTCTCGCCGTACTT
	SrIFIP	TTCCTACAGTTACTTAGATAATTCACTGTTAAGTATAGTATCAATTGTTAGG
	SrIBIP	CTAGTTATCAAAATTAAATTAAACTCGATTTTAACCTCTACAGGATTTCAC
	SrIFL	CACTGCGTCCCTCAATC
	SrIBL	AATAGTTGAAAACCTATATCCTAGA
16SrIII phytoplasmas	SrIIF3	GGATACCGCAGTGAACGTGAA
	SrIB3	GATTTCTCGTGTCTCGCC
	SrIFIP	GTATCAGGCTCTCCGGATTGTAACCTGCAGGAAAAGAAAGT
	SrIBIP	AACCAAATTGAAAGTTTTGGAAAACGATAACCCCTAGATTAACTTACCC
	SrIFL	GTCRCTACTRCCAGAATCGTTATT
	SrIBL	AACACCAAAGGAAGGTGATAGTCC
16SrXI phytoplasmas <sup>a</sup>	SrXIF3	AAGAAGGAGGGCCCTATAAGCTCAGT
	SrXIB3	ATATCGCTGTAAATTACGTC
	SrXIFIP	AGAAAAGATGACCTTTTCAGTTGGTGTGGITAGAGCACACGCCCTGATAAG
	SrXIBIP	CAAAGTAATAATAAAATCAAAGGACATCGGCTCTTAGTGCCAAG
	SrXFL	ATGGACTTGAACCATCGACC
	SrXBL	AAGGGCGTACAGTGGATGC

16SrXII phytoplasmas	SrXIF3 SrXIB3 SrXIEIP SrXIBIP SrXIFL SrXIBL	GGATGCCCTGGCACTAAG GTGTCCTACGCCGTACTTATC AACGGGGTTGTCCTCATCGGGCGATGAAGGACGCAATT TTCTGGTAGTAGTGACGGAGGATTACAGGACTGTCACCTCT AATCCACGGATCTCCACTTAT CGGAAGAGGCCTGATGCTATT
16SrXXII phytoplasmas <sup>b</sup>	SXXXIF3 SXXXIB3 SXXXIEIP SXXXIBIP SXXXIFL SXXXIBL	TAGAGGAAGGGCCCTAGCTCAGT GTATCGCCGTTAATTGCGTC TGAATAAGAGGAATATGGTATGGGTGTTAGAGGCACACGGCTTGATAAG TCCTCTAAATGACACACCAATGAAGGACATCGGCTCTAGTCACAAG GGACTTGAACACCATTGACCCG AAGGGCCGTACAGTGGATGC
Universal phytoplasma	UNIF3 UNIB3 UNIEIP UNIBIP UNIFL UNIBL	GAAGTCTGCAACTCGACTTC CCTTAGAAAGGAGGTGATCC ACGGGCCGGTTGTAACAAACGGAAATCGCTAGTAATCGCGAATC GTCTAAAGGTAGGGTCGATGACACTTCGGTAGGGATAAC CGAGAACGTTATCACCGCGAC GGGGTTAAAGTCGTAAACAAG
Universal plant ( <i>cax</i> gene)	COXF3 COXB3 COXFIP COXBIP COXFL COXBL	TATGGAGGCCGTTTTC AACTGCTAAGRGCATTCC ATGGATTGRCCTAAAGTTCAAGGGCAGGATTCACTATTGGGT TGCATTCTTAGGGCTTTCGGATCCRGCFTAAGCATCTG ATGTCGGACCAAAGATTTCACC GTATGCCACGTCGCATCC

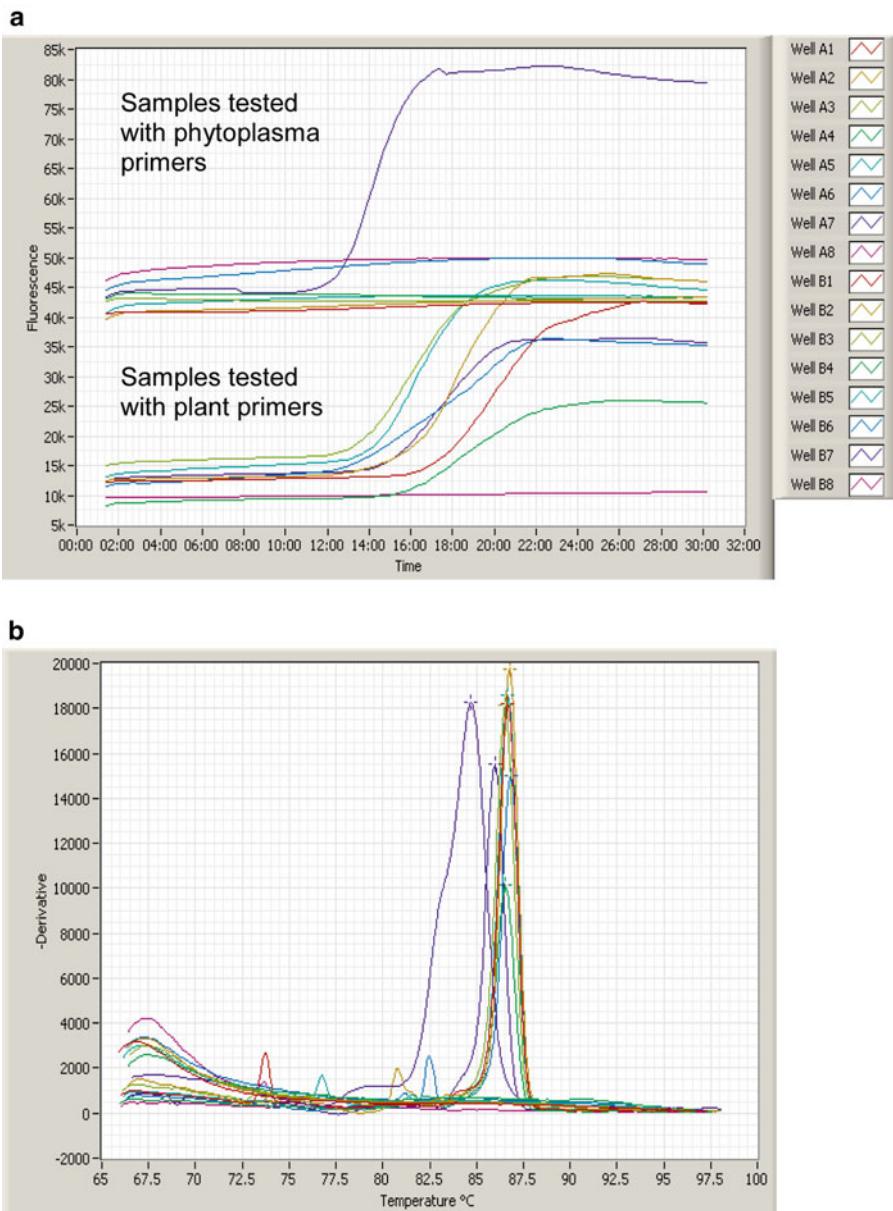
<sup>a</sup>This assay has been validated for the 16SrXI Napier grass stunt phytoplasma, and also detects the 16SrIV Bermudagrass whiteleaf phytoplasma. However, it does not detect 16SrXI rice yellow dwarf or 16SrXI sugarcane whiteleaf/grassy shoot phytoplasmas

<sup>b</sup>This assay has been validated for the Ghanaian Cape St Paul Wilt phytoplasma, but has not been tested on other possible 16SrXXII phytoplasmas

6. Using a pipettor, take 2 µL from the DNA extraction bottle for sample 1 and add this to tube 1 in the row of phytoplasma assay reaction mix tubes. Close the lid of the reaction tube. Take a further 2 µL from the DNA extraction for sample 1 and add this to the equivalent *cox* assay tube, closing the lid once added.
7. Repeat this procedure for the remaining five test samples.
8. For tube seven in the assay, add 2 µL of the positive control DNA sample for the particular 16Sr group assay that you are undertaking. If you are performing a generic phytoplasma assay, this DNA sample can be from any phytoplasma. If you are performing a specific 16Sr group assay, the positive DNA control must be from this same 16Sr group. Close the lids.
9. Finally, close the lids on negative control tubes (tube 8 in each strip of tubes).
10. Place the strips in the Genie II machine, phytoplasma primer tubes in block A, and plant primer tubes in block B and close the lid.
11. Following the manufacturer's instructions, program the Genie II machine to run at 63 °C for 30 min followed by an anneal analysis.
12. Run the machine through the amplification and anneal analysis. This will take around 35 min (*see Note 8*).

### 3.2 Analysis of Data

1. Analyze the data in real time by observing the amplification profiles (Fig. 1a). For most of the assays, amplification from positive samples will normally take between 10 and 20 min, although some samples may take longer if the phytoplasma titer is low. The plant primers take about the same length of time for amplification (*see Note 9*).
2. Analyze the anneal curves. The anneal temperature for positive samples should be within 2 °C of that of the positive control DNA sample (*see Fig. 1b* and **Note 10**).
3. Amplification and anneal analysis of the positive control DNA sample will confirm that the primer mixes are correct and that the experimental setup has been undertaken correctly. Positive phytoplasma detection in the test samples is then confirmed by a positive amplification and anneal curve at the correct temperature in the phytoplasma assay. Negative phytoplasma detection is confirmed if the assay for a sample has come up negative with the phytoplasma primers and positive with the plant *cox* gene primers. If a sample has come up negative with both phytoplasma and *cox* gene primers, it suggests the presence of enzyme inhibitors in the DNA extract, and the experiment will need repeating, perhaps using less plant material in the DNA extraction. If the water control samples have come up positive,



**Fig. 1** Typical results of real-time LAMP using fluorescence monitoring. **(a)** Results from the amplification stage on the Genie machines. Fluorescence is monitored in real time throughout the amplification process to build up the final amplification curve. The samples in wells A1–A8 have been tested with a phytoplasma-specific primer, and those in wells B1–B8 are the same samples tested concurrently with the plant *cox* gene primers. In both cases, well seven contains the positive control DNA, and well eight is the water control. Typical S-shaped amplification profiles can be seen for the positive control sample with the phytoplasma assay and for all samples (apart from the water control) with the plant assay, showing that all the tested samples contained DNA that supported LAMP, but none of the test samples (1–6) contained phytoplasmas. **(b)** The anneal curves for the same samples. The temperature on the x axis indicates the temperature at which annealing has occurred, in this case approx. 87 °C for the plant *cox* gene assay and approx. 85 °C for the phytoplasma assay

the results should be discarded and the experiment repeated with fresh mastermixes.

4. Tubes and plasticware should be disposed of appropriately, and once the first set of samples has been tested, further sampling can be undertaken in batches of 6. If necessary, DNA extraction bottles can be returned to the laboratory for further DNA purification and analysis (*see Note 1*). All data is stored on the machines and can be downloaded to a computer from the Genie machine for subsequent further analysis.

For a discussion of alternative reagent and assay methods, and for use of LAMP for other types of pathogens, *see Note 11*.

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## 4 Notes

1. Other buffers can be used, depending on the substrate being tested, and buffers including TE have been shown to work for substrates such as coconut trunk borings and *Catharanthus roseus* leaf tissue. The advantage of these simpler buffers such as TE or Na acetate buffer is that DNA can subsequently be extracted from the solutions using phenol-chloroform and ethanol precipitation, should further tests be required on the samples (e.g., should you wish to further purify the DNA in the laboratory, once detection has been confirmed, for conventional PCR and sequencing to determine the 16Sr subgroup).
2. The organization of primers in a DNA sequence for design into a LAMP assay is shown in Chapter 6 (Fig. 2). The internal primers FIP and BIP target two primer binding sites (F1c and F2 and B1 and B1c, respectively). LAMP reactions can be performed with external and internal primers only, but amplification is usually accelerated by the inclusion of loop primers as this allows all loops in the amplification product to act as primer binding sites. The primers described in Table 1 have been validated for particular phytoplasma assays. To design alternative primers or primers for other assays, software programs are available at PrimerExplorer (Eiken, Japan) (<http://primerexplorer.jp/e/>) or LAMP Designer (PREMIER Biosoft, USA). Primer design criteria are similar to those for PCR, and initially, sequences from target and nontarget species should be aligned. The primers should then be designed to target variation between the target and nontarget species and to mitigate against intraspecific variation. Where variation between target sequences cannot be avoided, degeneracies or synthetic bases such as inosine can be introduced into the primers. For inclusive detection of different targets, primers should be designed to position degeneracies or mismatches away from the 3' ends of the primer binding sites. Unlike PCR primers, LAMP

primers are distributed across the entire amplicon (200–300 bp), a characteristic which has the potential to confer a high degree of specificity to LAMP assays. Primers should be designed to position the most important mismatches at the 5' ends of the internal primers FIP and BIP. For maximum specificity, the 3' ends of all primers (F3/B3, FIP/BIP, and F-loop/B-loop) should also be used to target discriminatory mismatches if possible.

3. The eight-well strips recommended (OptiGene product numbers OP-0008-50 or OP-0008-500) are specifically designed for the battery-operated Genie II and Genie III machines. It is also possible to undertake closed-tube, real-time LAMP diagnostics in a laboratory setting using real-time PCR machines, in which case any tubes suitable for the real-time PCR machine can be used.
4. The isothermal mastermix for closed-tube real-time LAMP can be purchased from OptiGene (product numbers ISO-001 or ISO-002). These mastermixes contain optimized concentrations of the DNA polymerase, nucleotides, buffer, and double-stranded DNA intercalating dye for the fluorescence-based real-time detection system. It is possible to prepare LAMP reaction mixes from stocks and use non-real-time detection methods (*see Note 11*). Methods for preparing LAMP reaction mixes from stock solutions can be found in ref. [14].
5. The Genie II machine (OptiGene, UK) is a purpose-built, lightweight, battery-operated real-time LAMP machine, specifically designed for rapid in-field diagnostic tests. It can analyze 16 samples simultaneously. The Genie III machine, while only having eight wells, has dual-channel fluorescence excitation and measurement, and the machine incorporates a GPS system for direct recording of the location of field sampling. Other LAMP detection systems are available (*see Note 11*) but are less suitable or reliable for field use. For example, turbidity meters, which measure the magnesium pyrophosphate produced during the LAMP amplification, can provide evidence that an amplification has occurred but do not provide a means of undertaking a validation assay to confirm that the amplification product is specific and not an artifact. This is effectively the equivalent to undertaking a SYBR Green real-time PCR assay without undertaking the melt curve validation step. The advantage of the fluorescence-based real-time detection system and the Genie machines is that they have the anneal analysis built in to them to provide an amplification product validation step and therefore additional robustness to the assays.
6. LAMP reactions are very efficient and typically generate large amounts of amplification product. Care must therefore be taken at all stages to prevent contamination of facilities and

equipment with target DNA and amplification products, because carry-over of extremely small amount of amplification products can generate spurious results. Reagents and equipment for preparation of primer mixes and mastermixes must be rigorously separated from reagents and equipment used for DNA work; store and mix mastermix reagents in a dedicated area, and use only dedicated pipettors and stocks of molecular biology grade water. For on-site testing, the workflow contains as few steps as possible to reduce the risk of contamination. To reduce the risk of sample contamination further, do not open LAMP reaction tubes post-amplification.

7. For extraction of DNA from woody tissues, such as tree trunk borings, we recommend using a multipurpose drill bit and a cordless electric drill. Flame sterilize the bit using a propane torch and then cool it with a stream of deionized water. Sample by drilling into the trunk at a height above the ground of approx. 1 m, to a final depth of ~15 cm, using a back-and-forth motion to dislodge shavings. Passively collect approx. 0.5 g of the shavings into the DNA extraction bottle and extract by shaking as for other DNA samples. Once the trunk has been sampled, it can be sealed by tapping a piece of wooden dowel into the hole to reduce sap bleeding and provide a barrier against invasion by pests.
8. The standard LAMP protocol that we use is a 30-min cycle at 63 °C, since this has been found to be the optimal temperature for the phytoplasma assays, followed by the anneal assay. The assays tend to give amplification between 10 and 20 min, depending on the assay and the concentration of the phytoplasma DNA in the sample. For some assays, it may not be necessary to continue running the amplification part of the assay for the full 30 min, and it is possible to go straight to the anneal assay after 20–25 min. We have not found any need to run these phytoplasma assays for longer than 30 min, and doing this sometimes results in amplification artifacts. The artifactual nature of such products can be confirmed by the anneal analysis, since they give anneals at the wrong temperatures for the specific phytoplasma assays.
9. Using the direct DNA extraction method suggested for field analyses in this protocol, we have found that samples from some plant species, for example, Napier grass (*Pennisetum purpureum*), can result in autofluorescence. This results in an apparent increase in fluorescence in the first few minutes of the amplification, with an atypical shape to the amplification curve, and this can mask any subsequent typical amplification curve. However, we have noted that even when this masking of amplification does occur, the anneal analysis will work as normal, and in such situations, the anneal curve alone should be used as the validation for a positive or negative amplification.

10. After amplification using the real-time assay, products are typically analyzed by fluorescence monitoring during a slow annealing step in the Genie machines. This is based on the fact that the temperature at which the amplification product anneals/melts is consistent and characteristic for each assay (Fig. 1b). Using this analysis, specific amplification products can be distinguished from any nonspecific amplification artifacts. In addition, this approach can allow two or three amplification products to be resolved in the same reaction if the products have sufficiently different annealing/melting temperatures, which potentially allows assays to be developed that can detect multiple pathogens in a single reaction.
11. The methods described here are for real-time fluorescence-based LAMP assays that can be conducted in field settings and remote locations with minimal equipment and portable battery-operated machines. In laboratory settings, real-time fluorescence-based LAMP using intercalating dyes can also be carried out on platforms developed for real-time PCR, and in these situations, the assays can be combined with better automated DNA extraction methods for rapid high throughput analysis of samples. The advantages of these real-time detections systems are that no post-amplification steps are required, thus reducing the risk of carry-over contamination. As alternatives, LAMP products can be analyzed in the same ways as conventional PCR products, by gel electrophoresis, but this increases the risk of sample contamination and is generally not suitable for field use. The large amount of amplification product typically generated in a LAMP reaction also means that LAMP amplifications can be detected by alternative indirect means. Magnesium pyrophosphate generated as a by-product of polymerase activity reaches sufficiently high levels in LAMP reactions to allow direct observation of a white precipitate in positive reactions, or real-time or end-point turbidity measurement can be undertaken using simple instruments. Similarly, color change methods can be used to distinguish positive and negative reactions. For example, SYBR Green can be added at the end of the reaction and a color change from orange to yellow observed directly, or hydroxy naphthol blue (HNB) can be used as a color change indicator, and unlike SYBR Green (which inhibits LAMP reactions at the concentrations required for visual assays), HNB can be added to a reaction mixture prior to amplification, so it can be used in a closed-tube assay. However, the color change obtained using HNB is very subtle and difficult to discern. In addition, and as noted above, these indirect detection methods lack the anneal curve validation step that is built in to the fluorescence-based real-time detection systems.

The assays that have been described in this protocol are specifically for phytoplasma detection. However, there are numerous published LAMP assays for other plant, animal, and human pathogens and for other types of genetic analyses, and there is no reason per se why such primers and assays cannot be undertaken in the field using these real-time protocols. In related studies, we have found that DNA extractions work from fungal and other bacterial plant pathogens using the 2-min extraction methods as outlined, as do RNA extractions for plant virus detection assays. LAMP can also be used for detection of these RNA targets, and the isothermal mastermixes described in these protocols do have some reverse transcriptase activity, so it can be used directly in LAMP from RNA targets, although it is recommended that additional reverse transcriptase is added for more efficient amplification. Few changes are needed to the protocols for RNA-based LAMP assays although a short incubation at a lower temperature (approx. 50–55 °C) prior to the 65 °C amplification step has been shown in some assays to allow more efficient reverse transcription. It should be noted that reverse transcriptases tend to lack the thermal stability of the standard LAMP reagents, so if RNA-based assays are to be conducted in the field, it is recommended that the reverse transcriptase is transported to the field on ice and only added to the final reaction mixes on-site.

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# Chapter 9

## Diagnosis of Phytoplasmas by Real-Time PCR Using Locked Nucleic Acid (LNA) Probes

**Sabrina Palmano, Vincent Mulholland, David Kenyon, Gerry S. Saddler, and Colin Jeffries**

### Abstract

Phytoplasma infections are regularly reported worldwide, and concerns about their threats on agricultural production, especially in relation to global climate change, are increasing. Sensitive and reliable detection methods are important to ensure that propagation material is free of phytoplasma infection and for epidemiological studies that may provide information to limit the extent of phytoplasma diseases and to prevent large-scale crop losses. The detection method described here uses LNA chemistry in real-time PCR. It has been developed and validated for use on potatoes, and its sensitivity and specificity make it suitable for use in postentry potato quarantine and initiation of potato nuclear stocks to ensure that material is phytoplasma-free.

**Key words** Potatoes, *Solanum tuberosum*, Stolbur, Potato purple top wilt, 16SrDNA groups I, II, III, V, VI, and XII, “*Candidatus Phytoplasma americanum*”, “*Candidatus Phytoplasma australiense*”

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### 1 Introduction

For decades viruses were considered to be the only cause of “yellows disease” of plants. However, in 1967, a new group of pathogens was discovered as a possible cause of some of these diseases, also characterized by virescence, dwarfing, and witches’ broom. Electron microscope studies of infected plants [1] revealed the presence of microorganisms in phloem tissue with a strong similarity to the wall-less prokaryotes known in animals. Consequently they were called mycoplasma-like organisms (MLOs). The hypothesis that MLOs were causing these symptoms was supported by (1) the absence of fungi, bacteria, and protozoa in the infected plants, (2) the symptom remission after antibiotic treatment of infected plants with tetracycline, and (3) the transmission of the pathogens by grafting and insect vectors.

Several hundred plant diseases are now thought to be caused by MLOs [2, 3], but until the advent of molecular biology, the inability to grow these pathogens in axenic culture had seriously hampered their characterization and classification. Ribosomal rDNA sequencing has provided evidence that they constitute a large monophyletic group within the class *Mollicutes*. At the tenth International Congress of the “International Organization for Mycoplasmology” (1994), the scientific community officially accepted the trivial name of “phytoplasma” to indicate this group of monophyletic wall-less non-helical prokaryotes, and in 2004 [4], the provisional name of “*Candidatus Phytoplasma*” was proposed for this new taxon. Organisms which share more than 97.5 % identity in their 16S rDNA are considered to belong to the same species, and so far 32 species have been identified [5]. Affiliation of the many different phytoplasma strains to a defined specie is still a work in progress, so an alternative and more widely used system of classification is based on similarity coefficients derived from RFLP analyses, where phytoplasma strains are classified into 19 groups and more than 40 subgroups [6, 7] (see Chapter 10).

Phytoplasmas are intracellular, systemic, and obligate pathogens associated with phloem cells. They produce alterations to the plant ultrastructure; growth anomalies are also induced and there is interference with plant secondary metabolite production [8]. In plants infected by phytoplasmas, variations in the quantity of endogenous substances regulating growth (auxins and polyamines) and of other metabolites such as carbohydrates [9–12], polyphenols, and alkaloids [8, 13] may occur. Moreover, an inhibition of phloem loading and transport by callose and/or protein plugging, probably due to  $\text{Ca}^{2+}$  influx into sieve tubes, has been reported [14].

Phytoplasmas are transmitted in a persistent manner by sap-sucking insect vectors belonging to the families Cicadellidae and Psyllidae. Vectors acquire phytoplasmas while feeding in the phloem of infected plants; these bacteria replicate in the insect body and pass to the gut and then salivary gland basal lamina barriers before being regurgitated into healthy plants along with salivary fluids. Infected insects can transmit phytoplasmas for their entire life [15].

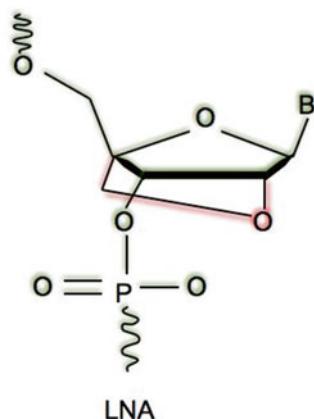
Typical symptoms observed in phytoplasma-infected plants (Fig. 1) are stunting, decline, leaf chlorosis (yellows) and curling, proliferation of auxiliary buds, and morphological changes in floral organs (phyllody and virescence) [7]. Phytoplasmas have a wide geographical distribution and host range and cause economic loss worldwide in important food crops such as maize (maize bushy stunt), rice (rice yellow dwarf), and potato (potato purple top wilt and stolbur). They can result in loss of production of fruit trees and grapevine on different continents (e.g., apple proliferation, pear decline, coconut lethal yellowing, flavescence dorée). They can cause significant damage to vegetables (e.g., the stolbur group) and ornamental plants (e.g., the aster yellows group) (see Chapter 10).



**Fig. 1** Examples of characteristic symptoms caused by phytoplasma infection in herbaceous and woody hosts. “European stone fruit yellows” (ESFY) phytoplasma-infected peach tree (**a**) close to healthy one (**b**); “aster yellows” phytoplasma disease on *Gaillardia* sp. (**c**) and healthy flower (**d**); stolbur phytoplasma disease on tomato (**e**) and the correspondent healthy plant (**f**). Flavescence dorée on grapevine (**g**) (photo by Loschi-Palmano)

They can also infect weeds and fodder plants which can become a “reservoir” for future infection of crop plants. These bacteria pose threats to crops worldwide, and many of them are classified as quarantine pathogens in some countries (<http://www.eppo.int/QUARANTINE/listA1.htm>; <http://www.eppo.int/QUARANTINE/listA2.htm>).

In the last few years, the number of phytoplasma diseases has increased, and reliable detection and identification is therefore needed. Routinely, phytoplasma are detected by PCR amplification using the conserved 16S rRNA gene as target [16]. Their detection is complicated by low phytoplasma titer and uneven spatial distribution in the plant [17]. Moreover, the sample matrix from plants rich in phenolics and polysaccharides may be inhibitory for *Taq* DNA polymerase activity [18]. To increase sensitivity, a nested PCR step with specific primers is often used [19, 20], but this increases the risk of contamination and false positives. To facilitate faster and more accurate detection, diagnostic assays based on real-time PCR procedures have been developed [21–25].



**Fig. 2** Molecular structure of a locked nucleic acid nucleotide (LNA<sup>TM</sup> Exiqon). The ribose ring is connected by a methylene bridge between the 2'-O and 4'-C atoms that lock the ribose ring in a stable conformation, ideal in hybridizations with complementary DNA or RNA strand

Improvement in real-time PCR sensitivity and specificity can be achieved using target-specific probes. TaqMan® technology [26] is most widely used, but different chemistries have been developed in order to improve the discriminatory power of the probes. Among the alternative probe technologies, locked nucleic acid (LNA) chemistry has been shown to be one with the highest sensitivity [27]. LNA nucleotides (Fig. 2) are nucleic acid analogues containing a bicyclic furanose unit locked in an RNA-mimicking sugar conformation [28]. This conformation improves probe binding affinity, stability of the duplex, and specificity by increasing the  $T_m$  1–8 °C for each incorporated LNA nucleotide [29]. LNA probes can therefore be much shorter and are especially appropriate when high specificity is required and when the design of a probe is difficult due to sequence characteristics. This last factor is quite a common for the AT-rich phytoplasma genome. LNAs have found many applications in molecular biology [30] but rarely in phytodiagnostics, except for fungi [31], viruses [32], and now phytoplasmas. This technology was applied to the detection of phytoplasmas in *Solanum tuberosum*. Potato stolbur and potato purple top wilt are recommended as quarantine pests for the EPPO region and are recommended for testing in postentry potato quarantine [33]. Phytoplasmas have been reported as affecting potatoes from different taxonomic groups: 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrX, and 16SrXII [34–42]; between them, only two “*Candidatus Phytoplasma*” species have been defined: “*Ca. Phytoplasma americanum*” [43] and “*Ca. Phytoplasma australiense*” [44].

Cross-reactivity of primers and probe with DNA from bacteria unrelated to phytoplasma has been reported especially if they are present in high concentration [21]. Indeed, the presence of

nonpathogenic bacteria of the genus *Paenibacillus*, ubiquitous in agricultural systems and occupying several niches in plants [45], has been found to interfere with phytoplasma detection, especially for samples with low titers of the target pathogen. Because of these limitations, a novel LNA probe-based real-time PCR procedure for a universal detection of all the phytoplasma groups reported in potatoes has been developed. Its high sensitivity and reliability make it suitable for testing in postentry potato quarantine, initiation of potato nuclear stocks, and potato certification.

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## 2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of  $18\text{ M}\Omega/\text{cm}$  at  $25^\circ\text{C}$ ) and analytical grade reagents.

### 2.1 DNA Extraction

1. Phytoplasma grinding buffer: 100 mM K<sub>2</sub>HPO<sub>4</sub>, 30 mM KH<sub>2</sub>PO<sub>4</sub>, 10 % sucrose, 0.15 % bovine serum albumin fraction V, 2 % polyvinylpyrrolidone-10, 25 mM ascorbic acid.
2. CTAB buffer: 2 % CTAB, 100 mM Tris HCl (pH 8), 1.4 M; NaCl, 20 mM EDTA (pH 8).
3. Chloroform-isoamyl alcohol (24:1).
4. TE buffer: 10 mM Tris HCl pH 8, 1 mM EDTA pH 8.
5. 3 M sodium acetate.
6. Isopropanol.
7. 70 % ethanol.
8. Absolute ethanol.
9. Sterile water.
10. Bioreba extraction bags «universal» 12 × 14 cm (catalog number 430100) (Bioreba AG, Reinach, Switzerland).
11. Ice bucket.

### 2.2 LNA Real-Time PCR

Store all reagents at  $-20^\circ\text{C}$ , otherwise specified.

1. 10× Buffer A (Applied Biosystems, Foster City, CA, USA) (*see Note 1*).
2. dNTPs 2 mM: prepare a mix containing 2 mM each nucleotide.
3. MgCl<sub>2</sub> 25 mM.
4. LNA probe 16S-370: sequence 5'-atgg**A**Ggaaa**C**Tctgac-Cga-3', modified with FAM-6 (6-carboxyfluorescein) at the 5' end and with TAMRA (tetramethylrhodamine) at the 3' end. The LNA-modified bases are in bold capital letters. Prepare 10 μM working solution in sterile distilled water.

5. Forward primer 16S-333F: sequence 5'—tcctacggaggcagcagta—3'. Prepare a 10 µM working solution in distilled sterile water.
6. Reverse primer 16S-396R: sequence 5'—tacttcrtcggtcacgcggc—3'. Prepare a 10 µM working solution in distilled sterile water.
7. AmpliTaq Gold enzyme 5 U/µl (Applied Biosystems).
8. Sterilized ultrapure water. Store at room temperature.
9. MicroAmp fast optical 96-well reaction plates (catalog number 4346906) (Applied Biosystems).
10. MicroAmp optical adhesive film (catalog number 4311971) (Applied Biosystems).
11. StepOnePlus™ real-time PCR system (Applied Biosystems) or other real-time PCR thermal cycler.

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### 3 Methods

#### 3.1 DNA Extraction

Here we detail a procedure that includes a phytoplasma enrichment step [16] (*see Note 2*).

1. Prepare the phytoplasma grinding buffer, adjust to pH 7.6, and keep refrigerated on ice. The buffer must be prepared fresh every time (*see Note 3*).
2. Cut 1 g of fresh infected plant material and place the samples in extraction bags (*see Note 4*).
3. Add 10 ml of phytoplasma grinding buffer, and keep the samples on ice until all the samples have been processed (*see Note 5*).
4. Homogenize the samples with a rolling ball or a press, keeping samples on ice when not being processed (*see Note 6*).
5. Transfer the liquid from the net side of the bags (opposite to where the leaf sample was placed) in 15 ml tubes and centrifuge at 4 °C for 5 min at 2,500×*g*.
6. Transfer the supernatant to clean and cold 15 ml tubes and centrifuge at 4 °C for 25 min at 18,000×*g*.
7. Dissolved the pellet in 1 ml of pre-warmed CTAB buffer at 65 °C.
8. Incubate samples for at least 20 min at 65 °C.
9. Transfer the samples to 2 ml microfuge tubes.
10. Add an equal volume of chloroform-isoamyl alcohol (24:1) and mix well.
11. Centrifuge at room temperature for 10 min at 12,000×*g*.

12. Transfer the aqueous phase in a new tube and add an equal volume of cold propan-2-ol.
13. Incubate on ice for 10 min.
14. Centrifuge for 10 min at 12,000  $\times g$ .
15. Dissolve the pellet in 400  $\mu$ l of TE.
16. Add 40  $\mu$ l of 3 M sodium acetate and 0.9 ml of 95 % ethanol.
17. Incubate for at least 1 h at -20 °C (*see Note 7*).
18. Centrifuge for 10 min at 12,000  $\times g$ .
19. Discard the supernatant and wash the pellet with 70 % ethanol.
20. Dry the pellet and dissolve it in 50  $\mu$ l of sterile water.

### **3.2 LNA Real-Time PCR**

1. Prepare the PCR mix as described in Table 1 (*see Note 8*).
2. Distribute 23  $\mu$ l in each well in the PCR plate (*see Note 9*).
3. Add 2  $\mu$ l of samples with a DNA concentration of 30 ng/ $\mu$ l to each well. For reference, a known negative and positive control must be included.
4. Seal the plate using the adhesive film. Set the reporter and quencher in the real-time PCR machine software as FAM and TAMRA, respectively.
5. Set amplification parameters as follows: 50 °C for 2 min, 95 °C for 10 min (AmpliTaq enzyme activation), and 45 amplification cycles, consisting in 15 s at 95 °C and 1 min at 67 °C.
6. Analyze real-time PCR data ensuring negative and positive controls have given the expected results (*see Note 10*).

**Table 1 Master mix reagents and final concentrations**

Reagents	Volume per PCR $\mu$ l	Final concentration
10× Buffer A	2.5	1×
25 mM MgCl <sub>2</sub>	5.5	5.5 mM
2 mM dNTP mix	2.5	200 $\mu$ M
Forward primer 10 $\mu$ M	0.75	300 nM
Reverse primer 10 $\mu$ M	0.75	300 nM
LNA probe 10 $\mu$ M	0.5	200 nM
AmpliTaq Gold 5 U/ $\mu$ l	0.2	1 U
Molecular grade H <sub>2</sub> O	10.3	Total vol 23 $\mu$ l

---

## 4 Notes

1. Do not use the “Gold Buffer,” which is also sold with the AmpliTaq enzyme by Applied Biosystems. Buffer A is only available in the “TaqMan 1000 RXN Buffer A Pack—catalog number 4304441.”
2. In order to make an accurate diagnosis, it is of fundamental importance to have a pure and concentrated quantity of phytoplasma DNA. Even if little variation in the results were obtained comparing different extraction methods, this procedure was demonstrated to be the most efficient one to increase phytoplasma concentration [46].
3. Stock solutions of the different component of the phytoplasma grinding buffer can be prepared in advance, filtered and stored at room temperature (1 M K<sub>2</sub>HPO<sub>4</sub>; 300 mM KH<sub>2</sub>PO<sub>4</sub>) or 4 °C (1,5 %BSA; 50 % sucrose) and then mix when needed. The PVP-10 is difficult to dissolve so add the reagent as solid once all the other ingredients are mixed.
4. The phytoplasma enrichment step requires fresh plant material. In case of frozen material, start the extraction from the CTAB step, homogenizing the sample with liquid nitrogen using a mortar and pestle.
5. The sample material may vary in quantity and quality of available tissues. Collect material from different leaves to ensure representative sampling in order to ascertain the phytosanitary status of the plant. Avoid sampling from dead or rotten tissues. According to the amount of the sample, add grinding buffer to each bag at 1:10 dilution. Do not use less than 0.3 g weight (adding 3 ml of buffer).
6. Care should be taken to avoid breaking the bags while grinding the sample.
7. You may stop the extraction at this step, leaving the sample at -20 °C overnight. Alternatively, you can make the procedure quicker by incubating the samples at -80 °C for 15 min or immersing the samples in liquid nitrogen for few seconds.
8. Real-time PCR master mix should be prepared in a laminar-flow hood or in a contamination-free environment.
9. Vortex briefly the PCR reagents before using and then spin for few seconds at maximum speed in a microcentrifuge.
10. It is recommended to include in the assay a fresh sample from healthy and infected periwinkle by at least one of the above-mentioned phytoplasma groups, maintained in controlled conditions, respectively as a negative and positive control of both extraction and amplification procedures.

## Acknowledgments

The corresponding author would like to thank colleagues in the Diagnostic and Molecular Biology Section and Plant Health Section (the UK Potato Quarantine Unit) for their help and especially Gerry S. Saddler for giving the opportunity to be hosted at SASA for a fruitful collaboration. This work was supported by the Scottish Government.

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# Chapter 10

## Q-Bank Phytoplasma: A DNA Barcoding Tool for Phytoplasma Identification

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### Abstract

DNA barcoding is an identification method based on comparison of a short DNA sequence with known sequences from a database. A DNA barcoding tool has been developed for phytoplasma identification. This phytoplasma DNA barcoding protocol based on the *tuf* gene has been shown to identify phytoplasmas belonging to the following groups: 16SrI, 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXIV, 16SrXX, and 16SrXXI.

**Key words** Phytoplasma, Identification, DNA barcoding, *tuf*, QBOL

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### 1 Introduction

Phytoplasmas are cell wall-less prokaryotes associated with diseases in several hundred plant species, including commercial crops such as cereals, grain legumes, fruit crops, root and tuber crops, ornamental plants and timber and shade trees [1, 2]. They are mainly transmitted by insect vectors, by propagation techniques such as grafting and cutting, and by micropropagation of shoots. Many of the economically important diseases affect woody plants including lime witches' broom, jujube witches' broom, coconut lethal yellowing, peach X-disease, grapevine yellows, and apple proliferation. The trivial name phytoplasma followed by the designation "*Candidatus Phytoplasma*" has been adopted for their nomenclature [3]. Plants infected by phytoplasmas exhibit an array of symptoms including virescence, development of green leaf-like structures instead of flowers (phyllody), proliferation of axillary buds resulting in a witches' broom behavior, abnormal internode elongation, and generalized stunting (Fig. 1a–f).



**Fig. 1** Phytoplasma symptoms in various plant species. (a) Cabbage infected with aster yellows phytoplasmas showing virescence and poor pod production, (b) tomato plant showing symptoms of little leaf and stunting in the apical portion due to infection by "stolbur" phytoplasmas, (c) *Echinacea purpurea* virescence due to phytoplasmas belonging to 16SrIX-C subgroup, (d) "bois noir" symptoms in grapevine leaves, (e) Japanese plum showing narrow leaves and witches' broom due to the presence of "*Candidatus Phytoplasma prunorum*," and (f) lime witches' broom due to "*Ca. P. aurantifolia*"

Phytoplasmas have some of the smallest genomes among bacteria and, like other members of the *Mollicutes*, their genome has a low G + C content (21–28 %), similar to mycoplasmas and to endosymbiotic bacteria. Many predicted genes are present in multiple copies; they contain two rRNA operons, and heterogeneity of these operons has been demonstrated in several phytoplasma strains. Phytoplasmas lack many genes involved in metabolism, but have a Sec transport pathway and contain many genes that are encoding transporter systems; glycolysis seems to be an important metabolic pathway for them.

The inability to fulfill Koch's postulates has severely restricted the understanding of the role of phytoplasmas in disease etiology and

plant-insect-phytoplasma interactions, although the disappearance of symptoms after tetracycline treatment provided evidence to support their association with diseases. Very recently phytoplasma growth in vitro on laboratory media has been reported [4], which is a step towards proving phytoplasma pathogenicity by fulfilling Koch's postulates.

Phytoplasma strains were initially differentiated and identified by their biological properties, such as symptomatology in infected plants, plant, and insect host range; however, the determination of biological properties was laborious and time-consuming, and often the results were inconclusive. Furthermore, experimentally determined plant host ranges and ranges of insect vector species were broader than those observed in nature and were therefore unsuitable for classification. Development of antisera and DNA probes clearly showed that phytoplasmas could be distinguished at the molecular level, and a differentiation was obtained on the basis of hybridization of phytoplasma-specific probes [5]. PCR assays coupled with RFLP or sequencing of the 16S rDNA of phytoplasmas later provided rapid and reliable phytoplasma classification. Later, several molecular markers have shown improved resolving power compared to 16S rDNA such as the ribosomal protein operon (*rp*), the protein translocase subunit (*secY*), or the translation elongation factor (*tuf*) gene sequences [6–9].

The geographic distribution and impact of phytoplasma diseases depends on their host range as well as the feeding behavior of their insect vector. Many phytoplasmas have restricted host ranges and oligophagous or monophagous insect vectors, which restrict their geographical distribution. Some of the main phytoplasma diseases worldwide are summarized below.

Aster yellows phytoplasma (16SrI group, '*Candidatus Phytoplasma asteris*') is widely distributed; it induces yellows and dwarfing in vegetables such as lettuce, carrot, onion, cabbage, celery, potato, and tomato, in small fruits such as blueberry and strawberry, and in ornamentals such as China aster, as well as clover. In Europe it is mostly associated with clover phyllody and strawberry green petal, and it is also an agent of diseases in gladiolus, hydrangea, primula, anemone, ranunculus, chrysanthemum and poplar. In Asia, aster yellows phytoplasmas are responsible for marguerite yellows, phyllody in hydrangea, and paulownia witches' broom. Witches' broom disease of lime (16SrII group, '*Ca. P. aurantifolia*') is responsible for major losses of Mexican lime trees (*Citrus aurantifolia* L.) and was first reported in Oman from where the disease has spread in the region where up to 30 % of the Mexican lime trees have been destroyed. Group 16SrII phytoplasmas are also present in Australia where they are associated with tomato big bud and sweet potato little leaf diseases. Western X-disease (16SrIII group, "*Ca. P. pruni*") is a serious phytoplasma disease for peach and cherry production in North and South America. It has also

been described in daisy, garlic, summer squash, tomato, China tree, cassava, coffee, and several other plant species. Coconut lethal yellows (16SrIV) phytoplasma is associated with the palm lethal yellowing (LY) disease vectored by *Myndus crudus* (American palm cixiid) and possibly also by *Cedusa* species of derbid planthoppers. LY infects and kills coconut palms as well as many other palm species in Florida (USA) and Mexico. “Flavescence dorée” (FD) phytoplasma is a quarantine pathogen of grapevine that is widespread in the main grapevine-growing countries in Europe. The phytoplasma is transmitted by *Scaphoideus titanus* and belongs to 16SrV group together with ‘*Ca. P. ulmi*,’ which is responsible for yellows of elm species in North America and Europe and with ‘*Ca. P. ziziphi*,’ the causal agent of jujube witches’ broom and cherry and peach decline in China, Korea, Japan, and India. Almond witches’ broom disease reported in Iran (16SrIX, ‘*Ca. P. phoenicum*’) has killed thousands of almond trees over the past 15 years in Lebanon. Phytoplasma diseases of fruit trees have been studied in detail in Europe due to their high economic impact; they include apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). Sequencing has shown that 16S rDNA of these phytoplasmas is identical or almost identical; however, psyllid vector transmissibility and host range specificity have allowed their differentiation. AP is present in almost all European countries and the psyllids *Cacopsylla picta* and *C. melanocheira* are responsible for transmission. PD was first reported in western areas of North America; today it is of importance mainly in European pear orchards. Known vectors are *Cacopsylla pyricola* and *C. pyri*. ESFY is affecting stone fruit species such as apricot, plum, and peach; the disease is characterized by rapid spread especially when the conditions are favorable for host plants and vectors (*Cacopsylla pruni*). The “stolbur” phytoplasma (subgroup 16SrXII-A, ‘*Ca. P. solani*’) infects a wide range of cultivated plants in Europe, the Mediterranean basin, and southern Russia such as solanaceous crops, grapevine, celery, sugarbeet, strawberry, and lavender. Grapevine yellows (GY) are widespread diseases; one of these is “bois noir” (BN), associated with phytoplasmas belonging to ribosomal subgroup 16SrXII-A. BN has symptoms that are indistinguishable from those of FD, and it is widespread in almost all viticultural areas worldwide. BN phytoplasmas are transmitted to grapevine by *Hyalesthes obsoletus*. The phytoplasma is also reported in the northern part of South America, in Chile, in the Arabian Peninsula, and in Asia.

Based on the 16S ribosomal gene (16Sr) sequences, in total over 30 groups have been established within the ‘*Candidatus Phytoplasma*’ taxon [3, 10]. Some of these phytoplasmas are quarantine organisms in the European Union (FD, palm lethal yellowing, apple proliferation, “stolbur” in potato) and must therefore

be tightly regulated by plant protection authorities. Spread of phytoplasma-associated diseases facilitated by migration of insect vectors due to global climate change and increased international movement of people and goods may result in emergence of phytoplasma diseases in regions previously not typical for the pathogen and leave plant protection inspectors not prepared for identification. Therefore, efficient protocols for phytoplasma identification are in great demand.

One of the most common phytoplasma identification methods is based on nested amplification of the 1,800 bp region of the 16Sr, including flanking regions, using P1 [11] and P7 primers [12], followed by amplification of a 1,200 bp fragment with R16F2n and R16R2 primers [13]. This amplicon is subsequently subjected to RFLP (*Restriction Fragment Length Polymorphism*) analysis. For finer differentiation, other genes, such as *tuf*, *secY*, *rp*, 16-23S intergenic sequence, and protein translocase subunit (*SecA*) [14–17], are also in use, but for discrimination of phytoplasmas within a particular 16Sr group, as the designed primers are often not universal. However, being by far the most popular way of phytoplasma identification, RFLP-based methods suffer from several problems: they require the use of expensive restriction enzymes, it is necessary to use reference strains or virtual profiles for comparison of restriction patterns, and finally they only consider a small part (restriction sites) of the available sequence information.

DNA barcoding is a technique in which short DNA sequences (up to 600 bp) are used for species identification [18, 19]: DNA from a sample suspected to be the phytoplasma infected is extracted, amplified with a set of generic primers, and sequenced, and finally the sequence is compared with database sequences for identification. This method is based on several assumptions: (1) the DNA barcode is present in all species; (2) it is possible to amplify the region in all species with just one set of generic primers; (3) sequence difference between species is much greater than that between the individuals of the same species; (4) the barcode is relatively short, so that it can be easily sequenced; and (5) taxonomy, to which DNA barcode are linked, has already been established. The main advantages of this method are that no morphological traits are needed for identification, DNA can be obtained from various sources, including environmental samples and museum specimens, and just one generic assay is used. A limitation is a variable detection limit, which depends on proper sample handling and a successful DNA preparation. Absence of sequences of particular species in the database may be another problem.

It was proposed to adopt DNA barcoding principles to the identification of plant pests and pathogens, and the QBOL (*Quarantine Barcode Of Life*) project, financed by the EU

commission, was launched [20]. This project, aimed at the development of a universal identification system for main groups of plant pathogens, including phytoplasmas, is focused on quarantine organisms. This includes obtaining high-quality reference barcode sequences for quarantine and regulated pathogens, development and validation of protocols, establishment of an online sequence database, and a collection from which reference pathogen strains could be obtained.

Several considerations had to be made when a DNA barcoding system for phytoplasmas was developed. Firstly, as phytoplasma DNA is present in a plant DNA background and, secondly, as phytoplasmas often coexist with other bacteria, the primers should not amplify plant DNA or unrelated bacterial DNA. Among many regions tested, two barcodes, based on *Tuf* and 16Sr genes, proved to be immune to these problems and hence were selected as final DNA barcodes for phytoplasma identification.

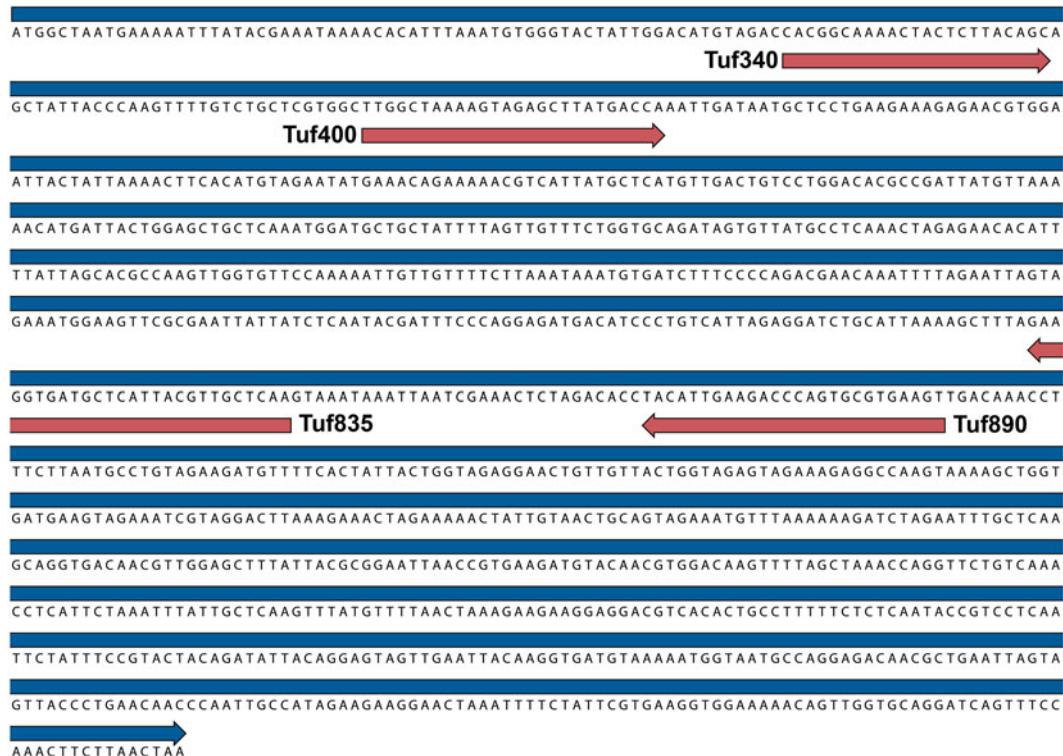
To develop the *tuf* barcode, all phytoplasma *tuf* genes available in the NCBI GenBank were aligned, and a conserved region within the *tuf* gene, present in all phytoplasmas, was found. Cocktails of primers (Table 1 and Fig. 2), which allowed for minor sequence variations between individual groups of phytoplasmas, were designed for the region, and the 420–444 bp long barcode was amplified from all phytoplasma strains tested (16SrI, 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXIV, 16SrXX, 16SrXXI groups). The phylogenetic tree, which was constructed based on these sequences, followed branching patterns of a tree constructed with full 16S sequences from the same strains, suggesting that the *tuf* barcode enables fine discrimination of phytoplasmas on both group and subgroup levels [9].

The current DNA barcoding tool for phytoplasma identification developed within the QBOL project includes two primer sets for nested amplification of the two DNA barcode regions (*tuf* and 16Sr genes); however, as *Tuf* has shown higher resolution, only the protocol for this region is presented here. An online sequence database with reference sequences for each DNA barcode for phytoplasma strains, covering the most important phytoplasma groups described so far, including quarantine phytoplasmas, and a reference strain DNA gene bank located at University of Bologna are also available. The identification pipeline includes the following steps: DNA extraction from plants suspected to be infected with phytoplasma, nested PCR amplification of DNA barcode, sequencing, sequence analysis and assembly, and online identification.

It should be emphasized that though this method was efficient for identification of phytoplasma strains both from lab-maintained collections and field-collected material, it still has some limitations. When mixed infections occur, cloning before sequencing is required to separately identify phytoplasmas present in the sample.

**Table 1**  
Primer sequences

Primer	Primer cocktail	Primer components	Primer sequence	Notes
Tuf first PCR	Tuf 340	Tuf/Tuf340a Tuf/Tuf340b	GCTCCCTGAAGAAARAGAACGTGG ACTAAAGAAGAAAAAGAACGTGG	Mix 1:1 to the final concentration 10 µM and use in the direct PCR as a forward primer mix
Tuf 890	Tuf 890ra Tuf/Tuf890rb Tuf/Tuf890rc		ACTTGDCCTCTTCKACTCTACCAAGT ATTIGTCCTCTTICWACACGTCCCTG ACCATTCCCTCAACACGTCCAGT	Mix 1:1:1 to the final concentration 10 µM and use in the direct PCR as a reverse primer mix
Tuf second PCR	Tuf 400	Tuf/Tuf400a Tuf/Tuf400b Tuf/Tuf400c Tuf/Tuf400d Tuf/Tuf400e	GTAAAACGACGGCCAGTGAACACAGAAAAACGTCAYTATGCTCA GTAAAACGACGGCCAGTGAACACTCTAAAGACATTACGCTCA GTAAAACGACGGCCAGTGAACACATCAAAGACAYTATGCTCA GTAAAACGACGGCCAGTGAACACAGAAAAAGACAYTATGCTCA GTAAAACGACGGCCAGTCAAAACAGCTAAAGACATTTATYCTCA	Mix 1:1:1:1 to the final concentration 10 µM and use in the nested PCR as a forward primer mix. M13F tag is attached to the 5' end and is indicated in <b>bold</b> . Use M13F primer for sequencing of the PCR product
Tuf 835	Tuf835ra Tuf835rb Tuf835rc		TAATACGACTCACTATAGGGAACACATCTTCWACHGGCAITTAAGAAAGG TAATACGACTCACTATAGGGAACACCTTCATAAGGCATTAAAAAWGG TAATACGACTCACTATAGGGAACACATCTTCTATAGGTAAATAAAAAGG	Mix 1:1:1 to the final concentration 10 µM and use in the nested PCR as a reverse primer mix. T7 tag is attached to the 5' end and is indicated in <b>bold</b> . Use T7 primer for sequencing of the PCR product



**Fig. 2** Phytoplasma *tuf* gene and primers used for the *tuf* barcode amplification

## 2 Materials

### 2.1 DNA Extraction

1. Liquid nitrogen.
2. Tissue homogenizer or sterile mortar and pestles for plant tissue disruption.
3. DNA extraction kit (*see Note 1*).

### 2.2 PCR

1. Forward and reverse primers (Table 1).
2. Positive and negative control DNA (*see Note 2*).
3. 20 ng/ $\mu$ l DNA from samples.
4. 5 U/ $\mu$ l GoTaq DNA polymerase (Promega).
5. 5 $\times$  colorless GoTaq Flexi reaction buffer (Promega).
6. 10 mM dNTPs.
7. 25 mM MgCl<sub>2</sub>.

### 2.3 Agarose Gel Electrophoresis

1. Agarose.
2. 1 $\times$  Tris Acetate EDTA (TAE) DNA electrophoresis buffer: Prepare a stock solution of 50 $\times$  TAE (Tris/ Acetate/ EDTA)

DNA electrophoresis buffer. For 1 l of the 50× stock TAE buffer, use 242 g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA, and double distilled water to 1 l. Adjust the pH to 8.5. To obtain 1× working solution of TAE, dilute the stock by 50× with double distilled water.

3. Ethidium bromide.
4. Loading dye (*see Note 3*).
5. 100 bp ladder molecular weight marker.
6. Gel electrophoresis equipment.
7. UV Transilluminator.

**2.4 Software for DNA Sequence Assembly and Alignments** CLC DNA Workbench 6 (CLC bio, Aarhus, Denmark) or any other sequence assembly software.

**2.5 Online QBOL DNA Barcoding Database** <http://www.q-bank.eu/>.

### 3 Methods

Please carefully read **Notes 4** and **5** before starting.

#### 3.1 DNA Extraction

Use preferred DNA extraction method to extract sample DNA (*see Note 1*).

Determine the DNA concentration by measuring absorbance ratio  $A_{260\text{nm}}/A_{280\text{nm}}$  with a spectrophotometer.

Store DNA at -20 °C (*see Note 6*).

#### 3.2 Nested PCR

##### 3.2.1 First PCR

Amplification of the 550 bp tuf region with Tuf340/ Tuf890 primers (Table 1 and Fig. 2).

1. Thaw at room temperature, gently vortex, and then briefly spin down the following reagents: reaction buffer, MgCl<sub>2</sub>, and primers (Table 1). Place them on ice.
2. Make calculations for the master mix according to Table 2. Remember to include reactions for no template control (NTC) and positive and negative controls.
3. Mix together water, MgCl<sub>2</sub>, reaction buffer, dNTPs, primers, and *Taq* polymerase. Vortex briefly, centrifuge, and aliquot 24 µl of the master mix per reaction into individual PCR tubes and return on ice (*see Note 7*).
4. Add 1 µl of DNA (20 ng/µl) and mix. Remember to include positive and negative control DNA and a no template control (NTC). Briefly spin down and return to ice.

**Table 2**  
**Master mix for PCR assays**

Components	Tuf 340/890 or Tuf 400/835
MQ water	10.3 µl
PCR reaction buffer	5 µl Promega 5× PCR buffer
25 mM MgCl <sub>2</sub>	1.5 µl
10 mM dNTPs mix (2.5 mM each)	2 µl
10 µM forward primer (Table 1)	2.5 µl
10 µM reverse primer (Table 1)	2.5 µl
Taq polymerase	0.2 µl Promega GoTaq polymerase, 5 U/µl

Volumes indicated below are per one reaction. The total volume of a single reaction is 25 µl. To prepare master mix, multiply the amounts indicated below by the number of reactions you are going to run plus a couple of extras

5. Place the PCR tubes in a thermocycler and program:  
94 °C 3 min and then 35 cycles (94 °C for 15 s., 54 °C for 30 s., 72 °C 60 s.) followed by 72 °C for 7 min.
6. Prepare 1:30 dilutions of the obtained PCR product for the nested PCR (Subheading 3.2.2).

### 3.2.2 Second PCR

Amplification of the 400 bp tuf region with Tuf400/ Tuf835 primers (Fig. 2).

1. Repeat steps 1–3 (see Subheading 3.2.1, steps 1–3).
2. Add 1 µl of the PCR product obtained in direct PCR, diluted 1:30 (Subheading 3.2.1, step 6), mix, briefly centrifuge, and return to ice.
3. Place the PCR tubes in a thermocycler and program as in Subheading 3.2.1, step 5.
4. Use 2.5 µl of the PCR product for gel electrophoresis. Refer to the Subheading 3.3.
5. Store the remaining PCR product at –20 °C until sequencing, Subheading 3.4.

### 3.3 Agarose Gel Electrophoresis

1. Prepare a 1.5 % agarose gel. For 200 ml of 1.5 % agarose gel, dissolve 3 g of agarose in 200 ml of 1× TAE buffer by heating the mixture in a microwave oven. Let it cool down to ~50 °C.
2. Pour the agarose into a tray, insert combs, remove any air bubbles, and let it set.
3. Place the gel in the electrophoresis chamber and pour 1× TAE buffer over, so that the buffer covers the gel completely.

4. Mix 5 µl of PCR product, 5 µl 2× loading dye.
5. Load the samples into the wells as well as 10 µl of the DNA ladder to 1 well.
6. Run the gel (*see Note 8*).
7. Stain the gel with ethidium bromide.
8. Analyze the gel on a transilluminator.
9. Choose samples with correct amplicon size for sequencing.

### **3.4 Sequencing**

1. Prepare samples according to the sequencing company's requirements (*see Note 9*).
2. Use M13F and T7R primers for sequencing the *tuf* barcode (*see Note 10*). Always sequence amplicons with both forward and reverse primers.

### **3.5 DNA Sequence Analysis and Assembly**

1. Carefully check chromatograms: multiple overlapping peaks may indicate presence of several sources of DNA in your preparations (due to mixed infection or contamination).
2. Assemble the two readings into one consensus sequence. We use CLC workbench for DNA assembly; however, other sequence assembly software packages may be used.
3. Identify primer regions and trim primer sequences and any flanking sequences.
4. Carefully solve nucleotide conflicts (if there are any). Use IUPAC codes for assigning ambiguous nucleotides.
5. Save the final consensus sequence and use it as a query in Subheading **3.6**.

### **3.6 Online Identification Using Q-Bank DNA Barcoding Database**

1. Go to <http://www.q-bank.eu>.
2. Paste your sequence into the query window on the ID page and start the alignment. A BLAST search against all sequences deposited in the Q-Bank database will be performed (*see Note 11*).

## **4 Notes**

1. We use the DNeasy extraction kit (Qiagen), but alternative protocols may be used for phytoplasma DNA extraction.
2. This can be any phytoplasma DNA sample which has been shown to be positive previously. Alternatively a reference strain may be obtained from the Q-bank phytoplasma strain collection (*see* <http://www.q-bank.eu/>*)*.
3. Commercially available loading dyes can be purchased from a number of manufacturers; however, make sure that you use the same loading buffer for the DNA ladder and for loading

samples, so they will migrate at the same rate and allow correct size estimation of amplicons.

4. Following good laboratory practice is essential for successful phytoplasma identification using this method. Take extreme care to not cross-contaminate your samples, especially as you are performing nested PCR assays. Always use filter tips and change gloves often and whenever contamination is suspected. It is advisable to spatially separate the areas where DNA samples are handled and PCR reactions are set up.
5. Certain techniques and reagents used in the current protocol may pose health hazards. Always wear proper protective clothing and conform to the local safety regulations. Familiarize yourself with MSDS for all reagents before use.
6. Storing DNA in small aliquots will help avoid repeated freeze-thaw cycles which have a negative effect on DNA preservation.
7. Keeping the polymerase on ice will improve stability. Keep *Taq* polymerase in a cooler, and add to master mix just before use.
8. Normally 5 V/cm will allow for good separation.
9. Post-PCR cleanup may be necessary at some companies, while others include it into their services. Post-PCR cleanup kits are widely available.
10. M13F and T7 tags were attached to the Tuf400 and Tuf835 primers, respectively, to facilitate sequencing.
11. You can change alignment parameters or use the default settings.

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# **Chapter 11**

## **High-Throughput Diagnosis of Potato Cyst Nematodes in Soil Samples**

**Alex Reid, Fiona Evans, Vincent Mulholland, Yvonne Cole, and Jon Pickup**

### **Abstract**

Potato cyst nematode (PCN) is a damaging soilborne pest of potatoes which can cause major crop losses. In 2010, a new European Union directive (2007/33/EC) on the control of PCN came into force. Under the new directive, seed potatoes can only be planted on land which has been found to be free from PCN infestation following an official soil test. A major consequence of the new directive was the introduction of a new harmonized soil sampling rate resulting in a threefold increase in the number of samples requiring testing. To manage this increase with the same staffing resources, we have replaced the traditional diagnostic methods. A system has been developed for the processing of soil samples, extraction of DNA from float material, and detection of PCN by high-throughput real-time PCR. Approximately 17,000 samples are analyzed each year using this method. This chapter describes the high-throughput processes for the production of float material from soil samples, DNA extraction from the entire float, and subsequent detection and identification of PCN within these samples.

**Key words** Potato cyst nematode (PCN), Real-time PCR, High throughput, Float material

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### **1 Introduction**

Potato cyst nematodes (PCNs) are economically important pests of potatoes belonging to the genus *Globodera*. There are two species *Globodera pallida* (Stone) Behrens and *Globodera rostochiensis* (Wollenweber) Behrens which originated from potato-growing areas in the Andes Mountains of South America. Outbreaks of PCN have now occurred in most of the potato-growing areas of the world mainly due to the unwitting transportation of infested soils by man in the nineteenth century [1]. The second-stage juvenile nematodes hatch from egg in the soil in response to exudates from growing potato roots. The nematode sets up a feeding site in the root where it stays, molting two further times to become an adult. The bodies of the sedentary females break through the root surface and are fertilized by the vermiform males. Eggs develop

within the females which eventually die, and their bodies harden to become a protective cyst which then drops off into the soil. These cysts can contain up to 500 eggs which are able to survive for several years until exposure to potato root exudate which starts the process again. Surveys have established that the two species of PCN are present in 64 % of the ware potato-growing land of England and Wales, with *G. pallida* present in 92 % of these infestations and *G. rostochiensis* present in 33 % [2]. In the UK, the mean annual yield loss attributed to PCN damage was estimated to equate to approximately £43 million between 1990 and 1995 [3]. An additional cost is the £8 million (estimated for 1999) spent in the UK each year on treating 28,000 ha of PCN infested land with granular nematicides [4].

EU Directive 2007/33/EC on the control of PCN came into force on 1 July 2010 replacing the previous directive which laid out a legislative framework appropriate for the control of a quarantine pest across the European Union [5, 6]. One of the key features of the new directive was the adoption of a harmonized method for soil sampling which meant a threefold increase in the number of samples requiring testing in Scotland.

Prior to 2010, the system in use at SASA was to wash soil samples by a modified Fenwick can method [7]; the presence of PCN cysts within the float material produced was determined by visual examination, using low-power microscopy. Cysts were then identified to species level by morphological and morphometric measurements, primarily of the J2 stylet and female perineal patterns [8]. Visual examination is a time-consuming and repetitive process requiring highly trained staff. The increased number of soil samples under the new directive meant it was impractical to continue using this method as too few trained staff were available to examine the increased number of samples. Therefore, to fulfill statutory requirements and to minimize the costs of PCN testing, high-throughput methods for soil washing and subsequent detection of PCN cysts were developed [9].

PCN cysts are extracted from the soil using a MEKU nematode carousel developed specifically for this method (Pollähne, Wennigsen, Germany). The carousel automates the process of cyst extraction, based on the traditional principles of sieving and flotation (as dried PCN cysts float on water). The samples are tracked using barcodes throughout soil collection, sample processing, DNA extraction, and PCR diagnosis.

Total DNA extraction from the float material is achieved by the use of a modified commercial plant DNA extraction kit in a 96-well plate format coupled with the extensive use of robotics.

While there are numerous conventional PCR diagnostic tests for the speciation of PCN [10–14], these have limitations for use in a high-throughput environment. Species identification by real-time PCR diagnostics offers much greater potential for high-throughput

screening [15–17]. Detection and identification of PCN in the samples are achieved by a real-time PCR assay, developed in-house, targeting the ITS1 region of the ribosomal DNA repeat, chosen due to the large amount of publically available sequence data. Primers and probes were designed after aligning 91 *Globodera* spp. sequences and three *Punctodera* spp. sequences (Table 1). A TaqMan MGB Probe was designed based on the *G. pallida* accession FJ212165. The *G. rostochiensis*-specific probe covers the same region as the *G. pallida*-specific probe [9]. Detection and identification of PCN are carried out in a two-step approach. Firstly, all samples are screened for the presence of PCN using a cocktail containing both PCN probes. Samples testing positive in this first detection assay are “cherry picked” into a new plate and subjected to two further simplex assays, one for *G. pallida* and one for *G. rostochiensis*, to identify the PCN species present in the soil sample.

In the 4 years since this process has been in operation at SASA, an average of 17,000 samples have been tested each year (in the 6–7-month period of the PCN testing season). Over this period there has been no significant change in the overall occurrence of PCN detected in samples from Scottish soils [18]. The increased sampling rate has resulted in an increase in the area of the tested land recorded as infested, from 2.0 to 5.3 % due to the increased sampling rate. In terms of cost efficiency, the 120 % increase in the quantity of soil tested since 2010 has been achieved with an increase in staff time of only 10 %, while the overall cost of testing a sample within the laboratory has decreased by 34 %.

**Table 1**  
Numbers of *Globodera* and *Punctodera* species used in alignment for primer and probe design

Species	No. of sequences in alignment
<i>Globodera pallida</i>	24
<i>Globodera rostochiensis</i>	42
<i>Globodera mexicana</i>	3
<i>Globodera tabacum</i>	12
<i>Globodera artemisia</i>	3
<i>Globodera achilleae</i>	6
<i>Globodera hypolysi</i>	1
<i>Globodera</i> sp.	3
<i>Punctodera chalcoensis</i>	1
<i>Punctodera punctata</i>	2

Accession numbers available from authors

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## 2 Materials

### 2.1 *Float Production*

1. MEKU nematode carousel (MEKU Pollähne).
2. Filter papers 24 cm round (Macherey-Nagel MN 751).

### 2.2 *DNA Extraction*

Unless stated, store all reagents at room temperature.

1. TissueLyser II with 2 × 24 adapter set (QIAGEN).
2. MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Life Technologies).
3. MICROLAB® STARlet Liquid Handling Workstation (Hamilton Robotics).
4. Liquidator™ 96 (Mettler Toledo).
5. Thermomixer comfort (Eppendorf).
6. Benchtop centrifuge fitted with a swing-out rotor capable of holding the S-Blocks supplied in the BioSprint 96 DNA Plant Kit (a 96 deep well plate) with speeds up to 3,000 × $\ddot{\text{g}}$ . For example, a Heraeus Multifuge 3S-R (Heraeus).
7. Rainin AutoRep E repeater pipette and various volume tips (Mettler Toledo).
8. Safe-Lock tubes™ 2 ml (Eppendorf).
9. Fast Funnel® MINI (S&S Concepts, Inc.)
10. Tungsten carbide beads 3 mm (QIAGEN).
11. BioSprint 96 DNA Plant Kit (QIAGEN). Follow kit storage instructions. N.B. Buffer RPW is supplied as a concentrate and prior to its first use should have 125 ml isopropanol and one vial of RNase A added. The solution should then be stored at 4 °C.
12. AP1 buffer (QIAGEN). Store at 4 °C.
13. Propan-2-ol.
14. 96 % ethanol.
15. PCR grade water (Sigma).
16. Tween 20 (Sigma).
17. Adhesive sealing sheets (Thermo Scientific).

### 2.3 *Real-Time PCR*

1. MICROLAB® STARlet Liquid Handling Workstation (Hamilton Robotics).
2. MixMate fitted with 96-well PCR plate holder (Eppendorf).
3. Centrifuge fitted with rotors adapted to accommodate 96-well and 384-well PCR plates, e.g., Microcentrifuge 5430 (Eppendorf).
4. 7900HT Fast Real-Time PCR System with 384-well block (Life Technologies).

5. MicroAmp® Optical 384-Well Reaction Plates and MicroAmp® Optical Adhesive Film (Life Technologies). FrameStar® 96 semi-skirted plates (4titude Ltd).
6. TaqMan® Environmental Master Mix 2.0 (Life Technologies). Store at -20 °C before first use, protected from light. After first use, store at 4 °C, protected from light (see Note 1).
7. TaqMan® Exogenous Internal Positive Control Reagents (Life Technologies). Store at -20 °C.
8. PCR grade water (Sigma).
9. Oligonucleotide primers (Eurofins MWG Operon). Store stock solutions in 100 µl aliquots at 100 µM at -20 °C.  
 PCN-FOR 5' CGTTTGTGTTGACGGACAYA 3'  
 PCN-REV 5' GGCGCTGCCRTACATTGTTG 3'
10. FAM™ labeled TaqMan® MGB Probes (Life Technologies). Store stock solutions in 20 µl aliquots at 100 µM at -20 °C.  
 Pallida probe 5' CCGCTATGTTGGGC 3'  
 Rostoc probe 5' CCGCTGTGTATKGGC 3'
11. *G. pallida* and *G. rostochiensis* control DNA; extracted from pure cysts at 100, 10, and 1 ng/µl dispensed in 5 µl aliquots in 1.5 ml microcentrifuge tubes. PCR grade water dispensed in 5 µl aliquots in 1.5 ml microcentrifuge tubes. Store at -20 °C.

#### **2.4 Tungsten Carbide Bead Recovery**

1. 0.4 M HCl.

### **3 Method**

#### **3.1 Float Production**

1. Soil samples are dried at 37 °C for a minimum of 2 days in constant temperature cabinets.
2. If required, dried soil samples containing larger clumps are broken up using a mallet which is washed between samples.
3. Samples are placed in a 1,000 µm sieve loaded onto the MEKU nematode carousel and the float material collected in a 200 µm sieve (see Note 2).
4. The samples are washed out of the final sieve onto filter papers and dried to await DNA extraction.

*See Notes 3 and 4 for further information.*

#### **3.2 DNA Extraction of Float Material**

The following method is for a float size of approximately 1 ml. For larger floats, split the sample into several tubes. We use a STARlet liquid handling workstation to fill the S-Blocks used in the MagMAX™ Express for all the solutions except the magnetic beads which we add using an AutoRep E repeat dispenser.

### 3.2.1 DNA Extraction

1. The dry float material is manually scraped off the filter paper into a Fast Funnel MINI placed over a 2 ml Safe-Lock tube. Add eight tungsten carbide beads to the tube (*see Note 5*).
2. Batches of 48 tubes are disrupted in the TissueLyser II set at 30 Hz for 30 s.
3. Remove tubes and tap on the bench to ensure the dust is at the bottom of the tube.
4. Add 1.5 ml of API buffer and shake again at 30 Hz for 30 s (*see Note 6*).
5. Centrifuge tubes at  $1,180 \times g$  for 5 min.
6. Transfer a minimum of 400  $\mu\text{l}$  of the supernatant using a pipette fitted with a wide-bore tip to an individual well of a 96-well S-Block containing 5  $\mu\text{l}$  RNase A, seal with an adhesive sealing sheet, and store at  $-20^\circ\text{C}$  (*see Note 7*).
7. Incubate S-Block at  $65^\circ\text{C}$  to thaw, and then continue to incubate for an additional 10 min (*see Note 8*).
8. Centrifuge at  $3,000 \times g$  for 5 min.
9. Prepare 5 S-Blocks, numbered 1–5, containing in each well:
  - S-Block 1, 400  $\mu\text{l}$  isopropanol and 30  $\mu\text{l}$  MagAttract Suspension G
  - S-Block 2, 400  $\mu\text{l}$  RPW buffer
  - S-Block 3, 400  $\mu\text{l}$  96 % ethanol
  - S-Block 4, 400  $\mu\text{l}$  96 % ethanol
  - S-Block 5, 500  $\mu\text{l}$  sterile PCR grade water (Sigma) containing 0.02 % (v/v) Tween 20
 Also prepare a microplate MP containing 200  $\mu\text{l}$  sterile PCR grade water (*see Note 9*).
10. Transfer 340  $\mu\text{l}$  of the supernatant from **step 7** to S-Block 1, taking care to leave as much of the float residue behind as possible (*see Note 10*).
11. Process the samples using a MagMAX Express using the BS96 DNA Plant program (*see Note 11*).
12. Remove the microplate MP (the DNA elution plate) and either process immediately or seal with an adhesive sealing sheet and store at  $-20^\circ\text{C}$ .

### 3.2.2 Tungsten Carbide Bead Recovery

The beads can be reused once cleaned. The tubes containing the beads can be stored up until sufficient numbers of samples have been processed.

1. Shake the tubes containing the beads (after the supernatant has been removed, **step 5** from the DNA extraction) (*see Note 12*).
2. Empty the contents of the tubes into a coarse sieve and wash with running tap water. Transfer the washed beads to a suitably sized Duran bottle.

3. Wash beads for 3 min with 0.4 M HCl.
4. Rinse with distilled water and air-dry.

### 3.3 Real-Time PCR

Make up a suitable volume of PCR master mix in a laminar-flow cabinet to protect from airborne contaminants. If more than one run is planned for the day, the additional master mix can be stored at 4 °C until required. When preparing the real-time PCR master mix, the volume is calculated to take account of the total number of samples to be tested as well as control reactions and any dead volume associated with pipetting and automated liquid handling (*see Note 13*). All real-time assays should include the following controls:

Non-template control (NTC): Molecular grade sterile water.

Positive control (PC): PCN DNA.

Extraction amplification control (EAC): DNA extract from known negative soil spiked with a PCN cyst.

Exogenous internal positive control (EIPC): Template DNA added to each sample to monitor efficiency of PCR amplification.

#### 3.3.1 Real-Time PCR “Detection Assay”

The amounts below are for 96 samples plus controls.

1. Remove all PCR components from freezer and thaw.
2. Dilute primers to the working concentration of 5 µM by diluting 7.5 µl of the 100 µM stock of each primer with 142.5 µl PCR grade water for each run.
3. Dilute the probes to the working concentration of 5 µM by adding 380 µl PCR grade water to the 20 µl stock tube.
4. In a 5 ml tube, make up the following master mix:
  - TaqMan® Environmental Master Mix 2,100 µl.
  - TaqMan® Exogenous Internal Positive Control primer mix 210 µl.
  - PCN-FOR (5 µM) 175 µl.
  - PCN-REV (5 µM) 175 µl.
  - Pallida probe (5 µM) 87.5 µl.
  - Rostoc probe (5 µM) 87.5 µl.
  - PCR grade water 644 µl.

TaqMan® Exogenous Internal Positive Control DNA 21 µl.

5. Divide the master mix between eight 1.5 ml sterile tubes—437 µl/tube.
6. Pipette 25 µl of the master mix into each well of a 96-well plate (Fig. 1). Pipette 25 µl of the master mix into the tubes containing the positive and negative controls (one 1.5 ml tube containing 5 µl of *G. pallida* DNA at 10 ng/µl, one 1.5 ml tube containing 5 µl of *G. rostochiensis* DNA at 10 ng/µl, and three 1.5 ml tubes containing 5 µl of PCR grade water).

Sample Plate layout

	Well											
	1	2	3	4	5	6	7	8	9	10	11	12
A	EAC	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

PCR Plate layout (384 well)

	Wells											
	1/2/3	4/5/6	7/8/9	10/11/12	13/14/15	16/17/18	19/20/21	22/23/24				
A	EAC	A3	A5	A7	A9	A11	P 10					
B	A2	A4	A6	A8	A10	A12						
C	B1	B3	B5	B7	B9	B11	R 10					
D	B2	B4	B6	B8	B10	B12						
E	C1	C3	C5	C7	C9	C11	NTC					
F	C2	C4	C6	C8	C10	C12						
G	D1	D3	D5	D7	D9	D11	NTC					
H	D2	D4	D6	D8	D10	D12						
I	E1	E3	E5	E7	E9	E11	NTC					
J	E2	E4	E6	E8	E10	E12						
K	F1	F3	F5	F7	F9	F11						
L	F2	F4	F6	F8	F10	F12						
M	G1	G3	G5	G7	G9	G11						
N	G2	G4	G6	G8	G10	G12						
O	H1	H3	H5	H7	H9	H11						
P	H2	H4	H6	H8	H10	H12						

**Fig. 1** Plate maps for the first assay showing where the samples in the wells of the sample plate go to in the 384-well PCR plate via the master mix plate. P10 is the *G. pallida* DNA control at 10 ng/μl. R10 is the *G. rostochiensis* DNA at 10 ng/μl. EAC is the extraction amplification control, NTC is the non-template control (water)

7. Pipette 5 μl of the DNA sample from the microplate MP into the corresponding well of the 96-well plate containing the master mix (Fig. 1).
8. Mix well and centrifuge at 1,000 × g for 1 min (see Note 14).
9. Aliquot the contents of each well in 9.5 μl lots into three wells of a 384-well plate (Fig. 1) and seal with optical adhesive film.
10. Amplify in a 7900HT Fast Real-Time PCR System with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95°C for 15 s, and 60 °C for 1 min.
11. For a sample to pass the internal control, it must yield a signal in at least two of the three wells (see Note 15).
12. Any samples positive for PCN are cherry picked into a new plate for second assay analysis.

### 3.3.2 Real-Time PCR “Identification Assay”

An identification assay is carried out when a maximum of 48 positive samples have been collected. The amounts below are for 48 samples plus controls.

1. Remove all PCR components from freezer and thaw.
2. Dilute primers to the working concentration of 5 μM by diluting 7.5 μl of the 100 μM stock of each primer with 142.5 μl PCR grade water for each run.

Master mix Plate layout

	Well											
	1	2	3	4	5	6	7	8	9	10	11	12
A	EAC	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

3. Dilute the probes to the working concentration of 5  $\mu\text{M}$  by adding 380  $\mu\text{l}$  PCR grade water to the 20  $\mu\text{l}$  stock tube.
4. In two 2 ml tubes, make up the following master mixes, one containing the Pallida probe and the other containing the Rostoc probe.
 

TaqMan® Environmental Master Mix 900  $\mu\text{l}$ .

PCN-FOR (5  $\mu\text{M}$ ) 75  $\mu\text{l}$ .

PCN-REV (5  $\mu\text{M}$ ) 75  $\mu\text{l}$ .

Probe (either Pallida or Rostoc) (5  $\mu\text{M}$ ) 75  $\mu\text{l}$ .

PCR grade water 375  $\mu\text{l}$ .
5. Divide the master mix between eight 1.5 ml sterile tubes—187  $\mu\text{l}/\text{tube}$ .
6. Pipette 25  $\mu\text{l}$  of the Pallida master mix into wells A1 to H6 of a 96-well plate. Pipette 25  $\mu\text{l}$  of the Pallida master mix into the controls (one 1.5 ml tube containing 5  $\mu\text{l}$  of *G. pallida* DNA at 100 ng/ $\mu\text{l}$ , one 1.5 ml tube containing 5  $\mu\text{l}$  *G. pallida* DNA at 10 ng/ $\mu\text{l}$ , one 1.5 ml tube containing 5  $\mu\text{l}$  *G. pallida* DNA at 1 ng/ $\mu\text{l}$ , and three 1.5 ml tubes containing 5  $\mu\text{l}$  of PCR grade water).
7. Pipette 25  $\mu\text{l}$  of the Rostoc master mix into wells A7 to H12 of a 96-well plate. Pipette 25  $\mu\text{l}$  of the Rostoc master mix into the controls (one 1.5 ml tube containing 5  $\mu\text{l}$  of *G. rostochiensis* DNA at 100 ng/ $\mu\text{l}$ , one 1.5 ml tube containing 5  $\mu\text{l}$  of *G. rostochiensis* DNA at 10 ng/ $\mu\text{l}$ , one 1.5 ml tube containing 5  $\mu\text{l}$  of *G. rostochiensis* DNA at 1 ng/ $\mu\text{l}$ , and three 1.5 ml tubes containing 5  $\mu\text{l}$  of PCR grade water).
8. Pipette 5  $\mu\text{l}$  of the DNA sample from the sample plate into the corresponding wells of the 96-well plate containing the master mixes. (The sample in well A1 of the sample plate goes into wells A1 and A7 of the master mix plate.)
9. Mix well and centrifuge at  $1,000 \times g$  for 1 min (*see Note 14*).
10. Aliquot the contents of each well in 9.5  $\mu\text{l}$  lots into three wells on a 384-well plate and seal with optical adhesive film. *See Fig. 2* for an example of the layout of the plates.
11. Amplify in a 7900HT Fast Real-Time PCR System with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95°C for 15 s, and 60 °C for 1 min.

### **3.4 Interpretation of Results**

#### *3.4.1 Detection Assay*

For a detection assay plate to pass, the EAC and PCs must both be positive for PCN and the NTCs negative. For a sample to test negative for PCN, the EIPC must give a Ct value (on average this is around 30), and the PCN must be undetectable in all three wells. If a Ct value is detected for PCN, then the sample is cherry picked and subjected to an identification assay.

Sample Plate layout

	Well											
	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6						
B	B1	B2	B3	B4	B5	B6						
C	C1	C2	C3	C4	C5	C6						
D	D1	D2	D3	D4	D5	D6						
E	E1	E2	E3	E4	E5	E6						
F	F1	F2	F3	F4	F5	F6						
G	G1	G2	G3	G4	G5	G6						
H	H1	H2	H3	H4	H5	H6						

Mastermix Plate layout

	Well											
	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A1	A2	A3	A4	A5	A6
B	B1	B2	B3	B4	B5	B6	B1	B2	B3	B4	B5	B6
C	C1	C2	C3	C4	C5	C6	C1	C2	C3	C4	C5	C6
D	D1	D2	D3	D4	D5	D6	D1	D2	D3	D4	D5	D6
E	E1	E2	E3	E4	E5	E6	E1	E2	E3	E4	E5	E6
F	F1	F2	F3	F4	F5	F6	F1	F2	F3	F4	F5	F6
G	G1	G2	G3	G4	G5	G6	G1	G2	G3	G4	G5	G6
H	H1	H2	H3	H4	H5	H6	H1	H2	H3	H4	H5	H6
	Pallida mastermix						Rostoc mastermix					

PCR Plate layout (384 well)

	Wells											
	1/2/3	4/5/6	7/8/9	10/11/12	13/14/15	16/17/18	19/20/21	22/23/24				
A	A1 (P)	A3 (P)	A5 (P)	A1 (R)	A3 (R)	A5 (R)	P 100 (P)					
B	A2 (P)	A4 (P)	A6 (P)	A2 (R)	A4 (R)	A6 (R)	R 100 (R)					
C	B1 (P)	B3 (P)	B5 (P)	B1 (R)	B3 (R)	B5 (R)	P 10 (P)					
D	B2 (P)	B4 (P)	B6 (P)	B2 (R)	B4 (R)	B6 (R)	R 10 (R)					
E	C1 (P)	C3 (P)	C5 (P)	C1 (R)	C3 (R)	C5 (R)	P 1 (P)					
F	C2 (P)	C4 (P)	C6 (P)	C2 (R)	C4 (R)	C6 (R)	R 1 (R)					
G	D1 (P)	D3 (P)	D5 (P)	D1 (R)	D3 (R)	D5 (R)	NTC (P)					
H	D2 (P)	D4 (P)	D6 (P)	D2 (R)	D4 (R)	D6 (R)	NTC (R)					
I	E1 (P)	E3 (P)	E5 (P)	E1 (R)	E3 (R)	E5 (R)	NTC (P)					
J	E2 (P)	E4 (P)	E6 (P)	E2 (R)	E4 (R)	E6 (R)	NTC (R)					
K	F1 (P)	F3 (P)	F5 (P)	F1 (R)	F3 (R)	F5 (R)	NTC (P)					
L	F2 (P)	F4 (P)	F6 (P)	F2 (R)	F4 (R)	F6 (R)	NTC (R)					
M	G1 (P)	G3 (P)	G5 (P)	G1 (R)	G3 (R)	G5 (R)						
N	G2 (P)	G4 (P)	G6 (P)	G2 (R)	G4 (R)	G6 (R)						
O	H1 (P)	H3 (P)	H5 (P)	H1 (R)	H3 (R)	H5 (R)						
P	H2 (P)	H4 (P)	H6 (P)	H2 (R)	H4 (R)	H6 (R)						

**Fig. 2** Plate maps for the second assay showing where the samples in the wells of the sample plate go to in the 384-well PCR plate via the master mix plate. (P) denotes the Pallida probe master mix; (R) denotes the Rostoc probe master mix. P100, P10, and P1 are the *G. pallida* DNA controls at 100, 10, and 1 ng/µl. R100, R10, and R1 are the *G. rostochiensis* DNA controls at 100, 10, and 1 ng/µl. NTC is the non-template control (water)

### 3.4.2 Identification Assay

For a sample to be called as a positive, it must yield a Ct value of 35 or less in at least two of the three wells (allowing for the occasional PCR failure) (see Note 16).

## 4 Notes

- Environmental master mix is used as it is specifically designed to amplify products in the presence of high levels of inhibitors such as those that may be present in the float material. [http://www.lifetechnologies.com/in/en/home.html?gclid=CPWnw8mTyMQCFYcnjgod9b8ALA&cs\\_kwcid=AL!3652!3!58515390982!e!!g!!life%2520tech&ef\\_id=VRUZ0QAABTvsDgNU:20150327085025:s](http://www.lifetechnologies.com/in/en/home.html?gclid=CPWnw8mTyMQCFYcnjgod9b8ALA&cs_kwcid=AL!3652!3!58515390982!e!!g!!life%2520tech&ef_id=VRUZ0QAABTvsDgNU:20150327085025:s)
- Alternatively, a Fenwick can method could be used for float production.
- Information on the nematode carousel can be found on the MEKU website (<http://www.meku-pollachne.de/Home/NEMATODE-instruments/Nematode-carousel/nematode-carousel.html>). The use of the carousel enables one person to process over 25 samples per hour compared to 7.5 samples per hour using the Fenwick can process. An additional benefit is

that the carousel uses only 21 l of water per sample c.f. 90 l for the Fenwick can.

4. Trials using soil samples seeded with a known number of PCN cysts demonstrated a cyst recovery rate for the carousel of 93 %. This figure compares well with the minimum extraction efficiency of 60 % recommended in a recent report covering proficiency tests carried out across a series of EU PCN testing laboratories [19].
5. We measure eight beads into each tube using a 1 ml disposable syringe barrel which has been cut off to hold eight beads at a time. This way one doesn't need to count the beads into each tube.
6. A precipitate can form in the AP1 buffer—if this happens, incubate at 65 °C for 20 min to redissolve. We use an AutoRep E pipette fitted with a 12.5 ml tip and set at 0.75 ml and add the AP1 in two lots to the sample. This helps to ensure the sample does not overflow the tube (in the case of larger floats). We add the RNase A to the wells of the S-Block in point 5 using an AutoRep E pipette fitted with a 100 µl tip and set at 5 µl.
7. Plates can be stored at –20 °C and further processed in batches for convenience.
8. We use a Thermomixer Comfort set at 65 °C to incubate the S-Blocks.
9. We use the STARlet liquid handling workstation to fill the S-Block and microplate MP with all solutions except the MagAttract Suspension G which is added using an AutoRep E pipette fitted with a 1.25 ml tip. Prior to using, ensure that the MagAttract Suspension G has been adequately resuspended using a vortex mixer.
10. We use a Liquidator 96 to transfer the supernatant from all 96 wells from one S-Block to another simultaneously. As 340 µl is greater than the maximum volume the Liquidator 96 can pipette at one time, we transfer 170 µl twice from one plate to the other.
11. The method would work well in a low-throughput environment using a manual version of the extraction method.
12. We do this by placing the tubes in an empty 2.5 l paint tin and shaking them in a commercial paint shaker. Smaller numbers of tubes could be shaken using a vortex mixer.
13. We use a STARlet liquid handling workstation to set up the PCR reactions and to “cherry pick” positive samples.
14. We mix the 96-well plates in a MixMate fitted with tube holder PCR 96 using the PCR plate setting.
15. We only pass samples if they have signal from the internal control in at least two of the three wells. The majority of samples have signal in all three wells, but occasionally, a well will fail mainly due to being empty, e.g., due to evaporation.

16. It is difficult to give a definitive concentration of PCN in the sample using this method as float material can contain variable amounts of DNA, and as the extraction method yields a finite amount of DNA, it may be that not all of the PCN DNA in a float sample is extracted.

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# Chapter 12

## Detection of Nepovirus Vector and Nonvector *Xiphinema* Species in Grapevine

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### Abstract

Fanleaf degeneration is considered the most damaging viral disease of grapevine. The two major nepoviruses involved are *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV) which are respectively and specifically transmitted by the dagger nematodes *Xiphinema index* and *X. diversicaudatum*. The methods described below are aimed at detecting four prevalent grapevine *Xiphinema* species: the vector species previously mentioned and two nonvector species *X. vuittenezi* and *X. italiae*.

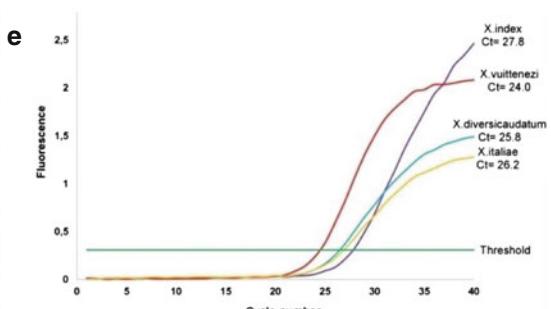
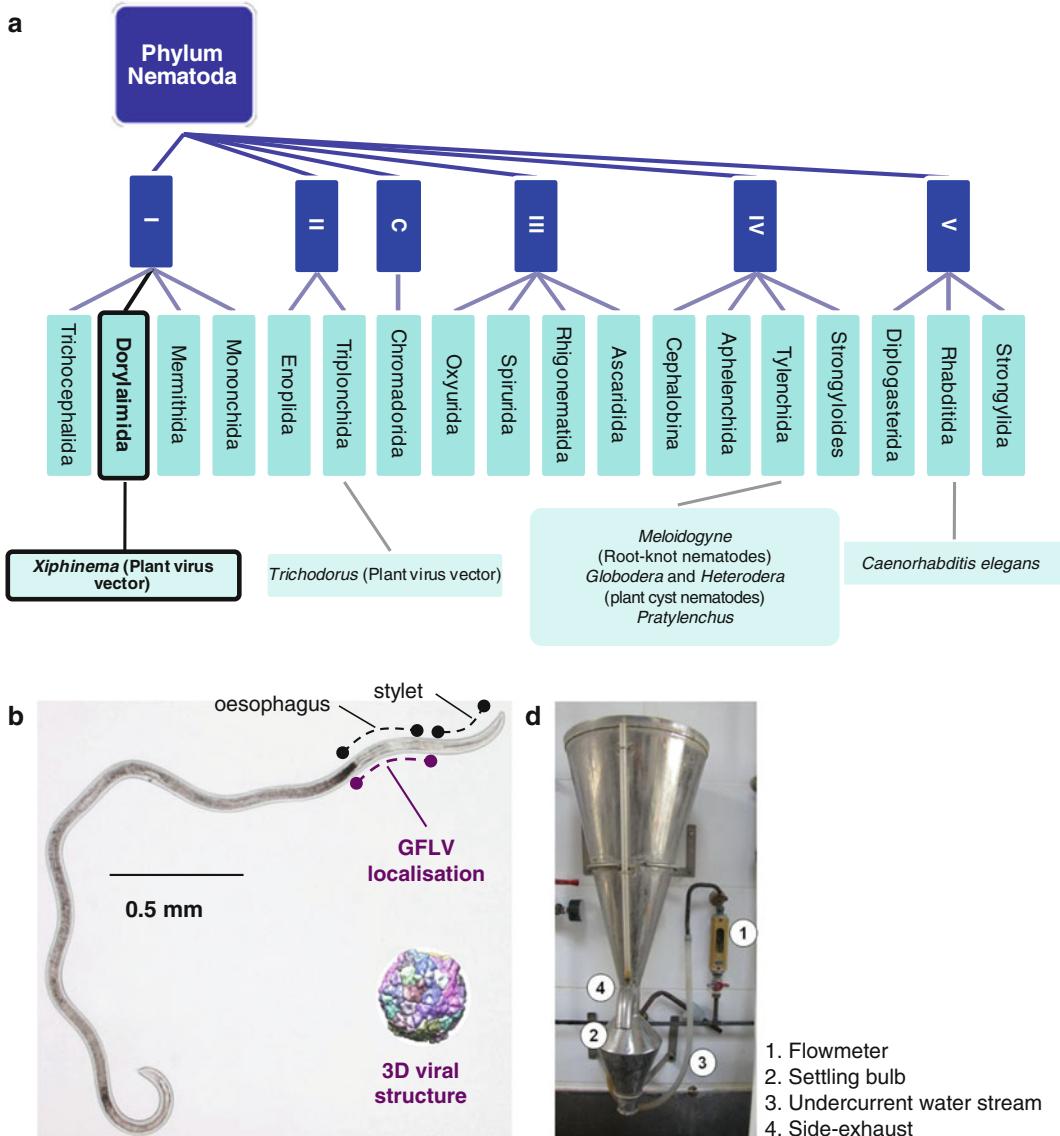
**Key words** ArMV, Detection, Fanleaf degeneration, GFLV, Grapevine, Nematode, Nepovirus, Real-time PCR, TaqMan®, *Xiphinema*, Vector

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### 1 Introduction

Fanleaf degeneration is one of the most damaging viral diseases occurring on grapevines worldwide. Sixteen viruses termed nepoviruses (for “nematode-transmitted polyhedral viruses”) may be involved in this degeneration. Among them, the two major viruses are *Grapevine fanleaf virus* (GFLV), considered as the first virus of grapevine in France and in the world [1], and *Arabis mosaic virus* (ArMV). These nepoviruses disrupt the plant metabolism and cause various symptoms (Fig. 1c) on leaves (deformation, yellowing, “panachure” mosaic on the limb), branches (shorter inter nodes), and bunches (reduction of their number and size, “coulure,” and “millerandage”), resulting in loss of yield, premature death of grapevines, and decrease of wine quality. In France, where the wine industry is substantial, this disease has an annual cost estimated between 350 M€ and 850 M€ [1]. Approx. 60 % of the grapevine plots are infected [2], and loss of production may rise up to 80 % in highly infected vineyards [1]. To date no natural resistance against these viruses has been reported.

Most of the plant parasitic nematode species described hitherto induce direct damage to plants and therefore cause huge economical



**Fig. 1** (a) Simplified scheme of the phylum Nematoda adapted from [13]; (b) nematode *Xiphinema index*, GFLV localization in the nematode, and GFLV particle illustrated by its X-ray viral structure (PDB 2Y7T, 2Y7U, 2Y7V, from [14]); (c) the characteristic symptom of yellowing due to GFLV infection of a grape “Ugni blanc” (*Vitis vinifera* “Ugni blanc”) (right, infected plant; left, noninfected plant); (d) Oostenbrink elutriator used for nematode extraction; (e) curves of real-time PCR detection for *X. index*, *X. diversicaudatum*, *X. vittenezi*, and *X. italiae*. Each positive sample is characterized by a cycle threshold (*Ct*) value

loss. These nematodes, belonging mainly to the order Tylenchida (Fig. 1a), display different modes of feeding. Those with the highest economic impact are endoparasites and either migratory (*Pratylenchus* spp.) or sedentary (root-knot nematodes, *Meloidogyne* spp.; cyst nematodes, *Heterodera* spp. and *Globodera* spp.). Other plant nematodes are ectoparasites (which means that they feed on the rootlets with all their life stages remaining outside the plant tissues) and have a lower direct economical impact on crops. Nevertheless among them some species from the orders Dorylaimida and Triplonchida may be responsible for severe agronomic problems due to their ability to transmit viruses to plants (Fig. 1a). GFLV and ArMV, the two major viruses involved in grapevine degeneration, are respectively and specifically transmitted by the Dorylaimida nematode species *Xiphinema index* and *X. diversicaudatum* (family Longidoridae). These species belong to the “dagger nematodes” due to the shape of their prominent stylet used to penetrate the apical root tissues. Adult stages feed on root tips and females lay independent eggs into the soil throughout their life. The first larval stages evolve in the egg, and hatching in the soil gives birth directly to the first-stage juveniles (J1) that will feed and evolve into the J2, J3, and J4 stages. In *X. index*, the life cycle can be completed within 2 months but is often longer depending on the environmental conditions. *Xiphinema* species inject their salivary secretions into the feeding cells and reabsorb the predigested cell content. Vector *Xiphinema* species acquire the virus when feeding on roots of virus-infected grapevines. The virus is retained by adhering on the surface of the cuticular lining in a specific region of the nematode esophagus (Fig. 1b), which allows its release by the saliva flow and thus its transfer to putative healthy grapes during subsequent feeding. Virus detection in the nematode relies on diverse techniques such as immunoassays [3], reverse transcription (RT) PCR [4], and real-time RT PCR [5].

Grapevines exhibiting degeneration symptoms provide optimal sites for sampling and identifying the presence of a *Xiphinema* sp. putative vector. In European vineyards, four morphologically and morphometrically close *Xiphinema* species may be detected: these are the vector species *X. index* (GFLV) and *X. diversicaudatum* (ArMV) previously mentioned and the nonvector species *X. italiae* and *X. vuittenezi*. For example, *X. index* and *X. diversicaudatum* can be confused, especially during the juvenile stages, with *X. vuittenezi*. This later species and *X. italiae* had been considered erroneously as vectors of *Grapevine chrome mosaic virus* (GCMV) and GFLV in grapevine, respectively [6]. *Xiphinema index* is present in many countries all around the world (as is GFLV) where it has been introduced from the Mediterranean vineyards with grapevine plants [7]. *Xiphinema diversicaudatum* is mainly distributed in western and northern European regions [8]. *Xiphinema vuittenezi* is prevalent in continental European regions [9], while *X. italiae* has a Mediterranean distribution area [10].

Thus, in European vineyards two or three *Xiphinema* species may be found together in the same grapevine plot and need to be accurately identified, primarily to establish the putative risk of virus transmission in the field. Moreover, the deep location and low field densities in the soil of the four *Xiphinema* species, *X. index*, *X. diversicaudatum*, *X. italiae*, and *X. vuittenezi*, make them difficult to identify from classical diagnostics when only juvenile stages or single or few adult individuals are detected. As only a single or a few individuals are sufficient to transmit the virus to a grapevine plant, the nematode detection and monitoring in contaminated fields require the development of techniques that are easy to use, reliable, and sensitive. In field as in pots under controlled conditions, the sole current way to characterize the vector and its effect relies on expert work for the detection and identification and on nematode counting, a highly time-consuming task.

Due to the specificity of virus transmission by its nematode vector, it remains crucial to distinguish the different species of *Xiphinema*. In recent years, the development of DNA-based molecular technology has provided attractive opportunities for improved plant nematode detection, identification, and systematics [11]. Real-time PCR allows to detect very low DNA quantities in diverse extracts and to quantify the amount of biological material (Fig. 1e). This article reports the development of the real-time PCR technique based on ribosomal DNA for the specific detection of *X. index*, *X. diversicaudatum*, *X. vuittenezi*, and *X. italiae*. This method has also been validated to estimate numbers of *X. index* in extracts from soil samples in a greenhouse assay on grape accessions of diverse host suitabilities. This was an example of its fast, sensitive, and reliable use as an alternative to the expert morphological measurements and time-consuming counting in the field or in experiments under controlled conditions.

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## 2 Materials

### 2.1 Soil Extraction Using Oostenbrink Method

1. Oostenbrink elutriator with a flowmeter (illustrated in Fig. 1d).
2. Sieve A (mesh 63 µm), sieve B (mesh 250 µm), sieve D (mesh 5 µm), 10 L buckets, conical measures.
3. Sieve C (mesh 1.5–2 mm). To prepare the extraction, put a facial tissue onto sieve C and wet it carefully in order to stick and stretch the tissue onto the sieve. Put the sieve into a bowl. Allow tap water to come up to the bottom of the sieve by adding water between the bowl and the side of the sieve.
4. Counting slide.
5. Stereo microscope.

## 2.2 Soil Extraction Using Alternative Method

1. Sieve A (mesh 63 µm), sieve D (mesh 5 µm), 10 L buckets, conical measures.
2. Sieve C (mesh 1.5–2 mm). To prepare the extraction, put a facial tissue onto sieve C and wet it carefully in order to stick and stretch the tissue onto the sieve. Put the sieve into a bowl. Allow tap water to come up to the bottom of the sieve by adding water between the bowl and the side of the sieve.
3. Counting slide.
4. Stereo microscope.

## 2.3 DNA Extraction

1. Sodium hydroxide solution: 0.5 M NaOH. Weigh 20 g NaOH, add 1 L of autoclaved water, and dissolve.
2. HCl solution: prepare 0.25 M HCl and add 10.3 mL of HCl concentrated 37 % in 489.7 mL of water.
3. Tris–HCl solution: prepare 200 mM Tris–HCl, pH 8. Weigh 24.2 g/L Tris and add water to a volume of 900 mL and dissolve. Adjust pH with HCl. Then make up to 1 L with water (*see Note 1*).
4. Triton X-100: 2 % solution in water.
5. Buffer A: Add 500 mL of 0.25 M HCl, 50 mL of 200 mM Tris–HCl pH8, 250 mL of Triton X-100 2 %, and 200 mL of water.
6. Lysis buffer: Mix 50 mL of Triton X-100 and 50 mL of 200 mM Tris–HCl pH 8 to 900 mL of water (*see Note 2*).
7. Proteinase K (Roche) 19.1 mg/mL.
8. Benchtop centrifuge.

## 2.4 Real-Time PCR

1. Positive reference material (*see Note 3*).
2. Molecular grade H<sub>2</sub>O.
3. Primers and TaqMan probes for *X. index*, *X. diversicaudatum*, *X. vuittenezi*, and *X. italiae* (*see Table 1*).
4. qPCR probe dilution buffer. 10 mM Tris–HCl, 1 mM EDTA, pH 8.
5. TaqMan Environmental Master Mix 2× (EMM 2.0) including ultrapure AmpliTaq Gold® DNA Polymerase, passive internal reference based on proprietary ROX™ dye, dNTPs with dUTP, optimized buffer components (Applied Biosystems).
6. MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems).
7. MicroAmp 96-Well Optical Adhesive Film (Applied Biosystems) or Optical Cap Strips.
8. Real-time PCR instrument (such as Applied Biosystems, 7900HT).

**Table 1**  
Primers and probes for real-time PCR

<i>Xiphinema</i> species	Forward primer	Reverse primer	TaqMan probe	Reporter dye	Amplicon size (bp)
SET 1 <i>X. diversicandatum</i>	Indiv-F TGAACCTGGAAAGGATCAT	Indiv-R TAGAGCCTAACCGGTAT	Indiv-div-VIC CGGTTTTTATCCGATCTGA GGCGAACCC	VIC	131
	<i>X. index</i>		Indiv-ind-FAM TTCTCGGTAAAATCTTGGGT CCGTGTGAA	FAM	107
<i>X. italiae</i>	Vuita-F CTGCGGAAGGATCATTATCGA	Vuita-R AAGGACAGTCGGTAGG TATCCA	Vuita-ita-VIC TAAAGGGTGGTAGAGGTCT ATCGGGGTTCTTCG	VIC	290
	<i>X. vittenezi</i>		Vuita-vui-FAM CGAGACTGATCGGTGGTCG AGTTTATTTCG	FAM	281
SET 2 <i>X. diversicandatum</i>	Div-F CGCGAATGTCACGGATCAAC	Div-R CCAAAGAAGGGAACGCTCCCT	Div-FAM TGACCTCGGGGATAAGTGTAA	FAM	176
	Ind-F ACGACCGCGTTAAGATTCGGT	Ind-R ACTAACATTCTCGTGCCTGGT	Ind-FAM TTCCCTGATCGTCTCCGGC	FAM	272
	<i>X. italiae</i>	Ita-F AGGGCCCGAAGACTAGTCGAT	Ita-FAM GCGTTTCTCGATAACCGC	FAM	137
	<i>X. vittenezi</i>	Vuit-F TCGATCGGAAAGACCGAAT	Vuit-FAM AACACACACGAGGAC	FAM	189
			CGCGAGAACGAGGTCGTAA		

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### 3 Methods

The methods described below outline extraction of nematodes from the soil (*see Subheadings 3.1 and 3.2*), total DNA extraction of the samples (*see Subheading 3.3*), and detection using real-time PCR (*see Subheading 3.4*). Ectoparasites can be extracted from the soil with these procedures. The undercurrent water stream has to be adjusted to 0.6 L/min for small nematodes and to 1.5–2 L/min for larger nematodes.

#### **3.1 Soil Extraction Using Oostenbrink Method**

1. Weigh maximum 2 kg of soil of the sample to test and homogenize it in approximately 5 L of water (*see Notes 4 and 5*).
2. Filter the sample through a fine mesh strainer (mesh approx. 1.5 mm) above a 10 L bucket and clean the remainder in the fine mesh strainer with a water jet.
3. Set the undercurrent water stream at 1.5 L/min allowing filling of the settling bulb with water (*see Note 6*).
4. When the water flows by the side exhaust, close it using a rubber plug and quickly pour the sample in the funnel of the elutriator.
5. Let the funnel fill with water and put a 10 L bucket below the side exhaust. When the elutriator is full, remove the plug from the side exhaust and let the bucket to fill.
6. Pass the whole content of the bucket through a sieve A (mesh 63 µm) (*see Note 7*).
7. Collect the material from the sieve A in a 500 mL conical measure by rinsing both sides.
8. Set a sieve B (mesh 250 µm) into a bowl and fill with tap water to approximately 1 cm above the bottom of the sieve.
9. Homogenize the content of the conical measure, and in the sieve B, pour the content carefully onto a spatula to spread the flow.
10. Mix gently and remove carefully the sieve B. Collect the material from the sieve B in a 500 mL conical measure by rinsing both sides, and pass the content of the conical measure through a sieve A in order to minimize the water quantity.
11. Gently transfer the content of the conical measure onto the extraction sieve C as described in Subheading 2.1 (*see Note 8*).
12. After 48 h, remove the sieve C and put it in a new bowl containing fresh water at the bottom; rinse the content of the bowl onto a sieve D (mesh 5 µm).
13. To ensure efficient nematode extraction, after 48 h, repeat from **step 12**.
14. Collect the material from the sieve D in a 15 mL corning by rinsing the sieve (*see Note 9*).

### **3.2 Soil Extraction Using Alternative Method**

1. Weigh maximum 0.2 kg of soil in a bucket and homogenize it in approximately 10 L of water (*see Notes 4 and 5*).
2. Agitate the solution vigorously with a spatula.
3. Let the solution rest for 1–2 min and transfer the supernatant onto sieve A (mesh 63 µm) (*see Note 7*).
4. Collect the material from the sieve A with a minimum of water in a 500 mL conical measure by rinsing both sides.
5. Add some water to the bucket and repeat **steps 2–4** twice.
6. Rinse the content of the conical measure onto a sieve D (mesh 5 µm) to concentrate the sample.
7. Gently transfer the content of the conical measure onto the extraction sieve C prepared as described in Subheading **2.1** (*see Note 8*).
8. After 48 h, remove the sieve C and place it in a new bowl containing fresh water at the bottom; rinse the content of the bowl onto a sieve D (mesh 5 µm).
9. To ensure efficient nematode extraction, after 48 h, repeat from **step 12**.
10. Collect the material from the sieve D in a 15 mL corning by rinsing the sieve (*see Note 9*).

### **3.3 DNA Extraction**

1. Centrifuge the sample obtained in Subheading **3.1** or **3.2** at  $3,000 \times g$  for 15 min (*see Notes 10 and 11*).
2. Pipette carefully the supernatant of the pellet allowing concentrating the sample in approximately 1 mL.
3. Add 1 mL NaOH 0.5 M and leave overnight at room temperature.
4. Heat the sample at 99 °C for 2 min.
5. Add 2 mL buffer A and mix the sample vigorously.
6. Heat the sample at 99 °C for 2 min, then mix the sample, and let the sample cool down at room temperature for 5 min.
7. Add 1 mL lysis buffer and 25 µL proteinase K. Mix the sample vigorously. The total volume is 5 mL approximately.
8. Heat the sample at 65 °C for 60 min and 95 °C for 10 min for proteinase K inactivation.
9. Centrifuge at  $3,000 \times g$  for 1 min to pellet the nematode fragments.

### **3.4 Real-Time PCR**

Real-time PCR master mixes should be prepared in a contamination-free environment. Positive controls (DNA extract of previously characterized nematode) and known negative control should be used such as molecular grade H<sub>2</sub>O and water coming from *Xiphinema*-free soil extraction to assess for contamination risks.

1. Make up the master mix as described in Table **2**, allowing for sufficient quantities to run each sample in triplicate.

**Table 2**

**Master mix reagents and final concentrations were appropriate for (A) multiplex detection using SET 1 (B) simplex detection using SET 2**

<b>(A) Reagent SET 1</b>		<b>Volume per reaction (<math>\mu\text{L}</math>)</b>	
		<b>Virus vector (<math>Xi/Xd</math>)</b>	<b>Non-virus vector (<math>Xv/Xita</math>)</b>
Molecular grade $\text{H}_2\text{O}$	8.5		8.5
TaqMan EMM 2×	12.5		12.5
Primer Indiv-F (7.5 pmol/ $\mu\text{L}$ )	1.0		–
Primer Indiv-R (7.5 pmol/ $\mu\text{L}$ )	1.0		–
Probe Indiv-div-VIC (5 pmol/ $\mu\text{L}$ )	0.5		–
Probe Indiv-ind-FAM (5 pmol/ $\mu\text{L}$ )	0.5		–
Primer Vuita-F (7.5 pmol/ $\mu\text{L}$ )	–		1.0
Primer Vuita-R (7.5 pmol/ $\mu\text{L}$ )	–		1.0
Probe Vuita-ita-VIC (5 pmol/ $\mu\text{L}$ )	–		0.5
Probe Vuita-vui-FAM (5 pmol/ $\mu\text{L}$ )	–		0.5
Sample (DNA extraction)	1.0		1.0

<b>(B) Reagent SET 2</b>		<b>Volume per reaction (<math>\mu\text{L}</math>)</b>			
		<b><math>Xi</math></b>	<b><math>Xd</math></b>	<b><math>Xv</math></b>	<b><math>Xita</math></b>
Molecular grade $\text{H}_2\text{O}$	9.0	9.0	9.0	9.0	9.0
TaqMan EMM 2×	12.5	12.5	12.5	12.5	12.5
Primer Div-F (7.5 pmol/ $\mu\text{L}$ )	–	1.0	–	–	–
Primer Div-R (7.5 pmol/ $\mu\text{L}$ )	–	1.0	–	–	–
Probe Div-FAM (5 pmol/ $\mu\text{L}$ )	–	0.5	–	–	–
Primer Ind-F (7.5 pmol/ $\mu\text{L}$ )	1.0	–	–	–	–
Primer Ind-R (7.5 pmol/ $\mu\text{L}$ )	1.0	–	–	–	–
Probe Ind-FAM (5 pmol/ $\mu\text{L}$ )	0.5	–	–	–	–
Primer Ita-F (7.5 pmol/ $\mu\text{L}$ )	–	–	–	–	1.0
Primer Ita-R (7.5 pmol/ $\mu\text{L}$ )	–	–	–	–	1.0
Probe Ita-FAM (5 pmol/ $\mu\text{L}$ )	–	–	–	–	0.5
Primer Vuit-F (7.5 pmol/ $\mu\text{L}$ )	–	–	–	1.0	–
Primer Vuit-R (7.5 pmol/ $\mu\text{L}$ )	–	–	–	1.0	–
Probe Vuit-FAM (5 pmol/ $\mu\text{L}$ )	–	–	0.5	–	–
Sample (DNA extraction)	1.0	1.0	1.0	1.0	1.0

2. Mix vigorously the nematode DNA extraction samples and centrifuge at  $3,000 \times g$  for 30 s.
3. Add 1  $\mu$ L sample (from the total DNA suspension obtained in Subheading 3.3) to 24  $\mu$ L TaqMan<sup>®</sup> reaction mix. The final volume is 25  $\mu$ L.
4. Seal the plate and perform amplifications in an Applied Biosystems 7900 HT Fast Real-time PCR system, under the following conditions: initial step at 50 °C for 2 min followed by 10 min at 95 °C and then 40 cycles at 95 °C for 15 s, 60 °C for 1 min.
5. Real-time PCR data analysis: After the PCR reaction, each amplification curve for each sample tested is analyzed and the threshold cycle number (*Ct* see Note 12) determined for each sample. A positive sample should have *Ct* values for all technical replicates above the set *Ct* threshold value as presented in Fig. 1e. Samples displaying *Ct* values below 37 are considered as positives.

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#### 4 Notes

1. Use a magnetic stir bar and warm the Tris solution to 37 °C to help the dissolution. Then use concentrated HCl (12N) to adjust the pH of the Tris–HCl to the required pH.
2. Buffer and stock solutions should be stored at 4 °C.
3. Positive controls are recommended for reference but not mandatory for experimental detection. Frozen specimen at -20 °C (-80 °C for long storage) can be used as positive control.
4. To increase the sampling efficiency and so detect ectoparasite nematodes, it is required to sample the soil close to the roots.
5. The method outlined here details the extraction of the nematode from soil samples. Usually *Xiphinema* are found in small numbers per kg.
6. The undercurrent water stream can be reduced to 0.6 L/min to extract smaller nematodes (e.g., *Pratylenchus*).
7. As the nematodes are present in the supernatant, avoid transferring the soil onto the sieve which could saturate the sieve and affect the quality of the samples. The presence of a higher amount of organic matter could interfere with the subsequent DNA extraction and real-time PCR experiments.
8. This method is interesting because it ensures the detection and quantification of living nematodes as only living nematodes will pass through the facial tissue.
9. To have an idea of the nematode diversity and quantity in the sample, collect 1 mL of the sample in a counting slide, and observe it with a stereo microscope.

10. Individuals can be picked manually and individually tested adjusting the volume of the DNA extraction to increase the detection sensitivity.
11. For individuals picked manually, adjust the condition as follows—put one or a few nematodes in 50 µL of water and follow the protocol mentioned in Subheading 3.3 with the following modifications: 50 µL NaOH 0.5 M; 100 µL Buffer A; 50 µL Lysis buffer; 2 µL proteinase K.
12. Vaerman et al. [12] defined *Ct* values as “fractional cycle numbers where amplification fluorescence levels reach a fixed threshold.”

## Acknowledgments

This study was made possible by the support of SASA (Science and Advice for Scottish Agriculture) facilities (Edinburgh, Scotland, UK) through the “Diagnostics, Wildlife & Molecular Biology” section.

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# Chapter 13

## Molecular and Serological Methods for the Diagnosis of Viruses in Potato Tubers

Christophe Lacomme, Ross Holmes, and Fiona Evans

### Abstract

Viruses cause important diseases to potato crops. Monitoring virus content in plant material for quarantine or seed certification scheme purposes is essential to prevent the spread of viruses and to minimize the impact of viral diseases. There are currently two main methods for virus diagnosis in potato tubers: growing-on ELISA testing which requires breaking tuber dormancy followed by an ELISA test on grown plantlets and direct real-time RT-PCR testing on tubers. This chapter will describe both methods that can be adapted for large-scale virus testing activities.

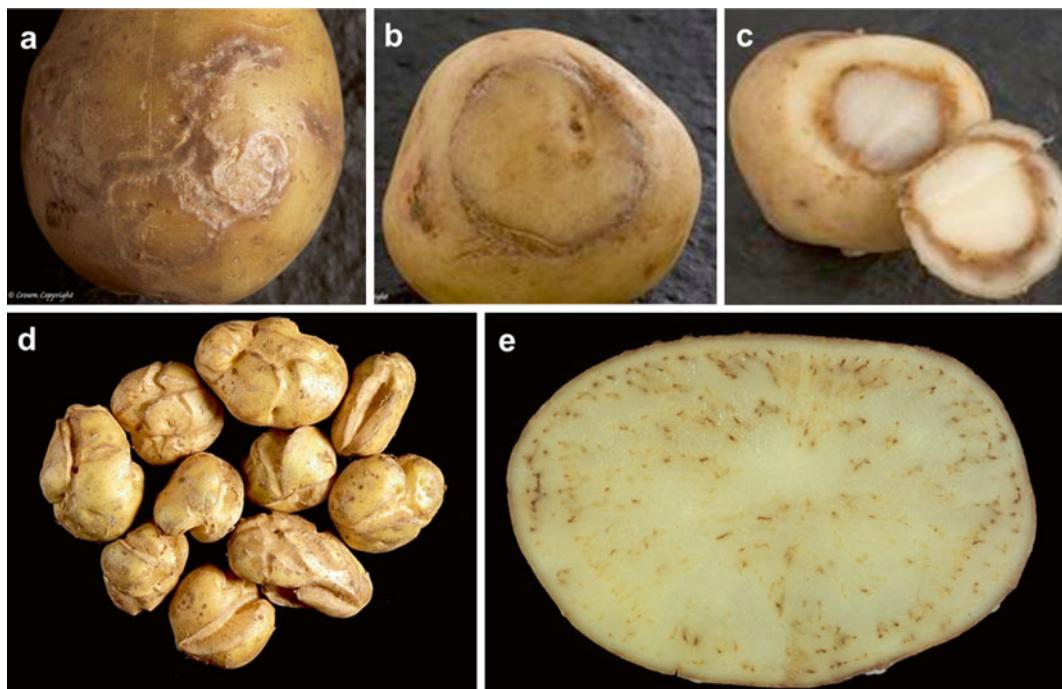
**Key words** Virus, Potato tubers, Growing-on, DAS-ELISA, Real-time RT-PCR

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### 1 Introduction

Currently, potato is the fourth major food crop worldwide increasing from 267 million tonnes in 1990 to 365 million tonnes in 2012 [1]. There are about 40 viruses that can infect cultivated potato; most of them are single-stranded positive-sense RNA viruses. Only a few viruses such as *Potato leaf roll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus A* (PVA), *Potato virus X* (PVX), and *Tobacco rattle virus* (TRV) can be found in most potato-growing areas worldwide, while the large majority are either more locally distributed or only occasionally found in cultivated potatoes (such as *Potato yellow dwarf virus* and *Potato yellow vein virus*, respectively, found in North America or South America) [2]. Many virus diseases of potato cause significant damages by either reducing crop yields, affecting tuber marketability (alteration of tuber shape and appearance), or rendering seed crop unsalable by failing to meet set tolerance level [2] (Fig. 1).

The most common potato-infecting viruses can be transmitted by a wide range of vectors including aphids (PLRV, PVA, *Potato virus V* PVV, *Potato virus S* PVS, *Potato virus M* PVM, PVY), free-living nematodes (TRV), and fungi (*Potato mop-top virus*



**Fig. 1** Tuber symptoms caused by potato viruses: (a) potato tuber necrotic ring spot disease (PTNRD) caused by PVY; (b, c) spraina caused by TRV or PMTV; (d) growth cracks caused by some potyviruses such as PVA, PVW, or PVY; and (e) net necrosis caused by PLRV

PMTV) or even mechanically by simple contact between plants (PVX). The large majority of potato-infecting viruses are aphid-transmitted belonging to different genera including *Carlavirus* (PVM, PVS), *Poliovirus* (PLRV), and *Potyvirus* (PVA, PVV, and PVY). They can be transmitted from plant to plant by aphids either in a persistent manner (e.g., PLRV and the peach potato aphid *Myzus persicae* Sulzer) or a non-persistent manner (*Carlavirus* and *Potyvirus* by more than 40 different colonizing and non-colonizing aphid species) [3]. Persistently transmitted virus, such as PLRV (a phloem-restricted virus), is acquired by the colonizing aphid *M. persicae* during feeding of phloem sap (within 30 min to 1 h) and transmitted to another plant during another feeding episode. The aphid will remain infectious and transmit PLRV during its whole life cycle [3]. While PLRV impact on potato production has significantly diminished over the past 20 years in countries that have developed seed certification program and efficient management measures (such as planting resistant varieties, developing efficient aphid monitoring and control using aphicide treatment), it is still a significant problem in developing countries [2]. Non-persistently transmitted viruses are distributed both in most cell layers of the plant and are transiently acquired and transmitted by a wide range of aphid species during leaf probing between plants [3]. This short

process (occurring within few minutes) makes non-persistent viruses and their vectors more difficult to control. PVY is a non-persistent virus and is widely recognized as the main virus causing seed potato rejection worldwide including North America and mainland Europe [2, 4]. PVY infection can dramatically affect yields (yield reduction can range up to 30 % to more than 60 %) depending on the potato cultivar, the source of infection (i.e., aphid borne or seed borne), and environmental conditions [5, 6].

Management of viruses is therefore important to ensure their impact on productivity and crop health is minimized. This requires the use of healthy virus-free potato seed, planting resistant varieties whenever possible, adopting prophylactic measures to control insect vectors and virus transmission (i.e., spraying insecticide and mineral oil), aphid monitoring, and surveying virus incidence. In many certification schemes, postharvest tuber testing of a sample of the crop is undertaken as a means to assess virus incidence and to determine if virus incidence falls within set tolerances for a given field generation. Diagnosis of viruses in dormant potato tubers is achieved in most of the cases either by growing-on DAS-ELISA or real-time RT-PCR methods. While growing-on DAS-ELISA is still widely used due to its relative simplicity, cost-efficiency, and robustness, it requires vast glasshouse space and a relatively long testing time (about 3–4 weeks), mainly for breaking tuber dormancy. Contrastingly, while real-time RT-PCR reagents are more costly, due to its higher detection sensitivity [7], real-time RT-PCR can be directly applied to tuber material on larger tuber bulks in multiplex assays reducing costs and shortening considerably testing time down to a couple of days.

The present chapter aims to provide the necessary information to apply both methods for the diagnosis of the main potato-infecting viruses (i.e., PLRV, PMTV, PVA, PVV, PVX, PVY, and TRV) that can be found in most potato-growing areas worldwide.

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## 2 Materials and Equipment

### 2.1 Samples and Controls

1. Potato tubers (*see Note 1*).
2. Leaves from infected plants to be used as positive control for the tested viruses.
3. Leaves from noninfected plants to be used as negative control.

### 2.2 RNA Extraction and Real-Time PCR

1. Potato tuber peelers.
2. Single-use homogenization bags (Bioreba).
3. Tissue homogenizer (Homex 6, Bioreba).
4. Set of pipettes and single-use tips.

5. Reagent and buffers for RNA extraction [8] (*see Note 2*):

- Grinding buffer: 1.5 % lithium dodecyl sulfate, 200 mM Tris-HCl, pH 8.5, 10 mM EDTA, 300 mM NaCl, 1 % sodium deoxycholate, 1 % Igepal CA-630, Antifoam B 0.05 %. 10 mM DTT and 5 mM thiourea are added prior to use.
  - 6 M potassium acetate (pH 6.5) (Sigma).
  - 4 M lithium chloride (Sigma).
  - Ethanol 70 % (Sigma).
6. Sterile/nuclease-free Eppendorf tubes.
7. Benchtop centrifuges for Eppendorf tubes.
8. Vortex mixer.
9. MicroAmp® Optical 96- or 384-Well Reaction Plates (Applied Biosystems).
10. MicroAmp® 96- and 384-Well Optical Adhesive Film (Applied Biosystems).
11. 2× JumpStart™ Taq ReadyMix™ (Sigma).
12. Forward and reverse primers and dual-labeled qPCR probes (Table 1).
13. Reverse transcriptase (M-MLV RT, RNA-dependent DNA polymerase, Promega).
14. Real-time thermal cycler (Applied Biosystems 7900HT or equivalent).
15. Molecular biology grade water (nucleic acid- and nuclease-free, Sigma) for RNA resuspension, real-time RT-PCR mix, and non-template control (NTC).
16. Software (e.g., SDS Software v2.4 for the Applied Biosystems 7900HT) for real-time PCR analysis.
17. Optional: MICROLAB® STARlet Liquid Handling Workstation (Hamilton Robotics), laminar flow cabinet.

### **2.3 Growing-On DAS-ELISA**

1. Melon scoop.
2. Water boilers.
3. Plastic seed trays (e.g., 240 mm × 380 mm × 60 mm depth or equivalent).
4. Compost mix for growing potato plantlets (80 l multipurpose compost, 25 g insecticide Intercept® 5GR, 4 l of perlite).
5. Gibberellic acid solution (final concentration 1 mg/l in purified water). Prepare a stock solution 100 mg/l, store at 3–6 °C up to 2 months. Dilute the stock solution in water 100-fold for eye-plug treatment, and use sufficient volume to cover all eye-plug bulks in each container.

**Table 1**  
**Sequence of primers and probes for detection of listed viruses and endogenous plant nucleic acids/RNA extraction control (see Notes 3–5)**

Assay	Primer/probe	Sequence	Virus/plant target gene	References
<i>Potato leaf roll virus</i> (PLRV)	PLRV-FOR PLRV-REV PLRV-Probe	GGCAATGCCGCTCAA TGTAAACACGAATGTCGCTTG CCTCGTCCTCGGGGAACTCCAGTT	Coat protein (CP)	[9]
<i>Potato mop-top virus</i> (PMTV)	PMTV-1948 F PMTV-2017 R PMTV1970-Probe	GTGATCAGATCCGGTCCT CCACTGAAAAGAACCGATTC ACCAGAACCTACGGTGCCGGTGC	PMTV RNA2, CP- read-through protein	[10]
<i>Potato virus A</i> (PVA)	PVA-FWD PVA-REV PVA-Probe	AGGTACTGCTGGGACTCATTCAG RACACTCTTACCTTGAGCATGG (R=A/G) CCACGCTTAAATCAATGACATCAA AAACTGACACT	CP	
<i>Potato virus V</i> (PVV)	PVV-FWD PVV-REV PVV-Probe	GAACAACCTGGCACGGTCACAA CCTTTGGTTGGCGCAATT CCGGCGCAATCAAGGCGATYTC (Y=C/T)	CP	[11, 12]
<i>Potato virus X</i> (PVX)	PVX 101-F PVX 101-R PVX 101-Probe	AAGCCTGAGCACAAATTGCG GCTTCAGACGGTGGCCG AATGGAGTCACCAACCCAGCTGCC	CP	
<i>Potato virus Y</i> (PVY)	PVY 411-F PVY 4/77-R PVY-Probe	GGGCTTAGGGTTGGTGCA CCGTCTATAACCCAACACTCCG TGAAAATGGAACCTCGCCAATGTCA	CP	[9]
<i>Tobacco rattle virus</i> (TRV)	TRV-1466 F TRV-1553R TRV-1489-Probe	CATGCTAACAAATTGCGAAAGC TACAGACAAACCATCCACAATTATT ACGTGTGACACCAACCATGTCAACT	TRV RNA1, 16 K gene	[10]
COX	COX-F COX-R COXSOL1511T-Probe	CGTCGCATTCCAGATTATCCA CAACTACGGATATAAGRRCRRAACTG AGGGCATTCATCCAGCTGAAGCA	<i>Cytochrome c oxidase</i> (nucleic acid extraction control)	[9]
NAD5	St-NAD-F St-NAD-REV St-NAD-Probe	GATGCTTCTGGGCTTCTGTT CTCCAGTCACCAACATTGGCATAA AGGATCCGCATAGCCCTCGATTATGTG	<i>NADH dehydrogenase</i> 5 (RNA extraction control)	[13, 14]

6. Insect-free temperature-controlled glasshouses (22–18 °C day-night, 16 h photoperiod).
7. Fume cabinet.
8. Tissue homogenizer (Homex 6, Bioreba) and single-use homogenization bags (Bioreba).
9. Microplate washer (MEKU, Erich Pollahne GmbH).
10. Set of pipettes, multichannel pipettes, and single-use tips.
11. Coating antibodies and corresponding conjugated antibodies coupled with alkaline phosphatase (commercially available monoclonal and polyclonal antibodies) against selected potato-infecting viruses: PLRV, PMTV, PVA, PVV, PVX, PVY<sup>O</sup>, PVY<sup>C</sup>, and PVY<sup>N</sup> (*see Note 6*).
12. Microtiter plates (Nunc MaxiSorp® 96-well plate).
13. Reagents and buffers for DAS-ELISA:
  - Wash buffer (PBST): NaCl 8 g, KH<sub>2</sub>PO<sub>4</sub> 0.2 g, Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 2.9 g, KCl 0.2 g. Dissolve in 1 l of purified water, and add 0.5 ml Tween 20. Store at room temperature. This buffer is used for washing microplates and for the preparation of extraction and conjugate buffers.
  - Extraction buffer for tuber tissue: Use PBST prepared as mentioned above. Weigh out 10 g egg albumin (ovalbumin), place in a flask with a magnetic stirrer, and mix with a small quantity of PBST. In a fume cabinet, add 20 g polyvinylpyrrolidone (PVP, MW 40,000) and dissolve in a small quantity of PBST. Add the egg albumin and make up to 1 l with PBST. Store at 3–6 °C.
  - Coating buffer: Dissolve 4.3 g Na<sub>2</sub>CO<sub>3</sub>·10H<sub>2</sub>O (sodium carbonate) and 2.9 g NaHCO<sub>3</sub> (sodium hydrogen carbonate) and adjust to 1 l of purified water. Store at 3–6 °C, and use within 2 days of preparation. This buffer is used for dilution of the first antibody for coating microtiter plates.
  - Conjugate buffer: Weigh out 0.2 g BSA (bovine serum albumin) and 2 g PVP (MW 40,000) in a fume cabinet. Add a small volume of PBST and dissolve using a magnetic stirrer. Make up to 100 ml with PBST. Store at 3–6 °C.
  - Substrate buffer: In a fume cabinet, dissolve 97 ml of diethanolamine in 800 ml of purified water and mix thoroughly using a magnetic stirrer. Adjust pH to 9.8 with concentrated HCl, and add purified water to 1 l. Store at 2–8 °C. Prior to use, add *p*-nitrophenylphosphate (pNPP, Sigma) to a final concentration 1 mg/ml to make up substrate for alkaline phosphatase.

14. ELISA microplate reader (Tecan Sunrise®, Infinite 500 or equivalent using XFluor program) for absorbance reading at OD<sub>405nm</sub>.

### 3 Methods

#### 3.1 Real-Time

##### *RT-PCR Virus*

##### *Diagnosis Method*

###### 3.1.1 Sampling of Tubers

Random Sampling of Tuber  
Tissue to Estimate  
Incidence of Viruses  
in a Sample

Sampling Symptomatic  
Tuber Tissues (Spraing or  
Tuber Necrotic Ring Spot  
Disease)

###### 3.1.2 RNA Extraction

1. Usually a 100–200 tuber sample is drawn from a crop (*see Note 1*).
2. Take off excess soil (brushing off or a brief wash).
3. Using a clean potato peeler, peel off skin (~2–3 mm thick) from the rose end (main eye) and the heel end (stolon end) of each tuber (*see Note 7*).
4. Proceed to the next tuber and for the whole bulk of 10 tubers. Place the peelings in a single labeled homogenization bag.
5. Place bag aside or store at –20 °C until homogenization.
6. Take a clean peeler (*see Note 8*) and proceed to the next tuber bulk.
1. Identify symptomatic tubers (Fig. 1). Spraing caused by TRV or PMTV appears as brown necrotic arcs or lines in the tissue (Fig. 1 b, c). Symptomatic tubers should be cut along the necrotic zone to confirm that necrotic arcs are observed in the flesh.
2. If necrotic arcs are present in the tuber flesh (Fig. 1b, c), flesh tissues should be taken from the necrotic area using a cleaned knife. Place sampled tissue in a homogenization bag.
3. If superficial necrosis is found (Fig. 1a), sample symptomatic tissues (tuber peelings from the symptomatic areas including rose end and heel end) using a potato peeler. Place peelings in a homogenization bag.
1. Freshly prepared tuber peelings are placed in a homogenization bag, or alternatively, peelings in homogenization bags are thawed just before grinding.
2. Add 3 ml of grinding buffer (*see Subheading 2.2*).
3. Grind thoroughly using a homogenizer.
4. Remove 500 µl of the homogenized sample and transfer into a labeled 1.5 ml microtube.
5. Add 500 µl of 6 M potassium acetate. Mix well by inverting and incubate on ice for 15 min.
6. Centrifuge at 16,000 ×*g* for 15 min.
7. Remove 700 µl of supernatant and transfer into a sterile labeled microtube.

8. Add 700  $\mu$ l of 4 M lithium chloride (LiCl). Mix well by inversion and incubate at 4 °C overnight.
9. Centrifuge at 16,000  $\times g$  for 25–30 min to pellet RNA.
10. Pour off supernatant into a suitable chemical waste container. Invert tube on absorbent paper and let stand to dry the pellet for few minutes.
11. Wash pellet in 250–300  $\mu$ l of 70 % ethanol. Vortex briefly to ensure the pellet is unstuck from the bottom of the tube.
12. Centrifuge at 16,000  $\times g$  for 10–20 min.
13. Discard supernatant. Invert tube and let stand for few minutes on absorbent paper to dry.
14. Add 75 to 100  $\mu$ l of nuclease-free water to the pellet. Vortex for 5–10 min to resuspend RNA (*see Note 9*). Sample can then be kept on ice or stored at –20 °C until use.

### 3.1.3 Real-Time RT-PCR

1. Prepare a master mix for each one-step real-time RT-PCR virus diagnosis assay, including an RNA/nucleic acid extraction control assay (singleplex or multiplex) all in triplicate (*see Note 10*). An example of real-time RT-PCR setup is presented in Table 2.
2. Multiply volumes according to the number of required wells with an extra 10–15 % or as necessary to compensate for pipetting error or to account for dead volume during automated liquid handling.
3. Set up the real-time RT-PCR in a 96-well or a 384-well plate with 8  $\mu$ l of master mix per well. Each plate should at least include non-template control (NTC), positive amplification controls (PAC) and test samples each in triplicate. Master mixes for a combination of primers and probes should be used to diagnose selected target virus species as well as endogenous

**Table 2**  
**Standard reaction conditions for one-step real-time RT-PCR assays**

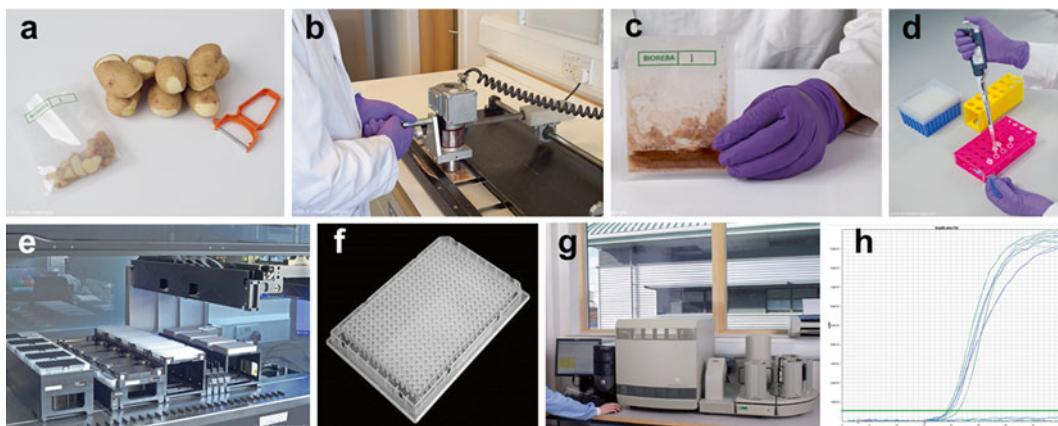
Reaction component	Concentration of stock	Volume per well ( $\mu$ l) (total volume 10 $\mu$ l)	Final concentration
Primer—FWD	7.5 $\mu$ M	0.4	300 nM
Primer—REV	7.5 $\mu$ M	0.4	300 nM
Probe	5 $\mu$ M	0.2	100 nM
Real-time PCR master mix	2×	5	1×
M-MLV RTase	200 U/ $\mu$ l	0.02	0.4 U/ $\mu$ l
ddH <sub>2</sub> O		1.98	
RNA		2	

plant target gene control (Internal Positive Control - IPC) to monitor efficacy of nucleic acid extraction.

4. Add 2  $\mu$ l of RNA sample per well. Seal the plate with optical adhesive film. Pulse spin the plate for 1 min, at  $1,000 \times g$ , and load the plate onto the real-time PCR cycler.
5. Launch the SDS Software, and complete plate template window, RT-PCR conditions, detectors with relevant fluorophores, and quenchers selected for each probe used. Real-time RT-PCRs are run using standard cycling conditions on the 7900HT (Applied Biosystems). Cycling conditions are as follows: 48 °C, 30 min, and 95 °C, 10 min; 40 cycles of 95 °C, 15 s, and 60 °C, 1 min.

### 3.1.4 Real-Time RT-PCR Data Analysis

1. Once the real-time RT-PCR run is completed, analyze the results using SDS Software. Results are collected ensuring that amplification baseline and threshold values have been appropriately selected so that a characteristic amplification curve (at least baseline and exponential phases) is observed (Fig. 2h). The SDS Software will calculate the cycle threshold ( $C_T$ ) value for each positive sample.
2. A real-time RT-PCR run is conclusive if the following conditions are met:
  - No amplification of target virus should be observed in NTC and negative control samples.
  - Amplification of target virus in positive control samples.



**Fig. 2** Steps involved in real-time RT-PCR diagnosis: (a) freshly prepared tuber peelings (at least heel end and rose end) are placed in a homogenization bag; (b, c) grind thoroughly using a homogenizer; (d) proceed with the rest of the RNA extraction process; (e, f) optional, aliquot the real-time RT-PCR reagents, RNA samples, and suitable controls in a 384-well plate using a liquid handling workstation; and (g, h) proceed with the real-time RT-PCR cycling and analyze results

- Amplification of endogenous target gene (*COX* or *NAD5*, see Table 2) for all tested samples including negative control and positive control in triplicate ( $C_T$  value above set threshold less than 40).
- Once all controls have generated appropriate results, positive- and/or negative-tested samples can be determined.

### **3.2 Growing-on DAS-ELISA Virus Diagnosis Method**

#### **3.2.1 Sampling of Potato Tubers**

At all stages, tubers, eye-plug bulks, trays, and plants are suitably labeled to ensure traceability.

1. Select 100–200 tubers from a randomly taken sample from a crop (see Note 1).
2. Eye plugs are removed from the rose end (sprouting end) of each tuber using a melon scoop (Fig. 3a, b).
3. Eye plugs are placed in a jar and immersed with 1  $\mu$ M gibberellic acid solution for 10 min. Eye plugs are taken out and allowed to dry overnight on labeled absorbent paper for traceability (Fig. 3c). The remaining tubers should be stored until testing is completed.
4. Dispose eye plugs in seed trays (Fig. 3d) and cover with compost. To aid subsequent testing, plugs are planted in rows of four eye plugs, seven rows to a seed tray (28 eye plugs per tray).



**Fig. 3** Steps involved in growing-on DAS-ELISA diagnosis: (a, b) remove eye plugs from the tuber rose end; (c) once treated with gibberellic acid, allow eye plugs to dry; (d) dispose eye plugs in seed trays ensuring individual bulks are separated and sample identification traceable and cover with compost; (e) once plantlets have reached suitable developmental stage, harvest leaves for each bulk in a homogenization bag; and (f, g) proceed with the ELISA test, and analyze results

5. Grow plantlets in a glasshouse (16-h photoperiod, 20 °C) (*see Note 11*).
6. After approximately 3–4 weeks growth, plantlets should be ready for testing by DAS-ELISA. Testing should be carried out when plantlets are approximately 20–30 cm tall (*see Note 12*).
7. Retain parent tubers and plantlets until ELISA test is completed.

### 3.2.2 DAS-ELISA

#### Plate Coating

1. Dilute coating antibody in coating buffer according to the supplier's recommendations (often between 1:100 and 1:1,000 dilution).
2. Mix well, and dispense a 200 µl aliquot of the diluted antibody into each well (*see Note 13*).
3. Place plates in boxes lined with moist tissue paper, cover top plate, put the lid on the box, and incubate at 37±2 °C for 4 h or overnight at 2–8 °C.
4. Wash plates thoroughly. To remove excess moisture, invert plate and pat dry on a pad of tissue paper.
5. Plates can be used immediately or placed in polythene bags and store at –20 °C.

#### Sample Preparation

1. Testing is carried out on bulked samples of four eye plugs: Harvest one leaf (one compound leaf near the apex) per plant for each bulk (usually four plants, i.e., 1 row of 4 plantlets equates to 1 bulk “sample”). Place the 4 leaves in a homogenization bag.
2. Grind up plant material in 5 ml of extraction buffer. For leaf material, add a further 5 ml of leaf extraction buffer depending on the number of viruses to be tested for.
3. Add 200 µl aliquots to allotted wells (at least two wells per sample).
4. Include positive control and negative control. Negative control material must be of the same type as the material under test.
5. 200 µl of leaf tissue of checked positive controls (potato leaf from infected plant with tested virus).
6. Incubate overnight at 2–8 °C.

#### Addition of Antibody Conjugate

1. Wash plates thoroughly (2×3 s, turning plates between washes) and pat dry.
2. Dilute antibody conjugate in conjugate buffer as recommended. Mix well and add 200 µl aliquots of the appropriate conjugate to each well using a multichannel pipette.

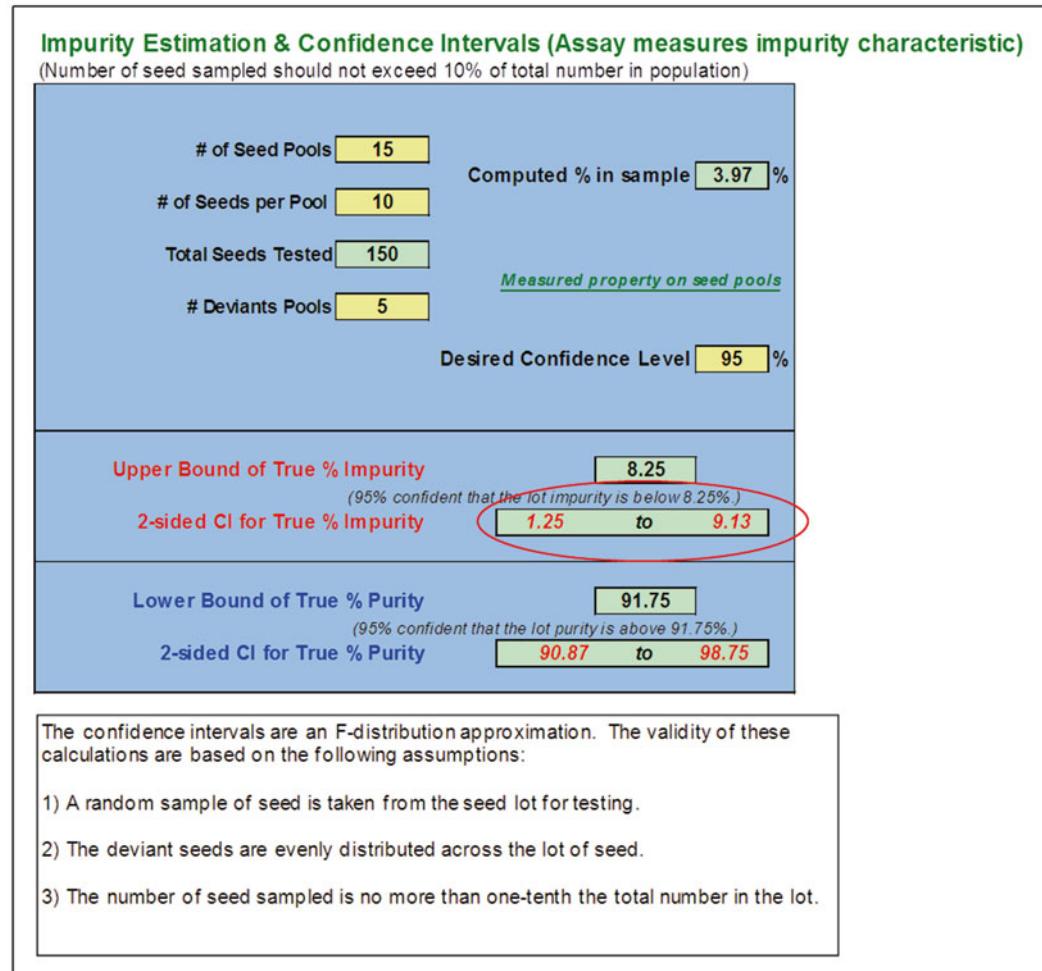
3. Place plates in boxes lined with moist tissue paper, cover top plate, put the lid on the box, and incubate plates at  $37\pm 2$  °C for 2 h.
- Addition of Substrate**
1. Wash plates thoroughly (turning plates between washes) and pat dry.
  2. Prepare substrate buffer. Add 200 µl aliquots to each well and incubate plates at room temperature.
- ELISA Plate Reading and Determination of Positives**
1. Read the absorbance levels at 405 nm using a plate reader.
  2. Record absorbance values at 405 nm after 1 h or longer if necessary.
    - Ideally, an OD<sub>405nm</sub> value for controls of at least 2.0 within 2 h of addition of substrate should be obtained.
    - If the positive or negative control samples fail to give the expected result, the test must be repeated using alternative control material.
  3. A positive threshold is set at a value of OD<sub>405nm</sub> >twofold the negative control.

### 3.3 Analysis

Analysis and expression of results depends on the type of sampling method used. If the test is aimed at identifying the nature of the virus species on individual tubers (i.e., “spot test” on symptomatic tuber samples), virus species (single or multiple mixed infection) found for each tuber tested are reported.

Alternatively, bulked analysis of a subset of randomly drawn tuber samples can be performed to estimate virus incidence (e.g., for seed certification classification purposes), to determine whether virus incidence of seed potato stocks falls within set tolerance levels (*see Note 14*).

1. Download the “Seedcalc8” software or equivalent from the International Seed Testing Association (ISTA) website <http://www.seedtest.org/en/statistical-tools-for-seed-testing-content---1--1143--279.html>.
2. Select worksheet “Quality Impurity Estimation.” Enter values in yellow boxes (Fig. 4) as follows:
  - (a) “# of Seed Pools” = number of seed bulks tested (usually between 10 and 20).
  - (b) “# of Seeds per Pool” = size of each bulk of tubers/plantlets (usually 4 plantlets for growing-on DAS-ELISA or 10 tubers for real-time RT-PCR).
  - (c) “# of Deviants Pools” = number of positive bulks for a single virus species or number of positive bulks for all virus species tested.



**Fig. 4** Example of *Seedcalc8* output for the estimation of virus incidence in bulked samples. In this example, five virus-positive bulks were identified from a total of 15 bulks of 10 tubers tested, giving a computed virus incidence of 3.97 % (confidence limit interval 95 % = 1.25–9.13 %) (circled in red)

3. The “Computed % age of virus in sample” should appear in the green box (Fig. 4 top right) as for the lower and upper confidence limit intervals at 95 % (Fig. 4 circled in red).

#### 4 Notes

1. Virus indexing can either be performed on individual symptomatic tubers by sampling the affected tissues or by drawing a random pool of tubers (usually between 100 and 200 tubers). Further guidance can be found in [http://www.unece.org/file/admin/DAM/trade/agr/standard/potatoes/S-1\\_2011\\_E.pdf](http://www.unece.org/file/admin/DAM/trade/agr/standard/potatoes/S-1_2011_E.pdf).

[pdfUNECE](#) UNECE Standard S-1 for seed potatoes, Edition 2005—Annex VI “Organizing the inspection of crops grown from sample lots of seed potatoes” and Annex IX “Sampling for virus testing.”

2. The RNA extraction method presented here is amenable for RNA extraction of tuber peelings from a large number of samples, as it does not require the use of liquid nitrogen for homogenization and of spin columns for nucleic acid purification which are likely to be clogged due to the presence of tissue debris in the homogenate.
3. The molecular diversity of PVY genome is extremely high, and an increasing number of PVY recombinant molecular types are being described [4, 15]. From our experience, the PVY primers and probes [9] can detect all PVY<sup>O</sup>, PVY<sup>N-Wilga</sup>, PVY<sup>EU-NTN</sup>, and PVY<sup>NA-NTN</sup> isolates tested so far; however, it is recommended to validate the use of this test (either *in silico* or experimentally) for a suitable number of PVY isolates that might be found in specific geographic areas where cultivated potatoes are being surveyed.
4. Due to the high variability of TRV RNA2 genomes and the occurrence of coat protein-less TRV NM strains lacking RNA2 (for a review see [16]), this implies that TRV NM variants cannot be detected by serological methods. Real-time RT-PCR is therefore recommended for accurate TRV diagnosis, as the selected primers and probes target the TRV RNA1 16 K gene.
5. Primers and probes should be validated to ensure minimum test performance criteria are observed and when necessary comply with relevant ISO standards. Guidance can be found in EPPO standard PM 7/98 [17]. Dual-labeled probes (5'-fluorophore/3'-nonfluorescent quencher) should be protected from light and thawed just before use.
6. Validated or commercially available source of antibodies should be always used. Diagnostic product manufacturers (Adgen; Agdia; Bioreba; DSMZ, Germany; Loewe Biochemica GmbH, Germany; Neogen Europe Ltd., UK; SASA, UK) supply ELISA reagents which include monoclonal coating and conjugated antibodies for a wide range of viruses (such as PLRV, PMTV, PVA, PVM, PVS, PVV, PVX, PVY<sup>C</sup>, PVY<sup>O</sup>, PVY<sup>N</sup>, etc.).
7. While virus distribution in tubers might vary depending on the virus species or the type of infection (early or late primary infection, seed-borne infection), tuber peelings were found to be suitable material for the diagnosis of a range of viruses such as PVY (all strains), PVA, PVV, PVX, PLRV, PMTV, TRV, PVS, and TSWV (this study, [12]).
8. To avoid risks of cross-contamination, gloves should be changed between each bulk. Decontamination methods have

been previously described [9]. As an alternative, potato peelers/knives can be washed with cleaning solution Micro-90 (Cole-Parmer), rinsed, immersed in boiling water for 5 min, and dried prior to use. A clean knife/peeler should be used between each bulk.

9. If insoluble matter has been carried out, pulse centrifuge to pellet insolubilized material, and pipette the supernatant.
10. Precautions should be taken to avoid contamination of samples and reagents. Ensure all reagents are thawed, mixed, and spun briefly before aliquoting. Some real-time PCR master mix will have different concentrations of MgCl<sub>2</sub>. Ensure that the final MgCl<sub>2</sub> concentration is within 4–6 mM or adjusted accordingly for optimal amplification of selected target(s) template.
11. Watering should be done sparingly until emergence to prevent rotting.
12. Premature testing on small plantlets can generate unreliable results; allow plantlets to develop sufficiently to display a suitable number of leaves.
13. Special care must be taken to prevent contamination of neighboring wells when adding the homogenate. Accurate pipetting with multichannel pipettes at every stage is required to ensure reagent volume is equally dispensed in all wells.
14. Examples of this testing regime have been described in the UNECE Standard S-1 for seed potatoes, Edition 2005—Annex IX “Sampling for virus testing” [18]. The testing regime presented allows the estimation of virus incidence (within a recommended confidence limit interval of 95 %). This process makes the assumption that (a) infected tubers are distributed homogeneously in the stock, (b) tubers are sampled randomly, and (c) the total of tuber samples does not exceed 10 % of the total tuber number. Validation of each testing method in relation to bulk size should be undertaken.

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# Chapter 14

## Immunocapture-Multiplex RT-PCR for the Simultaneous Detection and Identification of Plant Viruses and Their Strains: Study Case, Potato Virus Y (PVY)

Mohamad Chikh-Ali and Alexander V. Karasev

### Abstract

Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) is a sensitive, reproducible, and robust method for the detection and identification of RNA viruses. The IC step provides a simple method to isolate virus particles from plant tissue, particularly when inhibitory substances are present, and thus enables subsequent use of RT-PCR amplification for large-scale virus testing and typing. The multiplex format of the PCR is often used for the detection and identification of multiple virus/strain simultaneously to save time, labor, and cost. *Potato virus Y* (PVY) is one of the most economically important viruses infecting potato worldwide. PVY exists as a complex of at least nine strains and many more unclassified recombinants that vary in their genome structures, phenotypes, and their economic importance. In the current chapter, a detailed protocol of an IC-based, multiplex RT-PCR assay for the detection and identification of various PVY strains is described.

**Key words** Immunocapture, Potato virus Y, RT-PCR, Strain, Identification

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### 1 Introduction

Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) is a hybrid method that relies on the serological and genomic properties of a virus for the accurate and sensitive detection of RNA viruses [1, 2]. At the IC step, virus-specific polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs) can be used to selectively capture virus particles and isolate them from plant tissue and various, often inhibitory substances [1, 2]. At the subsequent RT-PCR step, specific primers targeting the genome RNA of plant viruses are used to produce complementary DNA (cDNA) by reverse transcriptase and amplify target regions using DNA polymerase [1, 2]. The combination of virus-specific antibodies in the IC step and specific primers in the RT-PCR step leads to an enhanced specificity of the test compared to the RT-PCR assay

alone that uses conventional nucleic acid extraction techniques to isolate total RNAs containing virus RNA and plant-derived RNAs. The use of IC helps to isolate virus particles from plant tissue that often contains inhibitory substances such as polysaccharides and polyphenolic compounds inhibiting the reverse transcriptase and DNA polymerase and reduce the sensitivity of the assay. In addition, the isolation of total RNA itself is a laborious and time-consuming process hindering the use of RT-PCR for large-scale testing, whereas the IC step uses a simple extraction method used for ELISA and hence enables the use of RT-PCR for large-scale testing for plant viruses. In the IC step, either PAbs or MAbs can be used depending on the objective of the virus test. In general, PAbs has broader specificity, but variation of antiserum quality from batch to batch can often be observed. Therefore, adjustments of the dilution of PAbs are expected when switching from one batch to another. On the other hand, MAbs give more consistent results and provide more stable source of reagents. A mixture of PAbs and MAbs can be also used to provide a wider range of specificity for a set of virus strains. The RT step is an intermediate step when cDNA is synthesized using reverse transcriptase. The RT step is followed by the PCR step where a thermostable DNA polymerase is used to amplify the cDNA under the control of at least two specific primers, forward and reverse. In the multiplex format of the RT-PCR, more than one primer pair is used for the amplification of more than one cDNA and/or multiple parts of a cDNA which typically allows the detection and identification of more than one plant virus/strain simultaneously, in order to save time, labor, and cost.

Plant viruses affect agricultural crops and lead to reduced quality and quantity of crops produced. The reliable and sensitive detection and timely identification of plant viruses and their strains in production areas are crucial for the effective control of diseases caused by plant viruses. Multiple formats of RT-PCR assays are being used for the detection and identification of plant viruses infecting crops and fruit trees [3–6]. *Potato virus Y* (PVY) is the most economically important virus infecting potato worldwide causing significant losses to potato tuber yield and quality [7]. PVY exists as a complex of at least nine strains and many more additional unclassified recombinants that vary in their genome structures, phenotypes, and their economic importance [7]. Some of PVY strains cause the potato tuber necrotic ringspot disease (PTNRD) in susceptible cultivars that affects the quality of potato tubers rendering them unmarketable (see Chapter 13). The strain composition of PVY circulating in production areas varies from one geographic area to another based on the environmental conditions, the set of predominant potato cultivars, and control practice. The ability to identify PVY strains accurately and in a timely fashion is crucial in any efforts to eradicate and control PVY in potato [8]. The necessity to have accurate and convenient methods to detect and distinguish PVY strains led to the development of a series of RT-PCR-based assays. The current

chapter discusses the detailed protocol of a previously described, multiplex IC-RT-PCR assay for the detection and identification of PVY strains [9, 10]. This IC-RT-PCR is able to detect and identify fourteen strains and recombinants of PVY simultaneously including PVY<sup>O</sup>, PVY<sup>N</sup>, PVY<sup>NA-N</sup>, PVY<sup>NTN</sup>-A, PVY<sup>NTN</sup>-B, PVY<sup>E</sup>, PVY-NE11, PVY<sup>N-Wi</sup>, PVY<sup>N-O</sup>, PVY<sup>NTN-NW</sup> (SYR-I, SYR-II, and SYR-III), and other rare recombinants.

## 2 Materials

### 2.1 Immunocapture

1. PVY-specific antibody (*see Note 1*).
2. 20 mM sodium carbonate buffer, pH 9.6.
3. Phosphate-buffered saline (1× PBS) with 0.5 % Tween 20 (PBST).
4. Sample extraction buffer [1× PBST, 0.3 % dry milk, 2 % polyvinylpyrrolidone (PVP)].

### 2.2 Reverse Transcription (See Note 2)

1. Reverse transcriptase buffer, 10 mM dNTP mix.
2. RNase inhibitor.
3. Mix of 0.3 μM oligo-dT and 2.7 μM random hexamer primers (*see Note 3*).

### 2.3 Polymerase Chain Reaction (See Note 4)

1. Heat-stable DNA polymerase, 10× buffer, 10 mM dNTP mix.
2. Primers.
3. Commercial DNA ladder to estimate the band size.
4. Prepare 1.5 % agarose gel in TAE buffer with ethidium bromide at 0.01 μg/ml final concentration.

### 2.4 Equipment

1. PCR 96-well plates or PCR tubes or tube strips (*see Note 5*).
2. Thermal cycler.
3. Electrophoresis machine.
4. UV illuminator and camera.

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## 3 Methods (See Note 6)

### 3.1 Immunocapture

1. Add 100 μl of antibody solution in 20 mM sodium carbonate buffer to each well/tube.
2. Incubate at 4 °C/overnight or at 37 °C/2–4 h.
3. Grind samples in extraction buffer at 1:10 (W:V) dilution rate.
4. Wash coated plates/tubes with PBST three times with dH<sub>2</sub>O intervals.
5. Add 100 μl of sample extract to each precoated well/tube.

6. Incubate at 4 °C/overnight or at 37 °C/2–4 h.
7. Wash plates/tubes with PBST four times with dH<sub>2</sub>O intervals.  
The last wash is done with dH<sub>2</sub>O.
8. Add 25 µl of RT mix to each well/tube.

### **3.2 Reverse Transcription (M-MLV RT, Promega)**

1. Prepare the following mix (total 25 µl for each sample):

M-MLV 5× reaction buffer	5 µl
10 mM dNTP mix	1.25 µl
Recombinant RNasin® Ribonuclease Inhibitor (40 U/µl)	0.625 µl
Oligo-dT and random hexamer primer mix	2.4 µl
M-MLV RT (200 U/µl)	1 µl
Nuclease-free water	14.725 µl

2. Add 25 µl of RT mix to each well/tube immediately after the last dH<sub>2</sub>O wash.
3. Incubate plates/tubes with the RT mix in a thermal cycler machine at the following program:
  - 25 °C for 2.5 min.
  - Increase temperature to 42 °C at 1 °C/30 s.
  - 42 °C for 45 min.
  - Increased to 60 °C at 1 °C/2 min.
  - Inactivate the reaction by incubation at 70 °C for 10 min.

### **3.3 Polymerase Chain Reaction (Taq DNA Polymerase, GenScript)**

1. Prepare the following mix for each sample (total 20 µl each):

GenScript 10× buffer	2 µl
10 mM dNTP mix	0.4 µl
100 µM primer mix (Table 1; see Note 7)	0.6 µl
cDNA	3 µl
GenScript Taq (5 U/µl)	0.3 µl
Nuclease-free water	13.7 µl

2. Incubate the PCR mix in a thermal cycler machine at the following program:
  - Denaturation at 94 °C for 4 min.
  - 10 cycles of:  
94 °C for 30 s.  
64 °C for 30 s.  
72 °C for 90 s.

**Table 1**  
**Primers used in the multiplex polymerase chain reaction (PCR)**

Primer name	Polarity	Sequence (5'-3')	Location	Reference
n156	Forward	GGGCAAACCTCGTAAATTGCAG	160–179	[9]
o514	Forward	GATCCTCCATCAAAGTCTGAGC	515–536	[9]
n787	Reverse	GTCCACTCTCTTCGTAAACCTC	770–792	[9]
n2258	Forward	GTCGATCACGAAACGCAGACAT	2260–2281	[17]
o2172	Forward	CAACTATGATGGATTGGCGACC	2169–2191	[17]
n2650c	Reverse	TGATCCACAACCTCACCGCTAACT	2627–2650	[17]
o2700	Reverse	CGTAGGGCTAAAGCTGATAGTAG	2678–2700	[9]
S5585m	Forward	GGATCTCAAGTTGAAGGGGAC	5578–5598	[17]
o6400	Reverse	GTAACTCCTAACAAATGGTGGTCG	6405–6430	[9]
n7577	Forward	ACTGCTGCACCTTAGATACTCTA	7582–7605	[9]
YO3-8648	Reverse	CTTTCCCTTGTTGGGTTGAC	8635–8657	[18]
SeroN	Reverse	GTTCCTCCTATGTCGTATGCAAGTT	8864–8888	[19]

- 10 cycles of:
  - 94 °C for 30 s.
  - 62 °C for 30 s.
  - 72 °C for 90 s.
- 10 cycles of:
  - 94 °C for 30 s.
  - 60 °C for 30 s.
  - 72 °C for 90 s.
- Final extension at 72 °C for 5 min.

### 3.4 Electrophoresis

1. Run the electrophoresis machine at 100 V for 45 min.
2. Visualize the gel under UV light and interpret the results (*see Note 8*).

---

## 4 Discussion

Following the emergence and epidemic spread of the recombinant PVY strains worldwide, RT-PCR techniques have continued to evolve to insure the reliable detection and identification of the newly emerged PVY recombinants including PVY<sup>NTN</sup>, PVY<sup>N-Wi</sup>,

and PVY<sup>N:O</sup> [9–17]. Each of these RT-PCR typing methods used natural nucleotide polymorphism around recombinant junctions (RJs) found in PVY recombinants. Most of these RT-PCR assays targeted only one RJ, and consequently were able to distinguish a limited set of the target strains, and were often unable to differentiate PVY strains with similar genomic structures (Table 2). On the other hand, the current multiplex IC-RT-PCR assay is a comprehensive method able to detect and identify fourteen strains and recombinants of PVY simultaneously, including PVY<sup>O</sup>, PVY<sup>N</sup>, PVY<sup>NA-N</sup>, PVY<sup>NTN</sup>-A, PVY<sup>E</sup>, PVY-NE11, PVY<sup>N-Wi</sup>, PVY<sup>N:O</sup>, PVY<sup>NTN</sup>-B, PVY<sup>NTN-NW</sup> (SYR-I, SYR-II, and SYR-III), and other rare types like 261-4 ([9, 10, 18], Table 2). This IC-RT-PCR assay uses a set of 12 primers ([9, 17–19], Table 1) targeting five RJs along the PVY genome [9, 10]. This sophisticated primer combination and multiple RJs targeted by this assay ensured the flexibility and efficacy of the assay that is able to differentiate some significant PVY strains/recombinants such as PVY-NE11, PVY<sup>E</sup>, and 261-4 even if they were not specifically targeted when this assay was developed in the first place (Fig. 1). These strains will be misidentified as PVY<sup>NA-N</sup>, PVY<sup>NTN</sup>, and PVY<sup>N:O</sup>, respectively, by the multiplex RT-PCR assay developed by Lorenzen et al. ([17]; Fig. 1). The reliability of this multiplex IC-RT-PCR assay was tested previously using well-characterized reference isolates representing various PVY strains and recombinants [9, 10]. The effectiveness of this RT-PCR was confirmed when used for both conventional RNA extracts and immunocaptured virus particles from potato and tobacco samples which make it applicable and suitable for large-scale PVY testing and strain typing [9, 10].

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## 5 Notes

1. It is recommended that prior to the use of a certain PVY-specific PAb or MAb for the IC step, serial dilutions in sodium carbonate buffer (such as 1, 5, and 10 K) are tested on reference isolates of PVY strains to find the optimal dilution rate. In our laboratory, we use one of the previously described polyclonal antisera, rabbit UID8 and goat G500 [20], at a 10 K dilution rate.
2. Any commercial reverse transcription kit can be used according to the manufacturer's instructions. We used PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) and M-MLV RT (Promega, Madison, WI, USA), successfully. The RT step is performed in the same plates/tubes used for the IC step.
3. Oligo-dT primer can be used without the random hexamer primer, though adding the latter would enhance the synthesis

**Table 2****The identification of *Potato virus Y* strains using published PCR assays**

Strain/ recombinant	Nie and Singh [12]	Nie and Singh [13]	Boonham et al. [11]	Moravec et al. [13]	Glais et al. [15]	Rigotti and Gugreli [16]	Lorenzen et al. [17]	IC-RT- PCR
O	O	?	O	?	?	O	O	O
N	N/NTN	?	N	?	?	N	N	N
NTNa (Z)	N/NTN	NTN	NTN	NTN	?	NTNa	NTN	NTNa (Z)
NTNb	?	NTN	NTN	NTN	?	?	NTN	NTNb
NA-N	NA-N	?	?	?	?	?	NA-N	NA-N
E	?	?	?	?	?	?	(NTN)	E
N:O	?	N:O/ N-Wi	?	?	N:O/ N-Wi	N:O	N:O/ N-Wi	N:O
N-Wi	?	N:O/ N-Wi	?	?	N:O/ N-Wi	N-Wi	N:O/ N-Wi	N-Wi
NE-11	?	?	?	?	?	?	(NA-N)	NE-11
SYR-I	?	?	?	?	?	(N:O*)	(NTN*)	SYR-I
SYR-II	?	?	?	?	?	(N-Wi*)	(NTN*)	SYR-II
SYR-III	?	?	?	?	?	(O*)	(NTN*)	SYR-III

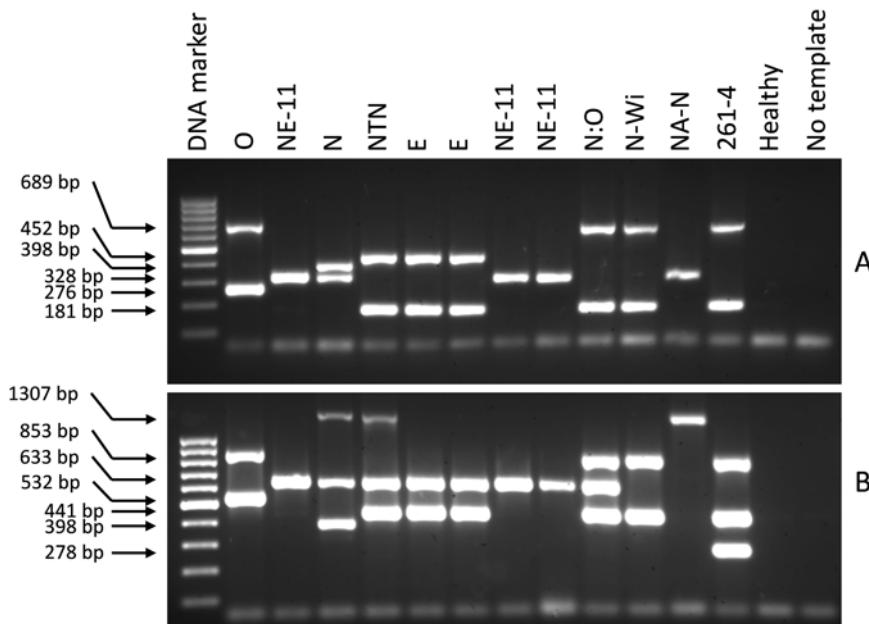
“?”: Strain type was not targeted when the PCR was developed and/or the reaction is not known

“\*”: Identification was predicted based on in silico PCR. Brackets refer to misidentification

“/”: Between strains indicate that the two strains cannot be differentiated by the corresponding method

of cDNA from low-quality RNA extracts or immunocaptured particles.

4. Any commercial DNA Taq polymerase can be used according to the manufacturer's instructions. We used TaKaRa Ex Taq™ kit (Takara Bio Inc., Shiga, Japan) and GenScript Taq DNA Polymerase kit (Piscataway, NJ, USA), successfully.
5. We used Eppendorf® twin.tec PCR plates (Eppendorf, Hamburg, Germany) for the IC-RT and VWR 96-well PCR plates (VWR, PA, USA) for the PCR.
6. Prior to the use of this IC-RT-PCR for large-scale testing, it is recommended to validate the system on a set of well-characterized reference isolates representing main PVY strains. From a technical point of view, it is very important to include control samples including water template sample, negative controls, and positive controls to make straightforward and reliable judgments on the results to avoid the possibility of background contamination of the reagents



**Fig. 1** Identification of Potato virus Y (PVY) strains using two IC multiplex RT-PCR assays reported previously. (a) IC multiplex RT-PCR reported by Lorenzen et al. [17]; (b) IC multiplex RT-PCR reported by Chikh Ali et al. [10]; names on the top refer to the PVY strains; numbers on the left side refer to the band sizes

used and nonspecific amplifications. Positive controls with banding patterns that cover all expected bands (Table 2) are very important in order to make sure that all primers are working properly.

7. For the multiplex PCR, 12 primers were used (Table 1). Primers should have very close annealing temperature and similar CG content percentage and be free of extensive secondary structures. It is strongly recommended to make a primer cocktail to decrease the chance of contamination that might be encountered during addition of 12 primers to the PCR mix separately.
8. The water template sample and negative controls should always produce negative results. Positive controls, on the other hand, should give the expected bands for the corresponding strain (Table 3, Fig. 1). When a new banding pattern appears, there is a possibility of a new genotype being found, and this needs to be clarified by sequence analysis. Special attention should be given to the bands with close sizes such as the 398 and 441 bp bands that differentiate PVY<sup>N</sup> from PVY<sup>NTN</sup> (Table 3, Fig. 1). Therefore, PVY<sup>N</sup>- and PVY<sup>NTN</sup>-positive controls, a 100 bp DNA ladder, appropriate agarose gel concentration, and sufficient electrophoresis time are necessary.

**Table 3**

**Primer combination and expected product sizes of polymerase chain reaction (PCR) and target Potato virus Y strains**

Primer combination	Expected product (bp)	Target strain
n156+n787	633	N, NTN(A), N:O, E, NE-11
n156+o514	278	SYR-III, 261-4
n2258+n2650c	398	N
n2258+o2700	441	NTN(A), NTN(B), N-Wi, N:O, E, 261-4
o2172+o2700	532	O
S5585m+o6400	853	O, N-Wi, N:O, 261-4
n7577+YO3-8648	1,076	SYR-I, SYR-II, SYR-III
n7577+seroN	1,307	N, NTN(A), NTN(B), NA-N

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# Chapter 15

## SNaPshot and CE-SSCP: Two Simple and Cost-Effective Methods to Reveal Genetic Variability Within a Virus Species

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### Abstract

The multiplex SNaPshot and the capillary electrophoresis-single-strand conformation polymorphism (CE-SSCP) procedures are here used for rapid and high-throughput description of the molecular variability of viral populations. Both approaches are based on (1) standard amplification of genomic sequence(s), (2) labeled primers or labeled single-stranded DNA, and (3) migration of fluorescent-labeled molecules in capillary electrophoresis system. The SNaPshot technology was used to describe the diversity of 20 targeted single nucleotide polymorphisms (SNPs) selected from alignment of viral genomic sequences retrieved from public database. The CE-SSCP procedure was applied to identify the polymorphisms of two small (<500 bases in length) genomic regions of viral genomes. The different steps of SNaPshot and CE-SSCP setup procedures are presented using *Potato virus Y* (PVY, *Potyvirus*) and *Plum pox virus* (PPV, *Potyvirus*) RNA viruses as molecular targets, respectively.

**Key words** Polymorphism, Genomic RNA, Viruses, Electrophoresis, Fluorescent-labeled ss-DNA

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### 1 Introduction

Many research fields require knowledge on biological, serological, and/or molecular diversities of populations. Comparisons of properties of viral entities can be performed using isolates collected from different locations or hosts [1–4]. However, these analyses can also be carried out at a single infected host level [5]. Indeed, viruses produce their progenies using a polymerase devoid of proofreading activity [6, 7]. Thus, the progeny obtained in an infected host can be considered as a population according to the genomic differences (mutations, deletions, and/or insertions) between individuals [8]. Characterization of the molecular diversity at different levels (single cell, single host, or community host) is a key step to study the structure of viral pathogens or to

understand ecological and evolutionary processes (e.g., emergence, maintain, and spread of new variants). Several well-documented sequencing procedures can be applied to the whole or to part(s) of the genome of members of a population. Most of these procedures are based on four main steps corresponding to (1) amplifying targeted genomic sequences, (2) cloning individuals, (3) sequencing their genome, and (4) making comparisons using alignments of produced data in order to describe similarities and differences between analyzed individuals. Even if these methods produce expected data sets (i.e., library of genomic sequences), they use time-consuming and expensive procedures. Alternative techniques such as SNaPshot [9, 10] and capillary electrophoresis-single-strand conformation polymorphism (CE-SSCP) [11], two simple and cost-effective methods, can be used to highlight genetic differences of individuals (e.g., isolates) within a defined population of a pathogen (e.g., virus species) [5, 12–16]. These two methods are based on standard amplification of genomic sequence(s) and on migration of labeled primers or labeled single-stranded DNA (ss-DNA) molecules in a polymer. The SNaPshot and CE-SSCP procedures make possible the rapid identification of targeted single nucleotide polymorphisms (SNPs) and the identification of polymorphisms of selected small (<500 bases in length) genomic regions of viral genomes, respectively. In the SNaPshot procedure, each fluorescent peak must be assigned to the appropriate targeted SNP. This assignment is based on the migration time associated to fluorescent molecules and has to be carefully calibrated prior sample analyses. In CE-SSCP patterns, each peak difference between two samples reflects a genetic variation within the analyzed population. However, the polymorphism described by the CE-SSCP procedure represents only part of the population diversity as this method is not able to detect sequence modifications that do not impact the single-strand conformation. Indeed, conformational polymorphisms of ss-DNA molecules depend on intrinsic parameters of nucleic acid sequence such as the length of the studied fragment, the number of nucleotide changes, their locations in the targeted sequence, and the four bases ratio in the analyzed nucleotide region.

In order to illustrate the use of SNaPshot and CE-SSCP procedures as methods to analyze the genetic variability within a virus species, these techniques were applied to *Potato virus Y* (PVY, *Potyvirus*; [17]) and *Plum pox virus* (PPV, *Potyvirus*; [18]) RNA viruses, respectively—two out of the ten most important plant viruses [19]. Based on the proposed protocols, these techniques make it possible to process up to 96 samples in less than 24 h from sampling to the description of genetic variations of tested isolates.

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## 2 Materials, Products, and Chemicals

### 2.1 General

1. Sterile RNase/DNase-free plastic wares (microtubes, tips, etc.).
2. Standard lab devices (pipettes, storage and sample racks, gloves, etc.).
3. Wet ice.
4. Deionized water.
5. Molecular biology quality (ultrapure) water.
6. RNase-free water.
7. Microtiter plate.
8. Absorbent paper.
9. Centrifuge.
10. Storage/incubating devices for -80 °C, -20 °C, +4 °C, +37 °C, and +42 °C.
11. PCR plates.

### 2.2 Sample Preparation

1. Sterile plastic pestle and sterile razor blade.
2. Single-use extraction bags (type ACC0930/0100, Agdia or equivalent).
3. Tissue homogenizer (type ACC0090000, Agdia) attached to a drill press.

### 2.3 Immunocapture

1. PVY and PPV polyclonal antibodies.

### 2.4 cDNA Synthesis

1. RNasin® [Promega].
2. AMV reverse transcriptase [Promega].
3. 10 mM dNTPs (2.5 mM each).
4. Triton X-100 (dilution 1.7 % in molecular biology quality water).
5. Random hexamers [Promega].

### 2.5 Production of Amplified Viral Sequence

1. Taq polymerase with buffer and MgCl<sub>2</sub> solution [Promega or Invitrogen].
2. DNA Smart Ladder [Eurogentec].
3. Thermocycler apparatus.
4. Agarose.
5. Ethidium bromide.
6. Agarose gel electrophoresis device.
7. Ultraviolet imaging system.

**2.6 Primer Extension**

1. Sephadex G50 or GenElute PCR Clean-up kit [Sigma] or commercial equivalent purification kit of PCR products.
2. ABI PRISM SNaPshot™ Multiplex Kit [Life Technologies].
3. Calf intestinal phosphatase (CIP) [Promega].

**2.7 Denaturation of Labeled PCR Products for CE-SSCP**

1. Tris-HCl 10 mM.
2. Glycerol.
3. Thermocycler apparatus or heated block.

**2.8 Capillary Electrophoresis for SNaPshot™ Analysis**

1. Hi-Di formamide [Life Technologies].
2. Thermocycler apparatus or heated block.
3. GeneScan™ LIZ®-120 size standard [Life Technologies].
4. POP-7™ polymer [Life Technologies].
5. ABI PRISM 3130XL Genetic Analyzer with 96 coated capillaries (effective length: 36 cm) [Applied Biosystems].
6. GeneMapper® [Applied Biosystems].

**2.9 Capillary Electrophoresis for CE-SSCP Analysis**

1. MegaBACE™ 1000 DNA analysis system with 96 coated capillaries (effective length: 40 cm) [Amersham Biosciences].
2. MegaBACE™ ET900-R size standards [Amersham Biosciences].
3. 6 % Short-chain linear polyacrylamide polymer (SPLA) prepared by Genomac International Ltd. (Prague, Czech Republic) according to [23]. The 10× running buffer used in the procedure is supplied with SPLA [Genomac International].

All homemade buffers, solutions, and mixtures have to be prepared with sterile deionized water or with molecular biology grade water. All primers and probes used in the presented procedures are described in Tables 1 and 2.

**2.10 Buffers**

1. Carbonate buffer: 1.59 g Na<sub>2</sub>CO<sub>3</sub>, 2.93 g NaHCO<sub>3</sub>, deionized water to prepare 1 l.
2. PBS buffer: 8 g NaCl, 2.9 g Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 0.2 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, deionized water to prepare 1 l.
3. Grinding buffer: PBS, 0.05 % (v/v) Tween 20, 0.2 % (w/v) polyvinylpyrrolidone (40 K).
4. Washing buffer: PBS, 0.05 % (v/v) Tween 20.

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**3 Methods**

SNaPshot is a fluorescent-based technique that allows the simultaneous identification of several (up to 20) nucleotides in targeted nucleic acid sequence(s). The SNaPshot procedure consists (1) in the incorporation of a single fluorescent-labeled ddNTP at the

**Table 1**  
**PCR primers and SNaPshot probe sequences for the SNP analyses of PVY**

	<b>Name</b>	<b>Sequence</b>	<b>Length</b>	<b>Position<sup>a</sup></b>
Reverse transcription	RT 3'ter	GTCCTCCTGATTGAAGTTAC	20	9682
Region 1	F1	ACTCGCMATGGCWACTTRCA	20	182
	R1	TTYGCTGGATCAATTTCCTC	20	1707
Region 2	F2	GTGCCAACAAATATGCCA	18	1208
	R2	TTCTGCYGCTGACACTCGTA	20	2701
Region 3	F3	TCACCTTTYGAGAAGGAGGA	21	2449
	R3	GTCATYACTGGCAARTRTA	20	4833
Region 4	F4	AYAATAGGAGCATWGCWTACAATAA	25	4658
	R4	TCAAYGGRCCCCACAACA	18	6910
Region 5	F5	TGGAARCATTGGATYGARA	19	6714
	R5	AATCTCCTGATYTGGTGYGTTA	22	8352
Region 6	F6	TCAGATCTTGGTTGAAYTATGATT	25	8134
	R6	ATAAAAGTAGTACAGGAAAAGCCAA	25	9427
SNaPshot	SNaP183	TTYGGTTCSWTTGAATGCAA	20	48
	SNaP363	(A)7-WGCTGGTGGAGAKCCWCC	25	339
	SNaP815	(A)10-AWTSTATGATGCACGTTCYA	30	815
	SNaP1281	(A)15-AATTCTTYTGAAAGGAAA	35	1281
	SNaP1677	(A)22-AARACARCCAGGGTSAG	40	1680
	SNaP1989	(A)27-GCTTGGAACCTGGCCAAC	45	1989
	SNaP2340	(A)30-AAGGGAATTITTAGRCCYAA	50	2340
	SNaP3039	(A)37-GCGYTTGCCAACTTTYGT	55	3039
	SNaP3603	(A)42-GTGGGAYCGRCAAATCCA	60	3603
	SNaP4008	(A)42-AAATTGATGARTGYCATGTTYT	65	4008
	SNaP4542	(A)52-RAGGYTGGGYCGTGTGG	70	4542
	SNaP5169	(A)54-GAGTCAAATTCAAGTCTCAT	75	5169
	SNaP5739	(A)60-TATGGRTTTGAYCCAACAGA	80	5739
	SNaP6261	(A)67-YTWCAATGGWTCCATGGA	85	6261
	SNaP6717	(A)70-TCKTGGGTWTATAAYCCAGA	90	6717
	SNaP7236	(A)75-GGAGCYATGTATGGWGCAA	95	7230
	SNaP7785	(A)80-CARCCTCTACYGTGTGGA	100	7779
	SNaP8325	(A)85-GATGAGGAGGARCTGARRGC	105	8316
	SNaP8712	(A)90-TACGACATAGGAGAAACTGA	110	8703
	SNaP9066	(A)95-CAAATGAAGGCCGCAGCWTT	115	9057

<sup>a</sup>Positions are according to Jakab et al. [22]

**Table 2**  
**PCR primers used for CE-SSCP analysis of *Plum pox virus* isolates**

<b>Region</b>	<b>Amplon size (nt)</b>	<b>Primer<sup>a</sup></b>	<b>Sequence (5'-3')</b>	<b>Position<sup>b</sup></b>
1	283	P3F (6-FAM)	CGATGGAAAGCTATTCTGC	3154–3173
		P3R (HEX)	AGGGTTCTCACCTTGCACAT	3418–3437
2	467	P3M (6-FAM)	ACATAGCAGAGACGGCACTC	8446–8465
		P4 (HEX)	TGCCTCAAACGTGGCACTG	8893–8912

<sup>a</sup>Fluorescent dyes 6-FAM and HEX, linked to PCR primers, are indicated between brackets

<sup>b</sup>Position on the PPV genome according to Palkovics et al. [24]

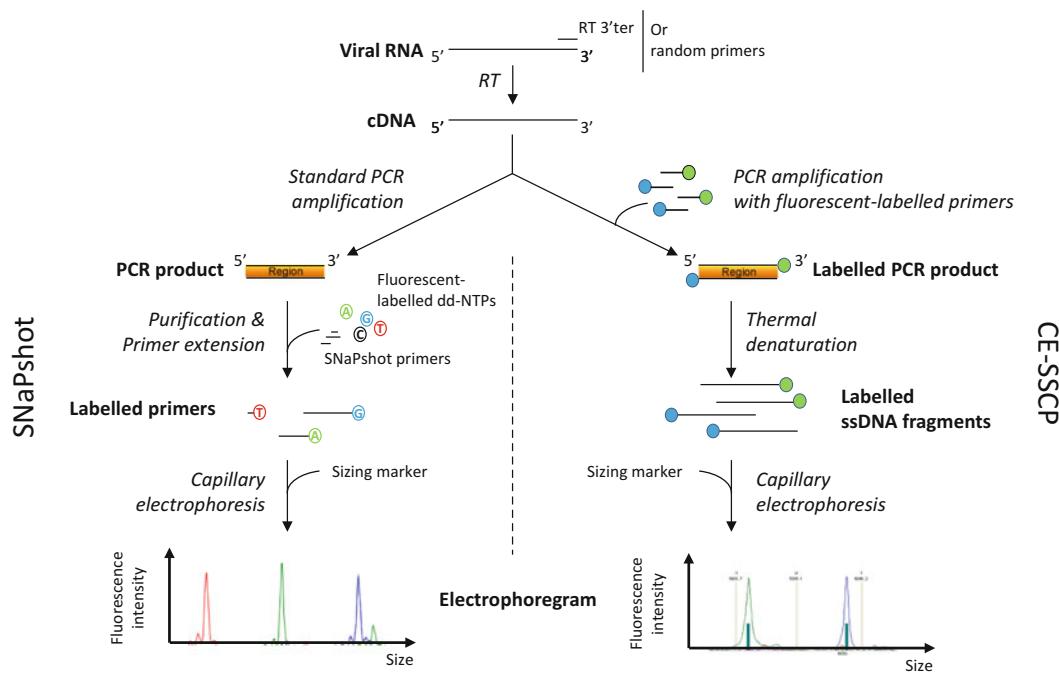
3'-end of a SNaPshot primer and (2) the detection of the targeted polymorphic nucleotide revealed by the fluorescent signal associated to the used SNaPshot primer. Indeed, the SNaPshot primer has to be designed to allow specific hybridization of its 3'-end one base upstream the targeted polymorphic site (SNP). As (1) each ddNTP used for the elongation step is linked to a specific fluorochrome and (2) primer elongation carried out using ddNTP leads to the production of a 3'-blocked fragment after a single nucleotide elongation, the characteristics (length and type of fluorescence) of the labeled SNaPshot primer make it possible to determine the identity (complementary to the fluorescent ddNTP linked to the primer) of the targeted SNP. The analysis of the labeled fragment is performed using a capillary electrophoresis procedure associated to fluorescence monitoring. Using primers with various lengths (one length per targeted SNP site), the SNaPshot procedure allows multiplex SNP interrogations.

Single-strand conformation polymorphism (SSCP) analysis is based on the principle that single-stranded DNA (ss-DNA) fragment with a single base substitution (or small insertion/deletion) gets a unique conformation and therefore migrates differently under non-denaturing electrophoresis conditions. Associating capillary electrophoresis (CE) to SSCP allows a high separation efficiency of the DNA fragments and a good reproducibility within a relatively short running time of the tested samples. The CE-SSCP procedure described here is based on the amplification of the targeted genomic region(s) by classical PCR using fluorescent primers (forward and reverse primers each being labeled with different fluorescent molecules). After a thermal denaturation, the produced fluorescent ss-DNA fragments are separated by capillary electrophoresis. The relative position of each of the two ss-DNA fragments is recorded and constitutes the “identity code” of a given sample.

The two procedures start with identical steps of sample preparation and cDNA production (Fig. 1). However, based on technological differences between SNaPshot and CE-SSCP, further steps of the two procedures are methodology specific.

### 3.1 Sample Preparation

As the studied material used in the presented experiments corresponds to RNA molecules (i.e., RNA viral genomes), the procedures (both SNaPshot and CE-SSCP) begin with the synthesis of cDNA molecules corresponding to the targeted viral genomic regions using a reverse transcription step. To produce the cDNA molecules, samples were grinded and virus particles immobilized in an antibody-coated plate using appropriate polyclonal antibodies. After disruption of the coat protein subunits coated in the plate wells (i.e., after heat treatment (5 min at 70 °C) of the plate), released viral RNA molecules were reverse transcribed into cDNA fragments using the appropriate procedure (*see* Subheading 3.1.3).



**Fig. 1** Schematic representation of SNaPshot (*left panel*) and CE-SSCP (*right panel*) procedures

Then, the produced cDNA was used as matrix for PCR amplifications of the viral sequences (*see Subheadings 3.2.2 and 3.3.2*).

### 3.1.1 Grinding Samples

All the samples should be stored at 0–4 °C during the complete grinding procedure and rapidly transferred into the immunocapture (microtitration) plate (Subheading 3.1.2) after grinding (*see Note 1*).

1. Depending on the type of plant material to analyze, collect leaf samples with a sterile razor blade or use a cap of a sterile microtube, such as perforating device. For some specific plant material (e.g., prunus flowers sampled in orchards), collect samples by hands, store them in plastic bag, and transfer them to the laboratory.
2. Crush samples in tube with a sterile pestle grinder in the presence of 200 µl of grinding buffer or put collected samples in grinding bags and use a tissue homogenizer in the presence of grinding buffer (1:10 weight/vol).
3. Store grinded material at 4 °C until use (avoid long-term storage of ground samples). If possible prepare and use samples the same day.

### 3.1.2 Immunocapture (IC)

The immunocapture step corresponds to the first step of standard ELISA procedure. This does not constitute a real nucleic acids extraction procedure. It allows the specific attachment of viral par-

ticles present in tested samples on antibody-coated wells of a microtiter plate. Particles contain viral genome that can be used as targets in the next steps of the presented procedure.

1. Prepare the appropriate (anti-PVY or anti-PPV) antibody solutions (1 µg/ml, 100 µl/well) in carbonate buffer (*see Note 2*).
2. Coat microtiter plate (IC plate) wells (or PCR plate if microtiter plate is not available, but PCR plates are less efficient for adsorption of antibodies) with 100 µl of diluted antibody solution and incubate for 2–4 h at 37 °C.
3. Wash the IC plate three times with sterile PBS–Tween buffer. Empty the IC plate after the last wash either by soaking on absorbent paper or by pipetting.
4. Load 100 µl of crude sap (ground samples) and incubate from 30 min at 37 °C to overnight at 4 °C (*see Note 3*).
5. Wash the IC plate three times with sterile PBS–Tween buffer and once with molecular biology grade water.
6. Empty the IC plate after the last wash either by soaking on absorbent paper or by pipetting.

### 3.1.3 Production of cDNA

The reverse transcription (RT) step can be performed immediately after the immobilization of the virus particles in the IC plate. Alternatively, the IC plate can be stored at –20 °C for several weeks to several months before further processing.

For samples analyzed by SNaPshot, the viral particles coated in the wells of the IC plate were used in the reverse transcriptase procedure without further manipulation of the viral material. Wells were filled with the RT reaction mixture and incubated at optimal temperature for AMV reverse transcriptase activity, i.e., 42 °C for an hour.

1. Prepare a RT mixture according to the following procedure (20 µl/well)

Enzyme buffer (5×)	4 µl
MgCl <sub>2</sub> (25 mM)	2 µl
dNTP (10 mM each)	2 µl
RT 3'ter primer (10 µM) (RT 3'ter sequence is presented in Table 1)	2 µl
RNasine (40 U/µl)	0.5 µl
AMV reverse transcriptase (10 U/µl)	0.2 µl
Nuclease-free water	9.3 µl

2. Incubate 1 h at 42 °C.
3. Maintain the plate at 4 °C for direct use of the produced cDNA in the SNaPshot procedure or store the plate at –20 °C for long-term storage before cDNA use.

For CE-SSCP analysis, the RT procedure is performed in two steps. The first step aims to disrupt the coat protein subunits at high temperature in presence of detergent (Triton X100), the second step being the reverse transcription per se. Total reaction volumes can be increased up to 50 µl if needed; however, the proposed procedure is designed for 25 µl. Random primers are used to produce a homogeneous production of cDNA fragments along the viral genomic RNA. The procedure was optimized to synthesize cDNA from rare viral RNA extracted from limited samples collected in orchards (e.g., petals of peach flowers and asymptomatic, potentially weakly infected leaves).

1. Prepare a denaturation mixture (volumes determined for the RT reaction carried out in 25 µl):

Triton X-100 1.7 %	3 µl
Random primers	1 µl
Nuclease-free water	8.1 µl

2. Incubate the 12.1 µl mixture in the well of the IC plate for 5 min at 70 °C, and then cool down quickly to 4 °C in a thermocycler machine.
3. Prepare the RT mixture:

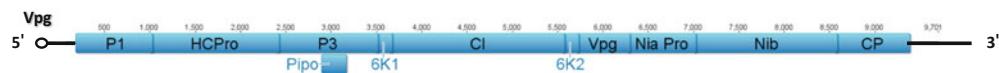
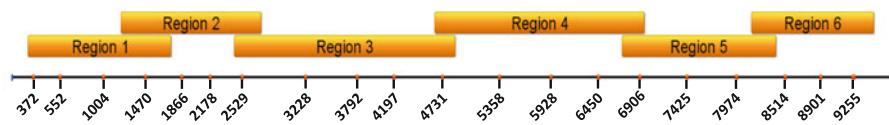
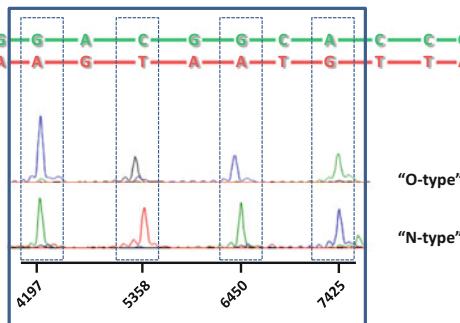
Enzyme buffer (5×)	5 µl
dNTP (10 mM each)	2.5 µl
RNAsine (4 U/µl)	1 µl
AMV reverse transcriptase (10 U/µl)	0.5 µl
Nuclease-free water	3.9 µl

4. Add the 12.9 µl of the RT mixture in the well of the IC plate.
5. Incubate 1 h at 37 °C followed by 5 min at 95 °C.
6. Store at –20 °C.

### 3.2 Multiplex SNP Interrogation Assay

#### 3.2.1 Design of Primer Pairs and SNaPshot Primers

The SNaPshot procedure includes an amplification step of the targeted genome (corresponding to a standard PCR) and a single base elongation step to produce fluorescent-labeled primers. These two reactions require the use of different types of primers. There are no special recommendations for the design of PCR primer pair(s) which are defined in a classical manner in conserved regions of the targeted sequence according to the knowledge on the viral genome variability. Thus, series of PCR primer pairs were designed in order to make it possible the production of amplified fragments overlapping the complete viral genomic sequence (Fig. 2 and Table 1). The SNaPshot primers used in the presented procedures were designed using consensus sequences obtained from PVY

**a****b****c****d**

**Fig. 2** Genomic organization of *Potato virus Y* (a), the six amplified regions overlapping the complete viral genome and the location of the 20 selected SNP positions (b), and identity of PVY<sup>O</sup>- and PVY<sup>N</sup>-type nucleotides for each targeted SNP site (c). As an example, electropherograms obtained for nucleotide 4197, 5358, 6450, and 7425 are presented for PVY<sup>O</sup> and PVY<sup>N</sup> referent sequences (d). Nucleotide positions according to [22]. Green, blue, black, and red peaks correspond to A, G, C, and T, respectively

alignments performed with PVY sequences retrieved from GenBank. Based on the resulting alignment, 20 PVY<sup>N</sup>/PVY<sup>O</sup> polymorphic nucleotides were selected in the different regions of the viral genome with an average distance of 500 nucleotides between two SNP sites. For each defined SNP, a primer able to hybridize one nucleotide upstream the targeted SNP site was designed (Table 1) with a specific length (from 20 to 105 nts).

To design SNaPshot primers, use the following guidelines:

- Fix the melting temperature (Tm) of the primers above 50 °C.
- If more than one SNP is targeted by the SNaPshot tool, the differences in length between two primers should be from 6 to 10 nucleotides for primers shorter than 36 nucleotides and from 5 to 8 nucleotides for longer primers. The use of primers with length differences corresponding to more than six nucleotides helps the analysis of raw data produced by the SNaPshot technology. The length of SNaPshot primers must not exceed 120 nucleotides.
- To maintain the melting temperature of the PVY homologous sequence of the SNaPshot primer around 50 °C, long primers (>30 nucleotides) were designed with their 5'-end constituted by

a polyA-tail (of appropriate number of “A”) to reach the expected lengths (*see Table 1*) without Tm modification (*see Note 4*).

- Use only HPLC-purified primers (*see Note 5*).

According to these guidelines, 20 primers with different sizes were designed to target the 20 selected PVY SNP sites. However, as the analysis of 20 labeled primers in a single capillary electrophoresis can lead to complex profiles, the SNaPshot assays can be processed using two batches of 10 SNaPshot primers per SNaPshot run. The two batches were made to optimize the length differences (10 nucleotides) between primers: batch #1 (primers SNaP183, SNaP815, SNaP1677, etc.) and batch #2 (primers SNaP363, SNaP1281, etc.).

### 3.2.2 Production of Amplified Genomic Viral Sequence

Six regions overlapping the complete PVY genome were amplified (Fig. 2) with appropriate couples of primers (e.g., F1 + R1 for the region #1).

1. Prepare the PCR mixture according to the following conditions:

Enzyme buffer (5×) [Promega]	10 µl
MgCl <sub>2</sub> (25 mM)	6 µl
dNTP (10 mM each)	1 µl
Primer F (10 µM, Table 1)	3 µl
Primer R (10 µM, Table 1)	3 µl
H <sub>2</sub> O mol. biol. quality grade	24.75 µl
Taq polymerase (5 U/µl) [Promega]	0.25 µl
cDNA	2 µl

2. Perform classical amplification steps as follows:

- 95 °C, 1 min.
- 40 Cycles: 95 °C 1 min, 57 °C 1 min, 72 °C 1 min 30 s.
- 72 °C, 10 min.

3. Check the quality of PCR amplification by electrophoresis on agarose gel 1 % (100 V, 30 min).

### 3.2.3 Primer Extension Procedure

According to the manufacturer’s recommendations, the primer extension procedure requires the use of purified PCR products as matrix (*see Note 6*). The purification procedure removes free dNTPs and subsequently the MgCl<sub>2</sub> and other compounds present in post-PCR fractions. Purification of PCR products can be realized using Sephadex G50 or commercial PCR purification kits (e.g., GenElute PCR Clean-up kit [Sigma]). For the presented multiplex analyses, the DNA matrix corresponds to multiple PCR

fragments scattered along the viral genome. Consequently, multiplex SNaPshot extension procedure requests a balanced mixture of the different purified PCR products (0.04–0.4 pmol each). At the end of the extension procedure, unincorporated fluorescent ddNTPs must be modified in order to avoid background of fluorescent signals during electrophoresis analysis. Thus, a phosphatase treatment (e.g., calf intestinal phosphatase (CIP)) is applied to labeled fractions prior being used for the electrophoresis step.

1. Prepare the extension mixture according to the following conditions (*see Note 7*):

SNaPshot™ multiplex reagent buffer	2 µl
SNaPshot primers (0.2 µM each)	1 µl
Purified PCR products (balanced mixture)	2 µl

2. Elongation: running cycle is constituted by three steps and repeated 25 times (*see Note 8*):
  - 96 °C, 10 s.
  - 50 °C, 5 s.
  - 30 °C, 30 s.
3. Add 1 U/well of CIP and incubate 1 h at 37 °C.
4. Inactivate CIP at 75 °C for 15 min.

During the primer extension step, a single base (ddNTP) is added at the 3'end of each hybridized primer. In consequence, the size of the resulting products corresponds to the length +1 (linked dideoxynucleotide) of the used primers. Moreover, the fluorescence of the labeled primer indicates the nature of the linked fluorescent ddNTP.

### 3.2.4 Analysis of Labeled Primers Using Capillary Electrophoresis

Capillary electrophoresis discriminates labeled primers present in tested samples based on their length and their fluorescence. To accurately identify each labeled SNaPshot primers by their respective size (theoretically primer length+1), samples are analyzed in the presence of a reference ladder made of a mixture of labeled standards (with a specific dye not used for the four fluorescent ddNTP) molecules (e.g., LIZ®-120 [Applied Biosystems]). Products are run on a capillary electrophoresis system (3130XL Genetic Analyzer, [Applied Biosystems]). Capillaries are filled with a POP-7™ matrix [Applied Biosystems] (*see Note 9*). The fluorescence of the labeled primers is detected by a laser, while the migration is running. Raw fluorescent data are analyzed using GeneMapper® software [Applied Biosystems] (*see Note 10*).

Labeled samples are denatured by heating in the presence of formamide. An internal standard (GeneScan™ LIZ®-120) is added extemporaneously in the mixture to allow the determination of the size of fragments at the end of the migration step.

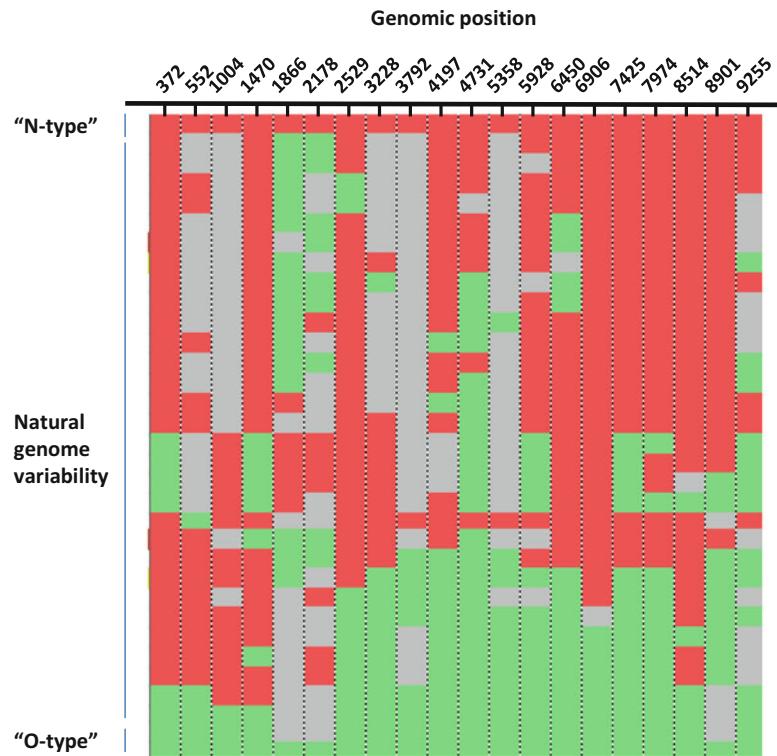
1. Prepare the capillary electrophoresis mixture (10 µl/reaction) in a microtube

Hi-Di formamide	8.5 µl
GeneScan™ LIZ-120 size standard	0.5 µl
Sample (labeled primers post-primer extension step)	1 µl

2. Heat the mixture at 95 °C for 5 min and place it immediately in wet ice for 1 min.
3. Place the microtubes on the tray of the 3130XL Genetic Analyzer [Applied Biosystems].
4. Run electrophoresis was performed at 15 kV for 3 h 10 min (including the preparation of the capillaries) for a 96-well migration.
5. Collect fluorescent data.
6. Analyze raw data using GeneMapper® software.

### 3.2.5 Data Analysis

During capillary electrophoresis, fluorescence data were recorded as a function of migration time. The software calculates the size of observed fluorescent fragments according to migration time of the labeled nucleic acids present in the LIZ-120 standard. Multiple SNP results can be manually scored according to labeled primer length and to the corresponding fluorescent signal. However, the GeneMapper® software [Applied Biosystems] can be calibrated (1) to automatically assign each peak of the crude results and (2) to combine all assigned peaks to define the SNPs targeted in the tested samples. However, it is important to note that calculated size associated to fluorescence peaks do not correspond to exact length + 1 of the primers as the fluorescent molecules linked to labeled primers slightly impact the length-dependant migration of primers in the polymer (*see Note 11*). Consequently, identification of peaks must be carefully carried out. The use of primers (in multiplex SNP interrogation assays) with length differences of 5–10 nucleotides facilitates assignation of peaks to the corresponding SNaPshot primers. Complete analysis of electropherograms (validation of each peak (color/nucleotide identity and size/genome position)) allows production of the full-length 20-SNP code. All the data are then aligned and compared to the SNP identity code for the reference PVY<sup>O</sup> and PVY<sup>N</sup> isolates (Fig. 3).

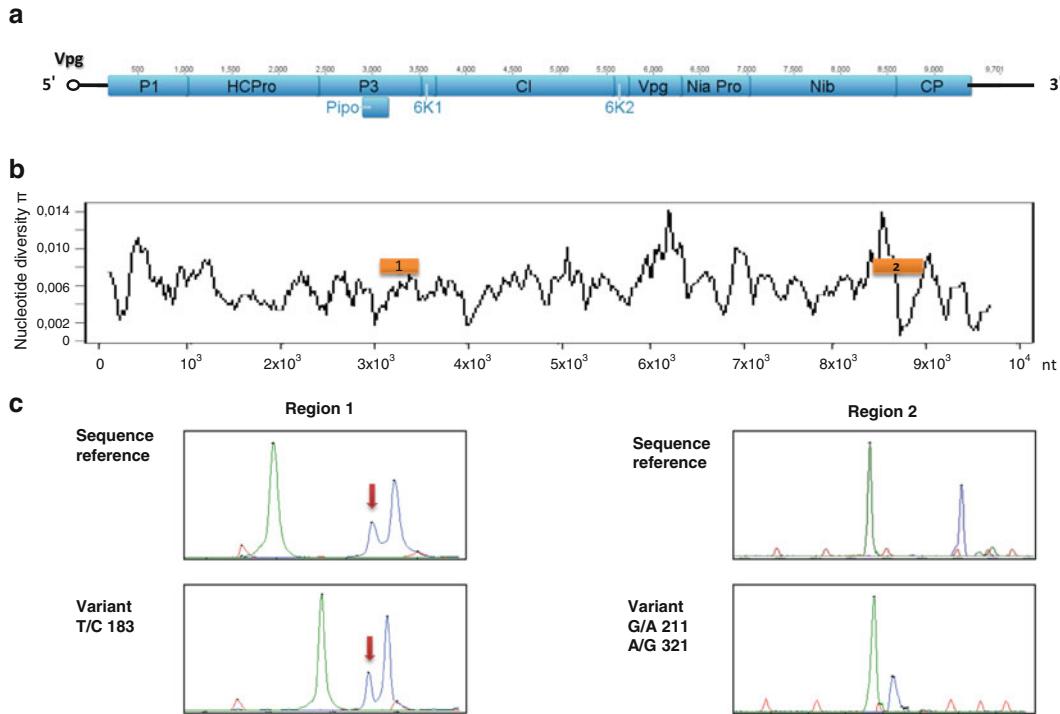


**Fig. 3** Illustration, using color code (red for PVY<sup>N</sup> type and green for PVY<sup>O</sup> type), of the polymorphism described with the 20 SNPs targeted by the SNaPshot procedure obtained for a collection of 31 PVY isolates sampled from field surveys. The referent N-type (full red SNaPshot code) and O-type (full green SNaPshot code) isolates are presented in the first and last line, respectively. SNP nucleotides identities not corresponding to the PVY<sup>N</sup> type and to the PVY<sup>O</sup> type are illustrated by a gray color code

### 3.3 CE-SSCP Analysis

#### 3.3.1 Primer Design and Analyzed Regions

The genomic regions to be analyzed were chosen based on the alignment of 18 full-length genome sequences of PPV-M isolates. Two regions displaying enough polymorphism but still flanked by sufficiently conserved positions for PCR primer design were selected (Fig. 4 and Table 2). For a good SSCP resolution, the analyzed fragments should be relatively short (<500 nucleotides long) as sensitivity of mutation detection by SSCP technique decreases with the increase of the length of fragments. The use of differently labeled forward and reverse primers allows analyzing the two ss-DNA fragments separately, which increases the chances of polymorphism detection. The CE-SSCP protocol presented here was adapted from the one developed by Bryja et al. [20].



**Fig. 4** Genomic organization of *Plum pox virus* (PPV) (a), genetic polymorphism among 18 PPV strain M full-length sequences (genetic diversity  $\pi$  computed within a sliding window of 100 nt. long) and positions of the two analyzed regions, denoted by orange boxes 1 and 2 (b), and examples of typical electropherograms corresponding to the most frequent detected variants for both regions (c). Green and blue peaks correspond to sense- and antisense-labeled ss-DNA molecules, respectively. The red arrows point to supplementary peaks corresponding to two different conformers of the same ss-DNA strand (see Note 12)

### 3.3.2 PCR Amplification with Labeled Primers

Prepare the PCR mixture according to the following conditions

Enzyme buffer 10× [Invitrogen]	2.5 $\mu$ l
MgCl <sub>2</sub> (25 mM)	0.75 $\mu$ l
dNTP (10 mM each)	2.5 $\mu$ l
Primer F 6-FAM (10 $\mu$ M)	0.5 $\mu$ l
Primer R HEX (10 $\mu$ M)	0.5 $\mu$ l
H <sub>2</sub> O mol. biol. quality grade	16 $\mu$ l
Taq polymerase (5 U/ $\mu$ l) [Invitrogen]	0.25 $\mu$ l
cDNA	2 $\mu$ l

Perform classical PCR amplification with a 30-min final extension step (guarantee the incorporation of all terminal nucleotides in the synthesized fragments) as follows:

For region 1 (283 nt. long):

- 94 °C, 5 min.
- 35 Cycles: 92 °C 20 s, 58 °C 20 s, and 72 °C 20 s.
- 72 °C, 30 min.

For region 2 (467 nt. long):

- 94 °C, 5 min.
- 35 Cycles: 92 °C 20 s, 55 °C 20 s, and 72 °C 40 s.
- 72 °C, 30 min.

Check the quality of PCR products by electrophoresis on agarose gel 1.5 % (100 V, 30 min) (*see Notes 13 and 14*).

### *3.3.3 Preparation of Labeled PCR Products for CE-Electrophoresis*

1. Prepare a sample plate with PCR products diluted tenfold in Tris-HCl 10 mM pH 8.5.
2. Mix 5 µl of each diluted PCR product with 5 µl of diluted size standards marker (0.4 µl of MegaBACE™ ET900-R and 4.6 µl of Tris-HCl 10 mM pH 8.5).
3. For each sample plate, prepare a plate of running buffer:
  - Add 5 ml of glycerol to 5 ml of 10x running buffer (Genomac International Ltd.) and adjust to 50 ml with deionized water.
  - Dispense 150 µl of the 1x running buffer in each well of the plate.
4. Incubate the sample plate at 95 °C for 2 min and cool down immediately on wet ice for at least 5 min. Centrifuge at 2,200 ×  $\varphi$  (10,000 rpm) for 1 min at 4 °C just before the injection into the capillaries (*see Note 15*).

### *3.3.4 CE-Electrophoresis of Labeled Fragments*

Place the sample and buffer plates in the MegaBACE™ DNA analysis system with capillaries filled with the SPLA matrix. Injection of the samples is carried out for 40 s at 6 kV. Electrophoresis is run at 9 kV for 90 min. The best resolution of peaks was obtained at 25 °C for region 1 and at 27 °C for region 2. A second run at 30 °C was needed for detecting minor variants regarding region 1.

### *3.3.5 Data Analysis*

During capillary electrophoresis, fluorescence data corresponding to ss-DNA molecule and to the size standards are recorded as a function of migration time. The electropherograms can be visualized and analyzed using the MegaBACE™ Genetic Profiler software (version 1.5 or later). The different runs are combined and the electropherograms aligned. The peaks of fluorescence corresponding to the ss-DNA fragments are manually or automatically detected, and their relative position can be determined using the internal size standards. Variants are determined according to their CE-SSCP profiles obtained with the two complementary strands from each analyzed region (*see Notes 16 and 17*).

Such a procedure was applied to the characterization of 879 PPV-M isolates collected from individual infected peach trees. For each variant identified in CE-SSCP, the PCR products of one or several samples were directly sequenced. The samples for which assignment was impossible because their CE-SSCP profile was not distinct enough from previously identified ones were also sequenced. CE-SSCP analysis resulted in the identification of 20 variants from region 1 (812 assigned samples) and 36 variants from region 2 (684 assigned samples). The specificity of assignment controlled by random sequencing of the identified variants was very high (close to 100 %).

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#### 4 Notes

1. Freezing homogenized sample at -20 °C is not recommended. The disruption of cell tissue releases chemical substances which degrade rapidly nucleic acids (Subheading 3.1.1). If absolutely necessary, short-term storage (several days) at -80 °C can be performed, but one can expect a loss of sensitivity in the following IC-RT-PCR steps.
2. The antibodies used under our experimental conditions were produced by FNPPPT/INRA (Le Rheu) and INRA (Montpellier) for PVY- and PPV-specific assays, respectively. As an alternative, validated or commercially antibodies should be used. Indeed, numerous diagnostic product manufacturers (e.g., Adgen, Agdia, Bioreba, DSMZ, Loewe Biochemica GmbH, etc.) supply ELISA reagents which include monoclonal coating and conjugated antibodies for a wide range of viruses including PVY and PPV.
3. In the described “immunocapture” procedure, the time for coating and sap incubation can be reduced to 30–60 min each. However, short incubation time could impair the efficiency of the IC step.
4. SNaPshot primers should be designed less than 120 nucleotides in length. Best results are obtained for probes in a range of 30–80 nucleotides.
5. Purification procedure of SNaPshot primers (SNP probes) is one crucial factor to obtain a reliable SNaPshot profile. During the development of a SNaPshot assay, PAGE purification of each used primer is highly recommended. However, for economic reasons, desalted primers could be tested, and if results fit expected ones, then this quality of primers can substitute for PAGE-purified primers for routine use of the assay.
6. Individual purification of PCR products is recommended, and if necessary, the purified PCR products can be mixed.

Purification of labeled primers shouldn't be omitted (use either CIP, SAP, or ExoI). Partial purification produces extra peaks on electropherogram or decreases the height of expected peaks.

7. SNaPshot primer concentration is optimal at 0.2 µM. However, this parameter can be optimized in case of high intensity of peaks. In this case, decreasing the quantity of PCR products has little or no effect.
8. After the primer extension step, the labeled products can be stored at 4 °C until 24 h before electrophoresis or at -20 °C for longer period of storage.
9. The SNaPshot protocol is described for ABI PRISM 3130XL Genetic Analyzer using 36-cm capillaries and POP-7™ polymer. Other instruments or materials are likely to impact the expected results, and a running test experiment is always necessary to adapt the current version of the SNaPshot procedure.
10. Weak fluorescence signals can be due to a low-temperature annealing of SNaPshot primer(s), low efficiency of the primer extension procedure, or inadequate SNaPshot primer(s) concentration(s).
11. Due to the structural characteristics of fluorescent dyes, the length of short-labeled primers is generally overestimated by the computer analysis. Indeed, the presence of the dye induces a delay in migration equivalent to few nucleotides. Such bias on primer length assignment modifies the crude results. However, the differences in length (from 5 to 10 nucleotides) between SNaPshot primers were fixed to avoid consequences of such a bias.
12. Depending on its nucleotide sequence, a DNA fragment can have several conformations leading to several fluorescent peaks (Fig. 4). This behavior can be observed with one or with the two complementary ss-DNA strands. Double peaks or more ("complex profiles") may also be detected if several variants occur in the same sample. Further analysis by cloning/sequencing evidenced that complex profiles detected on region 2 were mostly related to the co-occurrence of two or more sequences in the same sample with variable relative frequencies (the dominant sequence being found in no more than 60 % of the sequenced clones).
13. Under our experimental conditions, a 20-fold dilution of the PCR products was sufficient to eliminate artifacts due to unincorporated fluorescently labeled primers that can anneal to SSCP conformers (primers-ss-DNA fragments) during

denaturing and cooling prior to CE. If needed, PCR products can be purified using various methodologies and commercial kits. In preliminary analyses, we successfully tested the ExoSAP-IT™ procedure.

14. Low fluorescence signals may be due to poor PCR amplification or to poor injection due to large amount of competing PCR components (nucleotides, primers, and/or primer dimers) present in the non-purified PCR fractions. If such a problem occurs, it is recommended to proceed to the purification of the PCR products.
15. No formamide was added to the PCR products before thermal denaturation, as previously reported [20]. The use of formamide reduces the efficiency of electro-injection of the samples in the capillaries without increasing the efficiency of DNA denaturation and the stability of single-strand conformers [21]. A 90-min running time allows the detection of all peaks of the internal marker. However, based on the size of ss-DNA molecules and their migration in non-denaturing gel, electrophoresis can be easily shortened to 60 min or even 45 min for the detection of the sample peaks of studied PPV regions.
16. The addition of reference samples on each plate allows easier assignments of the samples and helps controlling slight inter-plates variability.
17. The CE-SSCP procedure is described for the MegaBACE™ DNA analysis system and with the 6 % SPLA matrix commercialized by Genomac International Ltd. The type of sieving matrix and the electrophoretic conditions (temperature and voltage) strongly influence the resolution of SSCP analysis. Any modification of those conditions and the use of other instruments will automatically require new optimizations of the described procedure. Applied Biosystems developed specific products and methodological procedures for CE-SSCP analyses on ABI PRISM® 3100 Genetic Analyzer, for example.

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## Acknowledgments

The authors thank Alexandra Blanchard and Lucie Mieuzet (INRA, Le Rheu, France) for their help during the setup of the SNaPshot procedure, Marise Guillet (FNPPPT/INRA, Le Rheu, France) for providing the PVY-specific antibody, and Maxime Galan (CBGP, Montpellier, France) for technical advices for CE-SSCP analysis and technical support with the MegaBACE™ DNA analysis.

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# Chapter 16

## Detection and Characterization of Viral Species/Subspecies Using Isothermal Recombinase Polymerase Amplification (RPA) Assays

Laurent Glais and Emmanuel Jacquot

### Abstract

Numerous molecular-based detection protocols include an amplification step of the targeted nucleic acids. This step is important to reach the expected sensitive detection of pathogens in diagnostic procedures. Amplifications of nucleic acid sequences are generally performed, in the presence of appropriate primers, using thermocyclers. However, the time requested to amplify molecular targets and the cost of the thermocycler machines could impair the use of these methods in routine diagnostics. Recombinase polymerase amplification (RPA) technique allows rapid (short-term incubation of sample and primers in an enzymatic mixture) and simple (isothermal) amplification of molecular targets. RPA protocol requires only basic molecular steps such as extraction procedures and agarose gel electrophoresis. Thus, RPA can be considered as an interesting alternative to standard molecular-based diagnostic tools. In this paper, the complete procedures to set up an RPA assay, applied to detection of RNA (*Potato virus Y*, *Potyvirus*) and DNA (*Wheat dwarf virus*, *Mastrevirus*) viruses, are described. The proposed procedure allows developing species- or subspecies-specific detection assay.

**Key words** Isothermal amplification, Specific primers, RNA viruses, DNA viruses, Rapid diagnosis

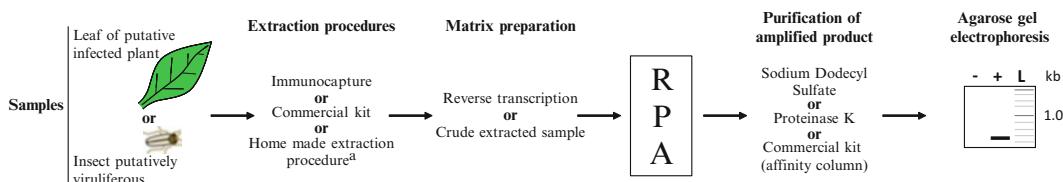
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### 1 Introduction

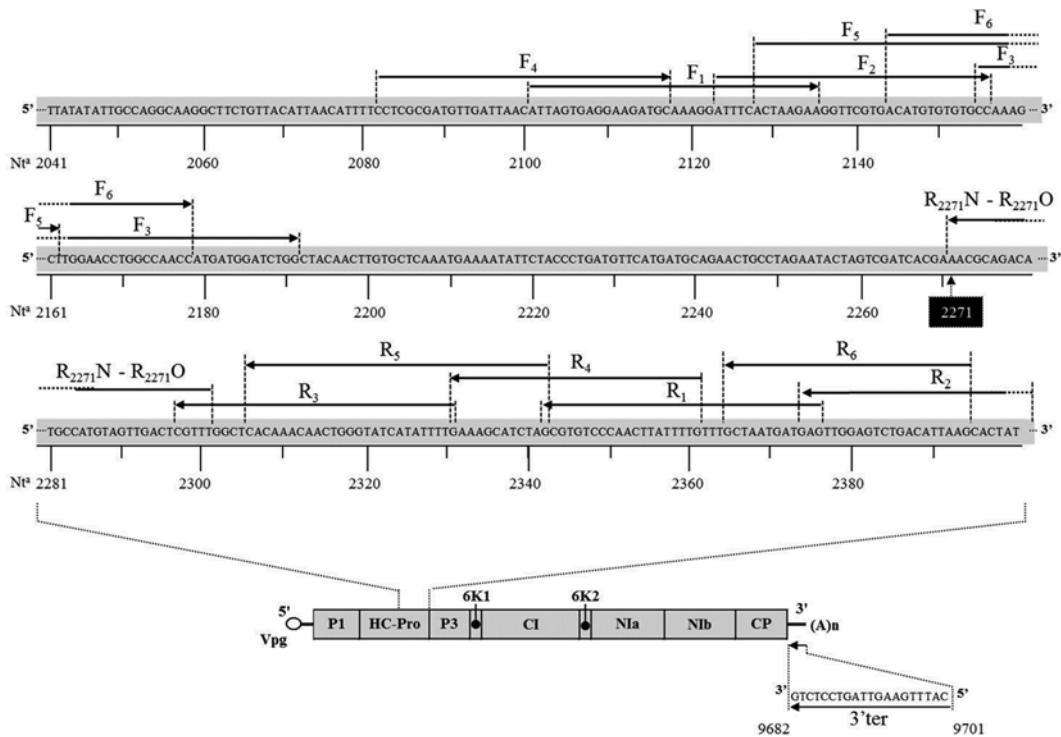
Detection and characterization of pathogens can be achieved by different techniques ranging from the observation of symptoms on putatively infected organs to the latest developed molecular-based tool (e.g., next-generation sequencing tools). Among the biological, serological, and molecular techniques available for pathogen detection [1, 2], the selection of the most appropriate tool is generally determined by (1) parameters associated to the characteristics of the pathogen itself and (2) the objectives of the research project. The sensitivity, specificity, and reliability of the different techniques are key selection criteria in deciding which method is fit for purpose. Finally, the complexity of the diagnosis procedure (e.g., sample preparation, requested facilities, and equipment) and

the total time of the assays from sampling to diagnosis result can also be taken into account by scientists to select the most appropriate diagnostic tool(s) for their researches. During the past 40 years, diagnostic techniques have continuously evolved, and new techniques have been developed in order to both improve and simplify the detection and the characterization of targeted organisms [3–7]. Reduction of the limit of detection from serological methods developed in the 1970s [1] to the latest PCR-based techniques [6] is an important aspect. However, the gain in sensitivity for pathogen detection was often associated with the use of costly technology (e.g., fluorescent-based detection techniques) requiring the use of expensive apparatus (e.g., real-time thermocyclers). The current challenge in the development of diagnostic tools is to combine sensitivity and specificity of detection with rapidity and cost-effectiveness. The recently developed recombinase polymerase amplification (RPA) technique represents one of the possible solutions for fast and simple molecular-based detection of pathogens.

In order to illustrate the isothermal RPA technique in viral diagnosis, we developed RPA assays for the detection of *Potato virus Y* (PVY, *Potyvirus*, aphid-transmitted single-stranded positive sense RNA virus infecting *Solanaceae*) [8] and *Wheat dwarf virus* (WDV, *Geminivirus*, leafhopper-transmitted circular single-stranded DNA virus infecting *Poaceae*) [9]. Moreover, versions of the RPA assays allowing the characterization of these pathogens at the subspecies level (PVY<sup>O</sup> vs. PVY<sup>N</sup> and WDV vs. WDV-Bar, respectively) are also presented in the manuscript in order to illustrate the possible applications of RPA in virus detection/characterization. A schematic representation of the diagnostic process and the genetic organization of PVY including targeted sequences (located within the HC-Pro gene) used to design RPA primers are presented (respectively, Figs. 1 and 2). Nucleotide A/C<sub>2271</sub> corresponds to a polymorphic site in the viral genome used to discriminate PVY<sup>N</sup> (using isolate 605 as reference for PVY<sup>N</sup> group) [10] and PVY<sup>O</sup> (using isolate 139 as reference for PVY<sup>O</sup> group) [11] isolates in the presented techniques.



**Fig. 1** From sampling to results: RPA, a rapid technique for reliable diagnosis of viral pathogens. The different steps of the RPA procedure are presented with a schematic agarose gel electrophoresis illustrating results obtained for RPA-based detection of *Potato virus Y* using healthy (lane “−”) and infected (lane “+”) samples. Protocol allowing direct noninvasive extraction of nucleic acids from insects is presented maintaining insect integrity for further entomological studies



**Fig. 2** Map of primers designed for the development of an RPA assay raised against PVY sequence. The nucleotide sequence of the 2041–2400 region from isolate PVY<sup>N</sup>-605 [10] is presented. Forward (F1 to F6) and reverse (R1 to R6) primers used in the first step of RPA are presented above their hybridization sites. The PVY<sup>N</sup>/PVY<sup>O</sup> polymorphic nucleotide 2271 is highlighted by a black box. This nucleotide has been used to design the R<sub>2271</sub>N and R<sub>2271</sub>O primers specific to PVY<sup>N</sup> and PVY<sup>O</sup> isolates, respectively (list of primer sequences available in Table 1). A schematic representation of the whole PVY genome is presented below to locate (1) the 2041–2400 nt region at the 3'-end of the HC-Pro gene and (2) the hybridization site of the 3' ter primer used for the reverse transcription step. <sup>a</sup>: nucleotide numbers according to Jakab et al. [10]

## 2 Materials

### 2.1 Required Materials, Products, and Chemicals

#### 2.1.1 General

1. Sterile pestle, mortar, and razor blades.
2. Sterile RNase/DNase-free plasticware (microtubes, tips, etc.).
3. Standard lab devices (pipettes, storage and sample racks, gloves, etc.).
4. Storage/incubating devices for -80 °C, -20 °C, +4 °C, +37 °C, and +42 °C.
5. Wet ice.
6. Absorbent paper.
7. Centrifuge.
8. Parafilm®.

**Table 1**  
**Sequence and position of primers used in the PVY RPA setup procedure**

	Name	Sequence (5'-3')	Position <sup>a</sup>
Reverse transcription	3'ter	GTCTCCTGATTGAAGTTTAC	9682
Generic PVY RPA assay	F <sub>1</sub>	ATTAGTGAGGAAGATGCAAAGGATTCACTAAGAA	2101–2136
	F <sub>2</sub>	ATTTCACTAAGAACGGTTCGTGACATGTGTGCC	2123–2156
	F <sub>3</sub>	CCAAAGCTTCCAACCTGGCAACCATGATGGATCTGG	2155–2191
	F <sub>4</sub>	CCTCGCGATGTTGATTAACATTAGTGAGGAAGATGC	2082–2117
	F <sub>5</sub>	ACTAAGAACGGTTCGTGACATGTGTGCCAAAGC	2128–2161
	F <sub>6</sub>	ACATGTGTGCCAAAGCTTGGAACCTGGCAACCC	2144–2178
	R <sub>1</sub>	CTCATCATTAGCAAACAAAATAAGTTGGGACACGC	2342–2376
	R <sub>2</sub>	CCAACTCTATAGTGTCTTAAATGTCAGACTCCAAC	2374–2408
	R <sub>3</sub>	CAAATATGATACCCAGTTGTTGTGAGGCCAAACG	2297–2331
	R <sub>4</sub>	CAAATAAGTTGGGACACGCTAGATGCTTTC	2331–2361
	R <sub>5</sub>	CTAGATGCTTCAAATATGATACCCAGTTGTTGTG	2306–2342
	R <sub>6</sub>	CTTAATGTCAGACTCCAACTCATCATTAGC	2365–2394
Specific PVY <sup>N/O</sup> RPA assay	F <sub>4</sub> O	TCTTGCAATGCTAAATTAAACATTGGCGAGGGAGGATGC	2082–2117
	R <sub>2271</sub> N	AAACGAGTCAACTACATGGCATGTCTGCGTT	2271–2301
	R <sub>2271</sub> O	AAACGAGTCAACTACATGGCATGTCTGCGTG	2271–2301

<sup>a</sup>Nucleotide position according to Jakab et al. [10]

### 2.1.2 Extraction of Nucleic Acid (ARN, ADN)

1. Deionised water.
2. Microtitration plate [NUNC PolySorp].
3. PVY polyclonal antibodies [FN3PT/INRA, Le Rheu].
4. Deep-well microtitration plate.
5. Absolute ethanol.
6. miVac DNA apparatus [GeneVac Ltd., Ipswich, UK].
7. DNeasy plant kit® [QIAGEN].
8. SV RNA extraction kit® [Promega].

### 2.1.3 cDNA Synthesis

1. AMV reverse transcriptase [Promega].
2. RNase-free water.
3. RNAsine® [Promega].
4. 10 mM dNTPs (2.5 mM each).
5. Primer (10 mM).

### 2.1.4 Isothermal Viral Sequence Amplification

1. Molecular biology quality (ultrapure) water.
2. PCR plates.
3. RPA primers (see Figs. 2 and 6; Tables 1 and 2).
4. TwistAmp® Basic RT [TwistDx].

**Table 2**  
**Sequence and position of primers used in the WDV RPA setup procedure**

	Name	Sequence (5'-3')	Position <sup>a</sup>
Generic RPA assay	1F	TAGTCGGCAAGAATATCGTGGACCGCGAACAA	998–1027
	2F	CAAAGGTTTGCCTGTACGACGGAGTGGATGAACAC	1035–1070
	3F	CAAGATAGGCCACATTAAGAAGGGAGCACTGTATC	1080–1114
	4F	GGTGGGTGTTACTGGTGACAGTGCTCTACGGCGTT	1132–1167
	R	GGCGAAGATGTCACTGCCTCGCAGTGACTGTCCTAG	1388–1423
	5R	TCTACTCCGTAAGCCTCGAATCCTATATCATTGTA	1881–1916
	6R	ATCCTATATCATTGTAACCTCCACTCCTGCGGATCA	1862–1897
	7R	GGATCAAGCGCAATCTGACTTAGAGTGGATGGACG	1833–1867
	8R	GGATGGACGATTATTCCAGGAGTCACCGGGGAGGC	1807–1841
Specific WDV RPA assay	W1F	CATCTTCGCCGGAGGCGAACGAGTAGTTGAT	1414–1444
	W2F	GACATCTTCGCCGGAGGCGAACGAGTAGTTG	1412–1442
	W3F	GTGACATCTTCGCCGGAGGCGAACGAGTAGT	1410–1440
	W4F	CAGTGACATCTTCGCCGGAGGCGAACGAGTA	1408–1438
	W5F	AGCAGTGACATCTTCGCCGGAGGCGAACGAG	1406–1436
Specific WDV- Bar RPA assay	Wb1R	GTTCATTATATGTATGAGGGTGAATCATTCTCG	1429–1432// 1445–1474
	Wb2R	GTTCATTATATGTATGAGGGTGAATCATTCTC	1430–1432// 1445–1474
	Wb3R	ATTATATGTATGAGGGTGAATCATTCTCG	1429–1432// 1445–1470

<sup>a</sup>Nucleotide position according to Kvarnhen et al. [18]

### 2.1.5 Purification of RPA Products

1. Sodium dodecyl sulfate 20 %.
2. Proteinase K (20 mg/mL) [Eurobio].
3. Wizard® SV Gel and PCR Clean-Up System [Promega] or commercial equivalent purification kit of PCR products.

### 2.1.6 Analysis of Amplified Products

1. Agarose.
2. Agarose gel electrophoresis device.
3. SybrSafe™ [Invitrogen].
4. DNA Smart Ladder [Eurogentec].
5. Ultraviolet imaging system.

### 2.2 Standard Solutions and Buffers

All homemade buffers, solutions, and mixtures have to be prepared with sterile deionized water or with molecular biology grade water. Primers used in the presented procedure are described in Table 1 (for PVY) and Table 2 (for WDV).

1. TBE buffer, pH 8.0: 10.78 g Tris, 5.50 g boric acid, 0.58 g EDTA, deionized water to prepare 1 L.
2. 3 M NaOAc, pH 5.5: 36 mL acetic acid, 114 mL sterile water, 24 g NaOH. Complete to 200 mL with sterile water.
3. PBS buffer: 8 g NaCl, 2.9 g Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 0.2 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, deionized water to prepare 1 L.

4. Carbonate buffer: 1.59 g Na<sub>2</sub>CO<sub>3</sub>, 2.93 g NaHCO<sub>3</sub>, deionized water to prepare 1 L.
5. Washing buffer: PBS, 0.05 % (v/v) Tween 20.
6. Extraction buffer: PBS, 0.05 % (v/v) Tween 20, 0.2 % (w/v) polyvinylpyrrolidone.
7. Ethanol 70 % solution must be prepared using absolute ethanol.
8. TNES buffer: 50 mM Tris–HCl pH 7.5, 400 mM NaCl, 20 mM EDTA pH 8.0, SDS 0.5 % (w/v).

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### 3 Methods

The methods described below outline the following: (1) the viral or total nucleic acid extraction procedures from leaf samples and from leafhoppers and (2) the isothermal recombinase polymorphism amplification (RPA) assay.

#### 3.1 Nucleic Acid Extraction

The first required step to perform any molecular-based detection procedure is to extract from the original sample a fraction containing nucleic acids. Different procedures, adapted according to the infected biological material to analyze, make possible such extraction step. Among them, viral RNA extraction procedure from plant tissues by immunocapture (IC) and viral DNA extraction from insect are described. As an alternative to these extraction procedures, commercial kits for nucleic acid extractions exist and can be used. The benefit of these kits is presented. Finally, homemade extraction procedures (e.g., phenol/chloroform extraction) can also be applied to extract total nucleic acids for RPA-based diagnosis.

##### 3.1.1 Immunocapture

The immunocapture (IC) corresponds to the first step of standard ELISA procedure [12]. This does not constitute a bona fide nucleic acid extraction procedure. It allows the specific attachment of viral particles on antibody-coated wells of a microtitration plate. Viral RNA or DNA can then be extracted from captured virus particles.

1. Collect, in a sterile microtube, leaf fragments (0.2 g) from plant using sterile razor blade or the cap of the microtube as a perforating device.
2. Grind collected sample using a sterile plastic pestle (adapted to microtubes) in the presence of 200 µL of sterile extraction buffer to produce crude sap extract.
3. Prepare the anti-PVY antibody solutions (1 µg/mL) in sterile carbonate buffer.
4. Coat the wells of a microtitration plate with 100 µL of PVY polyclonal antibody, and incubate the plate for 2 h at 37 °C.

5. Wash the plate three times with sterile washing buffer. Empty the IC plate after the last wash either by soaking on absorbent paper or by pipetting.
6. Load 100 µL of crude sap extract in a coated well, and incubate overnight at 4 °C (*see Note 1*).
7. Wash plates three times with sterile washing buffer, and discard the buffer after the last wash either by soaking on absorbent paper or by pipetting.
8. Store Parafilm®-covered plates at -20 °C until used.

### 3.1.2 DNA Extraction from Insect

Total nucleic acid extraction procedure on insects is performed using a noninvasive method. Live insects (sampled from fields or from rearing systems) are placed in absolute ethanol before nucleic acid extraction.

1. Transfer the insects from absolute ethanol to sterile microtubes in presence of 500 µL ethanol 70 %, wait 5 min, and repeat this step once.
2. Transfer the insects onto a microtitration plate wells (one insect per well), and dry the wells at room temperature (to reduce time needed to dry the plate, this step can be performed using a miVac DNA apparatus).
3. Add 150 µL of TNES buffer, supplemented with 2 µL of Proteinase K (20 mg/mL), in each well, and incubate the plate overnight at 55 °C.
4. Centrifuge at 1,800 ×*g* for 1 min at 20 °C and transfer supernatant into wells of a clean deep-well microtitration plate.
5. Wash the insect bodies left at the bottom of the wells of the first plate twice with deionized water, empty the wells by pipetting, add 100 µL of ethanol 96 % (or absolute ethanol) in each well, and finally, stored Parafilm®-covered plates at -20 °C until used.
6. Add 45 µL of cold NaCl (5 M) to the 150 µL fraction present in wells of the deep-well microtitration plate and homogenate gently the mixture.
7. Centrifuge at 5,000 ×*g* for 10 min at 4 °C and transfer the resulting supernatant in a new deep-well plate already filled with 500 µL/well of cold absolute ethanol.
8. Place the plate for 20 min at -80 °C (or for a couple of hours at -20 °C), and centrifuge at 5,000 ×*g* for 10 min at 4 °C.
9. Turn (with a gently move) upside down the plate to discard the supernatants (nucleic acids should stay as pellets at the bottom of the well following centrifugation); in case of any doubt on the stability of the nucleic acids pellets, remove supernatant by pipetting.

10. Wash wells with 250 µL ethanol 70 %, remove ethanol as recommended above, and dried empty plate at 30 °C using the miVac DNA.
11. Resuspend dried pellets in 50 µL of sterile water and store Parafilm®-covered plates at -20 °C until used.

### 3.1.3 Commercial DNA and RNA Extraction Kits

Numerous DNA and RNA extraction kits are available commercially to prepare purified nucleic acids fractions. Some kits propose DNA extraction procedures (e.g., DNeasy plant kit®, [QIAGEN]), while others are suited for RNA extraction (e.g., SV RNA extraction kit®, [Promega]). All these kits are based on successive steps corresponding to grinding, lysis, clarifying, nucleic acid–matrix interactions, washing, and elution. A DNase/RNase-free (in RNA extraction kits) or an RNase/DNase-free (in DNA extraction kits) enzymatic activity is generally included either within one of the previously listed steps or as an additional step. Differences between kits lie in the time required to perform the complete extraction protocol, the requirement of centrifugation or vacuum devices, the maximal nucleic acids yield, and the purity (quality) of eluted fractions. They must be tested and compared for each application to identify the most adapted kit for the material to be tested.

## 3.2 Reverse Transcription (RT)

The reverse transcription (RT) is performed after the immobilization of the viral particles in the microtitration plate (*see Subheading 3.1.1*). If IC plates have been stored at -20 °C, transfer plates from -20 °C to dry ice and remove Parafilm®. IC plates can then be used in the reverse transcriptase assay without further manipulation of the viral material. Wells were filled with the RT reaction mixture (20 µL/reaction) and incubated at optimal temperature for AMV reverse transcriptase activity, i.e., 42 °C for an hour. The cDNA corresponding to PVY genome was produced using a reverse primer complementary to the 3'end of the viral sequence (Fig. 2 and Table 1, 3'ter primer).

1. Maintain plate, enzymes, and dNTPs in wet ice during the RT preparation steps.
2. Prepare RT mix (20 µL/sample) and adapt the total volume to prepare according to the number of samples to test. Final concentrations in RT mix are: 1× buffer, 1 mM of dNTPs, 2.5 mM MgCl<sub>2</sub>, 20 U of RNasin® ribonuclease inhibitor, 2 U of reverse transcriptase, and 1 mM appropriate primer.

5× buffer (provided with enzyme)	4 µL
10 mM dNTPs (2.5 mM each)	2 µL
10 mM primer (3'ter)	2 µL
Sterile RNase-free water	4.3 µL

(continued)

40 U/ $\mu$ L RNasine®	0.5 $\mu$ L
10 U/ $\mu$ L AMV reverse transcriptase [Promega]	0.2 $\mu$ L
25 mM MgCl <sub>2</sub>	2 $\mu$ L
Total nucleic acids extract ( <i>see Note 2</i> )	5 $\mu$ L

3. Incubate 1 h at 42 °C.
4. Maintain the plate at 4 °C for direct use of the produced cDNA in the RPA procedure or store the plate at -20 °C until used.

### 3.3 Isothermal Recombinase Polymerase Amplification (RPA)

RPA technology uses two enzymes which promote (1) pairing oligonucleotide primers with homologous sequence in duplex DNA and (2) simultaneously synthesize DNA and separate double-stranded DNA molecules. The advantage compared to the classical PCR technique is the fact that RPA operates at 37 °C and amplification of the targeted genomic region is achieved after incubation of 30–60 min. RPA was performed using the TwistAmp® Basic kit (TwistDx, Cambridge, UK). The RPA assay is performed in a 50  $\mu$ L volume in the presence of 500 nM forward and reverse primers, 14 mM magnesium (Mg) acetate, 2  $\mu$ L of extracted nucleic acids, DNase-free water, and TwistAmp® (RPA) enzymes. These products, except Mg acetate and TwistAmp® enzymes, were prepared in a mastermix. The latter is distributed in tubes (supplied by the TwistAmp® Basic kit) containing dried enzymes. Nucleic acid template is added to the tubes prior to Mg acetate as addition of this compound will activate RPA enzymes and is added last to start the amplification process.

1. Prepare the following RPA mixture (50  $\mu$ L/reaction):

Rehydration buffer	29.5 $\mu$ L
10 $\mu$ M forward primer	2.4 $\mu$ L
10 $\mu$ M reverse primer	2.4 $\mu$ L
25 mM dNTPs	1 $\mu$ L
Viral template ( <i>see Note 3</i> )	2 $\mu$ L
Mg acetate (280 mM)	2.5 $\mu$ L

2. Incubate 30–60 min at 37 °C (*see Notes 4–6*).
3. At the end of incubation time, go to purification steps.

#### 3.3.1 Purification of Amplified RPA Products

At the end of the amplification step, the DNA fragments produced by the RPA system are associated to the enzymes promoting the amplification of the targeted genomic regions. These DNA–protein complexes prevent the direct use of DNA molecules for further analysis. Moreover, the DNA–protein complexes result in stable large polymers that cannot be easily separated in

agarose gel electrophoresis. Consequently, to be able to accurately analyze the RPA results and/or to use the amplified DNA fragments in further molecular biology steps, the isothermal amplification procedure must include a purification step to remove these proteins. Three different purification procedures were tested. In routine use, only one out of the three purification methods should be used. The choice of the most appropriate method depends on criteria such as the time and the cost associated to the purification procedure and the aim of the RPA assay (diagnosis or more complex molecular biology procedure).

#### Proteinase K Treatment

Proteinase K is a serine protease used in molecular biology to digest proteins in samples [13]. While Proteinase K is described to be fully efficient in its specific buffer (*see* Proteinase K provider's recommendations), it will be active in most of enzymatic buffers used in molecular biology. Thus, it can be used directly in the RPA mixture after the amplification step.

1. Add 1  $\mu$ L of Proteinase K (20 mg/mL) to the RPA mixture.
2. Incubate 1 h at 55 °C.
3. Check the quality of purified RPA products by electrophoresis on agarose gel 1 % (100 V, 30 min).
4. Store the mixture at 4 °C (short-term storage) or at -20 °C (long-term storage) until used.

#### SDS Treatment

Sodium dodecyl sulfate (SDS) is a strong detergent commonly used in preparing proteins for denaturing polyacrylamide gel electrophoresis (PAGE) technique [14]. SDS is able to interact with proteins by disrupting non-covalent bonds involved in protein–protein interactions. Consequently, in the presence of SDS, proteins lose their native conformation. In the purification procedure of RPA products, SDS can be used to alter molecule–molecule interactions and facilitate DNA migration in the agarose gel electrophoresis.

1. Add 2  $\mu$ L of SDS (20 %) to the RPA mixture.
2. Check the quality of purified RPA products by electrophoresis on agarose gel 1 % (100 V, 30 min).
3. Store the mixture at 4 °C (short-term storage) or at -20 °C (long-term storage) until used.

#### Commercial PCR Clean-Up Kits

PCR clean-up kits (e.g., Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) are designed to extract and purify DNA fragments directly from post-PCR amplification mixtures by passage (fixation, wash, and elution of nucleic acids) through anionic columns. The different kits propose similar protocols to purify DNA fragments. The protocol applied to RPA samples is according to manufacturer's recommendations. Here, a simplified version of the

PCR Clean-Up System is presented. For detailed PCR Clean-Up System procedures, please refer to manufacturer's information.

1. Add an equal volume of membrane binding solution to the RPA mixture (i.e., 20 µL).
  2. Load the 40 µL mixture on the minicolumn (from the kit), and incubate at room temperature for 1 min.
  3. Centrifuge at 16,000 ×*g* for 1 min at room temperature.
  4. Discard flow through and add 700 µL of membrane wash solution to the center of the PCR Clean-up minicolumn.
  5. Centrifuge at 16,000 ×*g* for 1 min at room temperature.
  6. Repeat **step 4** once with 500 µL membrane wash solution and centrifuge at 16,000 ×*g* for 5 min at room temperature.
  7. Transfer the PCR Clean-up minicolumn in a sterile microtube.
  8. Add 50 µL of nuclease-free water to the center of the PCR Clean-up minicolumn.
  9. Incubate 1 min and centrifuge at 16,000 ×*g* for 1 min at room temperature.
  10. Discard the PCR Clean-up minicolumn.
  11. Check the quality of purified DNA molecules by electrophoresis on agarose gel 1 % (100 V, 30 min).
- Or
12. Store eluted DNA fraction at 4 °C (short-term storage) or -20 °C (long-term storage) until used.

### **3.4 Data Analysis**

#### *3.4.1 Design and Selection of Most Appropriate Primer Pairs for RPA Assay*

The selection of the targeted region of the viral genome is important in the procedure as this region will be used to design primers for routine detection of the pathogen. In the presented procedure, the selection of the HC-Pro region was determined by both data available on genomic variability of PVY genomes and the previously identification of single nucleotides (e.g., A/C<sub>2271</sub> from HC-Pro region) involved in one of the main biological properties of PVY isolates, i.e., the necrosis capacity [15–17].

In the region overlapping nucleotides 2041–2400 (3'-end of HC-Pro gene) of the PVY genome, 6 forward and 6 reverse primers were designed according to the following guidelines:

- Primers must be in the range 30–35 nucleotides long.
- Long tracks of guanines at the 5'-end (first 3–5 nucleotides) should be avoided.
- Cytidines (and in general pyrimidines) may be beneficial at the 5'-end of primer sequence.
- Primer with guanines and cytidines at their 3'-end (last 3 nucleotides) tends to improve performance of RPA assay.

- Avoid long tracks of one particular nucleotide and large number of small repeats and sequences that favor secondary structure of primers, primer–primer interactions, and hairpins.
- Maintain GC content in the range 30–70 %.
- Select forward and reverse primer sites to amplify fragments that not exceed 400 bp in length.

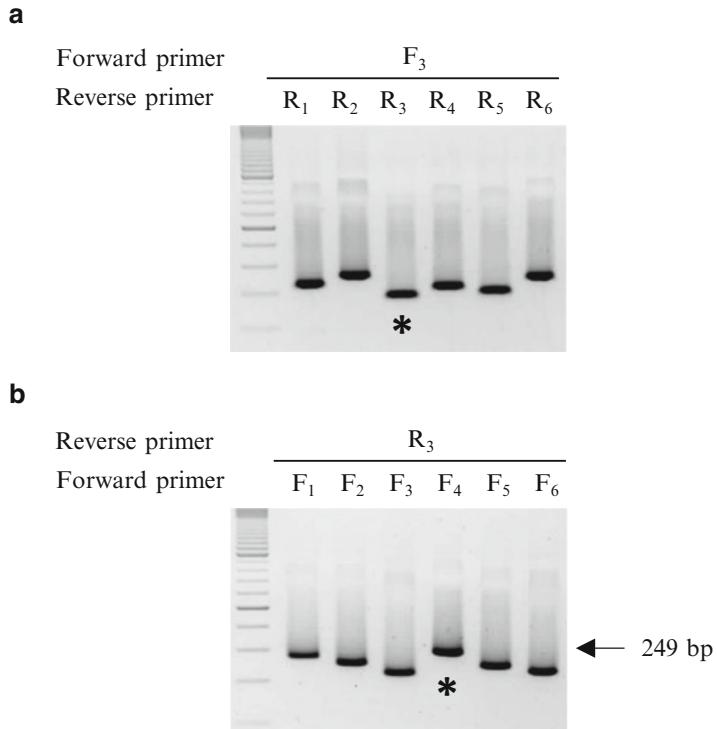
Based on these guidelines, standard RPA primers have been designed (Figs. 2 and 6; Tables 1 and 2) and used in different forward/reverse primer combinations to identify the “best” primer pair for RPA assay.

The selection of the optimal primer pair was achieved using a two-step procedure. First, the 6 reverse primers were tested in the presence of the forward primer F<sub>3</sub> (randomly selected from the available forward primers) for their ability to amplify PVY sequence. As no obvious difference was found between amplified DNA fragments obtained with the six tested primer pairs, reverse R<sub>3</sub> primer was chosen for the next step of the RPA setup (Fig. 3a). Then, the six forward primers were tested, associated to R<sub>3</sub> primer, for their ability to amplify PVY targeted region. Results associated to this second step do not highlight differences between the tested forward primers (Fig. 3b) for their ability to produce expected DNA fragments. Consequently, F<sub>4</sub> primer was randomly selected out of the six forward primers. Future assays carried out with the RPA-based PVY detection tool will be performed with F<sub>4</sub>/R<sub>3</sub> primer pair. Efficient PVY detection will correspond to the amplification of a DNA fragment of 249 bp (Fig. 3b) (*see Note 7*).

### 3.4.2 Effect of RPA Purification Steps

Analysis of the RPA products at the end of the amplification step can be carried out by agarose gel electrophoresis. Several actions (including purification of RPA products) must be performed to analyze unambiguously RPA results (i.e., amplification of a DNA fragment corresponding to the targeted genomic region). The three purification methods (Proteinase K, SDS treatment, and commercial PCR clean-up kit) tested during the setup procedure gave similar results (Fig. 4). The most appropriate purification method must be determined according to the intended use of the RPA-amplified fragments. For diagnostic purpose, the SDS treatment (cheap and fast purification procedure) is more appropriate, whereas for further use of the DNA fragments in molecular biology procedures, commercial purification kits are recommended.

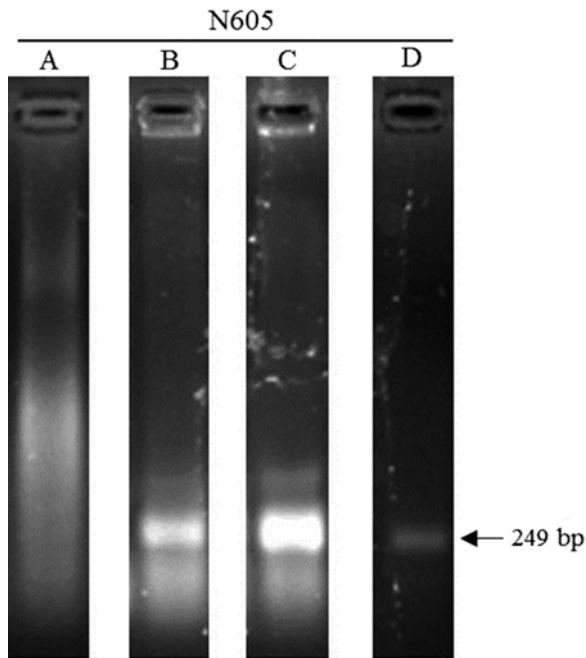
The presented RPA procedures for PVY detection and associated purification step (Subheading 3.3.1) have been tested on a library of 12 PVY isolates from France, Ireland, Canada, Poland, the Netherlands, Slovenia, Belgium, and Greece. The developed RPA-based PVY assay allows detection of all PVY isolates from these different sources.



**Fig. 3** Selection of RPA forward and reverse primers. The different reverse (a) and forward (b) primers were tested for their capacity to amplify PVY sequence with RPA procedure carried out in the presence of the forward  $F_3$  primer (selection of the “best” reverse primer) or reverse  $R_3$  (selection of the “best” forward primer), respectively. PVY<sup>N</sup>-605 cDNA was used as matrix in the presented experiments. Asterisks indicate the reverse ( $R_3$ ) and forward ( $F_4$ ) primers selected for the PVY RPA assay. The next steps of RPA setup will be carried out with  $F_4/R_3$  primer pair giving an amplicon of 249 bp

### 3.4.3 RPA Assay for PVY Subspecies Discrimination

The main rationale of this assay is to diagnose necrotic and non-necrotic isolates by specifically targeting nucleotide 2271 (number according to Jakab et al. [10]), one of the known single nucleotide polymorphisms involved in the necrotic ability of PVY isolates. For this purpose, two reverse primers ( $R_{2271}N$  and  $R_{2271}O$ ) (see Fig. 2 and Table 1) were designed to allow specific hybridization of their 3'-end on nucleotide A/C<sub>2271</sub>. The sequences of  $R_{2271}N$  and  $R_{2271}O$  specific primers were determined according to the necrotic PVY<sup>N</sup>-605 (accession number: X97895) and the non-necrotic PVY<sup>O</sup>-139 (accession number: U09509), respectively. In the PVY<sup>N</sup>- and PVY<sup>O</sup>-specific RPA assays, these primers were used in association with  $F_4$  and  $F_4O$  forward primers, respectively. The  $F_4$  forward primer corresponds to the primer used in the RPA-based PVY detection assay described above (see Subheading 3.4.1). The  $F_4O$  primer is a modified version of the  $F_4$  primer with nucleotide



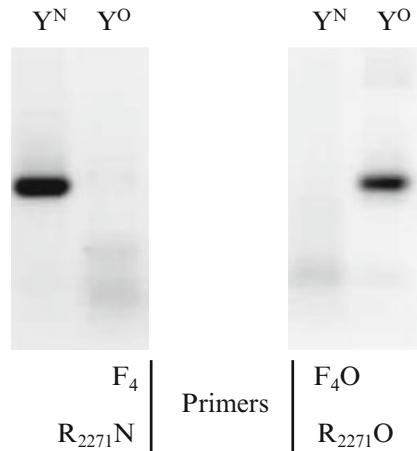
**Fig. 4** Comparison of different methods for purification of RPA products. PVY<sup>N</sup>-605 RPA products obtained by amplification with F<sub>4</sub>/R<sub>3</sub> primer pair (**a**) were purified with either Proteinase K (**b**), SDS 20 % (**c**), or commercial PCR clean-up kit (**d**)

sequence corresponding to PVY<sup>O</sup>-139 sequence. The use of F<sub>4</sub>O primer in RPA-based PVY<sup>O</sup> assay instead of the previously designed F<sub>4</sub> (type-N) primer was decided to improve the specificity of the assay. Applied to reference isolates, the two assays allow specific detection of the targeted PVY isolate (Fig. 5).

The RPA method is able to specifically detect and identify isolates of PVY<sup>N</sup> or PVY<sup>O</sup> strain groups. Due to its convenience and ease of implementation, this technology should be useful for researchers and diagnostic laboratories.

#### 3.4.4 Application of RPA Assay to Other Viral Species

An RPA assay was developed to diagnose *Wheat dwarf virus* (WDV) barley and wheat strains. WDV possesses a circular single-stranded DNA genome of 2.75 kb. Two WDV wheat and barley strains have been characterized and can be discriminated by targeting a polymorphic 12 nucleotides motif (nt 1433–1444, from WDV Enköping1 isolate accession number: AJ311031) [18] located in the replication-associated protein encoding region of the WDV barley strain genome. Alignments of WDV sequences retrieved from public databases helped to determine the best sites (most conserved genomic regions) for primer design. Thus, forward and reverse RPA primers were designed upstream the 12 nucleotides deletion in order to allow amplification of both wheat

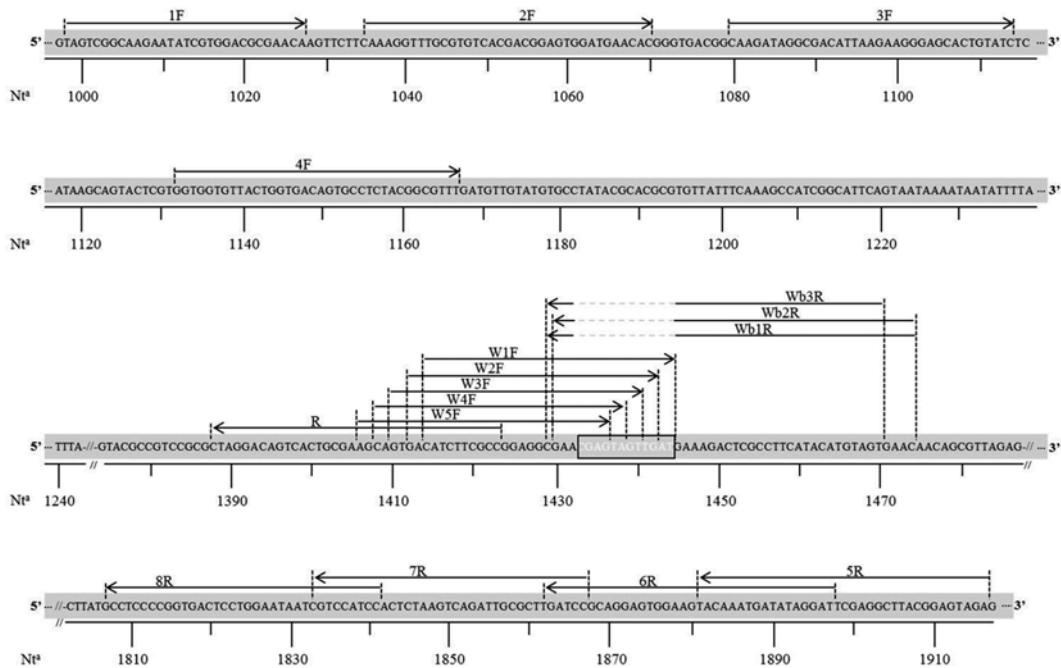


**Fig. 5** Strain-specific PVY RPA assays. The forward and reverse primers used for the PVY<sup>N</sup> (gel on the *left*)- and PVY<sup>O</sup> (gel on the *right*)-specific assays are indicated below the gels. Samples containing PVY<sup>N</sup> (Y<sup>N</sup>) or PVY<sup>O</sup> (Y<sup>O</sup>) cDNA were used as DNA matrix in the presented experiments. List of primer sequences is available in Table 1

and barley strain isolates (Fig. 6; Table 2). The different primer pair combinations were tested, and as no difference was observed between the primer pairs, the 2F/R primer pair was selected for routine detection of WDV isolates (Fig. 7a). For the development of strain-specific RPA assays, the sequence deleted in WDV barley strain genome was used to design series of strain-specific primers (*see* Fig. 6 and Table 2). The three WDV barley strain reverse primers and the five WDV wheat strain forward primers were associated to the 2 F forward and the 7R reverse primers, respectively (Fig. 7b, c). The primer pair selection procedure allows the identification of the optimal primer pair for the two WDV strain-specific assay. Based on the amplification profile observed on agarose gel electrophoresis (Fig. 7b, c), W1F/7R and 2F/Wb3R primer pairs were selected for the RPA-based specific detection of wheat and barley strain of *Wheat dwarf virus*, respectively.

#### 4 Notes

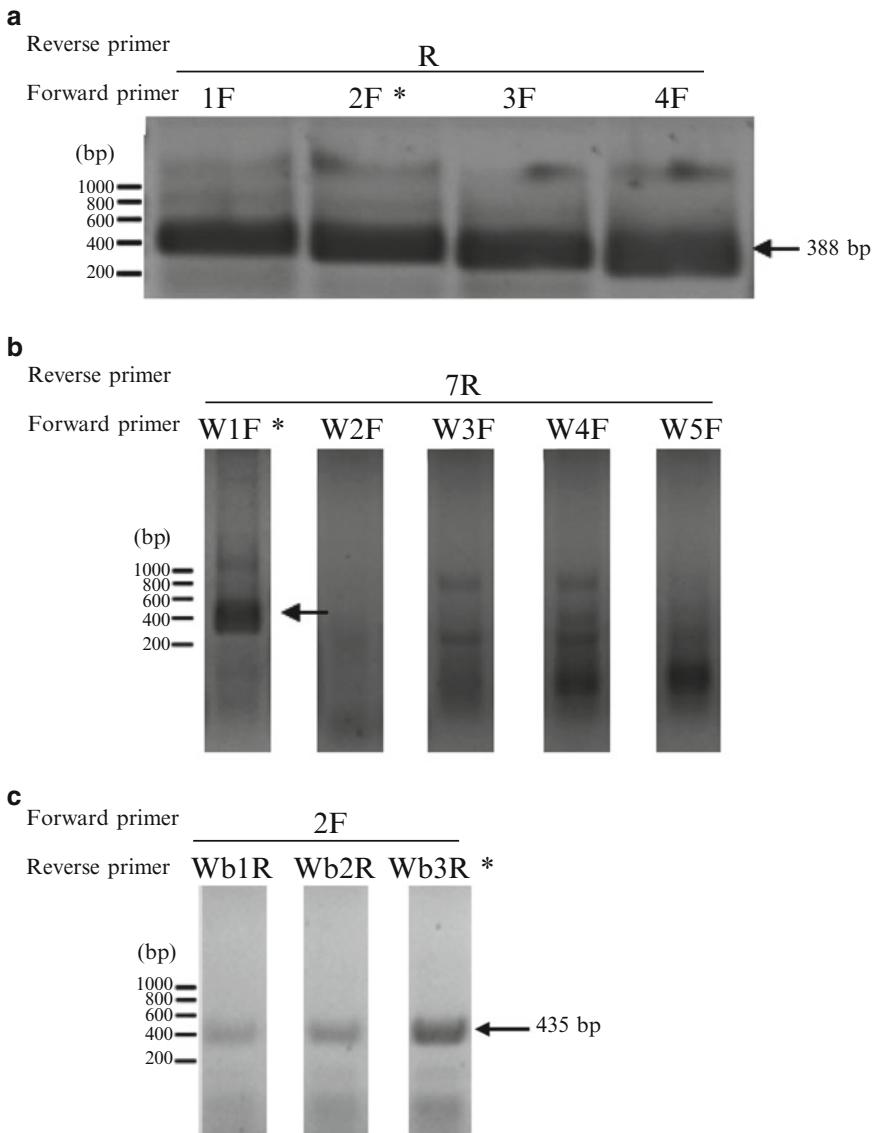
1. In the described “immunocapture” procedure, the crude sap extract is incubated in a coated well overnight at 4 °C, but this time can be reduced to 2 h at 37 °C. However, according to the virus load in sample, this new procedure could reduce the efficiency of virus extraction.
2. For samples prepared using the immunocapture procedure, total nucleic acids extract corresponds to fixed material in coated well of ELISA plate, and volume used is then 0 µL.



**Fig. 6** Names and positions of primers designed for the development of an RPA assay to detect all WDV strains (1F to 4F; 5R to 8R), specific to WDV wheat strains (W1F to W5F) or to barley strains (WDV-Bar) (Wb1R to Wb3R). A partial nucleic acid sequence (nt 997–1917) from Enkoping1 isolate (accession number: AJ311031) is presented [18]. Gaps, represented by //, are introduced within this sequence. The 12 nucleotides deletion (nt 1433–1444) differentiating WDV barley from wheat strains is boxed surrounded. Primers designed for specific detection of WDV-Bar strains (Wb1R, Wb2R, Wb3R) are represented by arrows cut at the deletion region (nt 1433–1444). List of primer sequences is available in Table 2. <sup>a</sup>: nucleotide numbers according to Kvarnveden et al. [18]

Thus, the mix must include 5 µL of nuclease-free water to substitute the volume of nucleic acids extract. RT reaction occurs directly in the well of microtitration plates.

3. Viral template corresponds to either cDNA produced from immunocapture- RT steps applied to leaf samples (infected or healthy samples) or total nucleic acids extracted from insects (viruliferous or virus-free).
4. To set up RPA assay, some parameters could be changed from the standard protocol such as amplification time and reaction temperature.
5. In the described assay, the RPA amplification of the targeted genomic regions is achieved in our conditions after 30–60 min incubation. However, according to the primers used (sequence, length) and the amplicon length, it is recommended to validate the RPA efficiency by increasing the amplification time from 30 min to overnight.



**Fig. 7** Selection of RPA forward and reverse primers for the generic amplification of WDV species (a) or specific amplification of WDV subspecies (b) or of WDV-Bar subspecies (c). Asterisks indicate the selected primers. All details relating to primers are summarized in Table 2

6. The optimal temperature applied in RPA protocol ranged from 37 to 42 °C. A higher temperature could impair the enzymatic activities, and a lower temperature does not allow the system to operate at maximum efficiency.
7. The analysis of the RPA products obtained during the primer selection procedure was not possible by direct load of RPA fractions on agarose gel. To obtain the high-quality gel images presented in Fig. 3, the RPA assay was extended from 30 to

60 min to overnight incubation at 37 °C. This long amplification process seems to lead to the production of molecules (DNA fragments) compatible with migration in agarose gel. However, as the results associated to long-term incubation were not repeatable (the quality of amplification signal, i.e., band on agarose gel is highly variable from the expected band to a “smear”), we recommend to use one of the purification method presented.

## Acknowledgments

The authors thank Maxime Assous-Dupont (INRA, Montpellier, France) and Isabelle Naas (INRA, Rennes, France) for their help during the setup of the RPA; Isabelle Abt and Romain Mabon (INRA, Montpellier) for providing the leafhoppers; and Maryse Guillet, from the FN3PT, for providing the PVY polyclonal antibodies.

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# **Chapter 17**

## **Virus Testing by PCR and RT-PCR Amplification in Berry Fruit**

**Stuart MacFarlane, Wendy McGavin, and Ioannis Tzanetakis**

### **Abstract**

Berry fruit crops are prone to infection by a wide range of viruses, with the list expanding every year, primarily because of the expansion of the crops to new geographic regions. Although some methods allow for virus detection in a nonspecific manner, the advent of cheap and effective nucleic acid sequencing technologies has allowed for the development of species-specific tests. This chapter describes methods for extraction of nucleic acids for molecular testing from a range of different berry fruit crops and lists oligonucleotide primers that have been developed for amplification of a large number of berry fruit viruses.

**Key words** Plant virus, Berry fruit, Strawberry, Raspberry, Blueberry, Elderberry, Detection, RT-PCR, RNA extraction, DNA extraction

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### **1 Introduction**

The most commonly grown berry fruits, also sometimes referred to as small fruits, include strawberry, blackberry, raspberry, blueberry, elderberry and various currants (such as blackcurrant, redcurrant, and gooseberry). These crops are grown worldwide, primarily in temperate climates, and the appreciation that they contain high levels of antioxidants and other biochemicals that might confer health benefits has increased their popularity among consumers and growers. These plants are perennial in habit and may have a productive life of more than 20 years (and more than 60 in the case of blueberry plants), although there is a shift to more frequent replacement of plants in some growing regimes to capture the advantages of the new, more productive cultivars. A major driver for the replacement of berry fruit plants is their increasing accumulation of diseases, particularly of viral cause, as they continue to grow in the field. It is also becoming clear that, for viral diseases, often the infection of a plant with a single virus produces no or very few visible symptoms. As the plant becomes further infected with other viruses, disease symptoms become more



**Fig. 1** Symptoms of Raspberry leaf blotch virus infection

apparent (Fig. 1) and fruit yield decreases, even leading, in the case of some virus complexes, to plant death.

A good example for plant decline and yield reduction is the raspberry crumbly fruit disease. This is one of the most important raspberry diseases worldwide, causing a reduction in fruit drupelet number that severely affects fruit yield and quality. Although virus infection is associated with this disease, other factors, both genetic and environmental, may also play a role. The involvement of raspberry bushy dwarf virus (RBDV), a pollen transmitted virus, in crumbly fruit disease has been known for more than 40 years [1]. As methods for virus identification and characterization have become more sensitive, a better understanding of virus disease complexes has arisen, so that in a recent study, three viruses, RBDV, raspberry leaf mottle virus (RLMV), and raspberry latent virus (RpLV), were found to be associated with crumbly fruit in the Pacific Northwest of North America [2]. Interestingly, by precisely measuring virus levels in the plants it was found that the presence of RLMV increased the level of RBDV 400-fold [3]. In addition, plants containing all three viruses had more severe symptoms and produced fruit of the lowest quality.

To reduce the incidence and spread of plant diseases, including those caused by viruses, various organizations have devised and published recommended procedures and processes to facilitate the production of healthy (virus-tested) planting material, and to prevent introduction and movement of infected material into and within participating countries and regions. The European and Mediterranean Plant Protection Organization (EPPO) produces phytosanitary guidelines on behalf of 50 member countries (<https://www.eppo.int/>) and publishes a collection of standards that includes diagnostic protocols for regulated plant pests including a number of viruses that infect berry crops (<http://>

[archives.eppo.int/EPPOStandards/diagnostics.htm](http://archives.eppo.int/EPPOStandards/diagnostics.htm)). The UK has devised the Plant Health Propagation Scheme (PHPS), which follows the EPPO guidelines and is a certification scheme to ensure the production of healthy planting stocks of various berry fruit and other crops (<http://www.fera.defra.gov.uk/plants/plantHealth/phps.cfm>). This scheme publishes many documents detailing requirements for propagation of berry plants, including explanatory leaflets that set out lists of disease agents that must be tested for in the various crops, what testing methods should be used and detailing testing protocols for some specific pathogens (<http://www.fera.defra.gov.uk/plants/feesForms/plantHealth.cfm>). In the United States, through the National Clean Plant Network and the development of national certification standards for berry crops (<http://www.ncpnberries.org/>), there have been significant steps towards the development and dissemination of material that is tested for all known pathogenic viruses of berry fruit crops. This practice has the potential to drastically minimize the spread of systemic pathogens in berry fruit nursery material in North America.

The guidelines for berry virus testing referred to above include a range of different testing procedures. The simplest test is a visual observation for disease symptoms. This can be appropriate in some instances, although symptom production may require the presence of more than one virus, and can vary depending on variety, seasonal or environmental conditions and plant nutritional status. For some viruses, for example nepoviruses, mechanical inoculation of infected plant extracts to indicator plants may result in obvious disease symptoms such as necrotic or chlorotic lesions or mosaics on leaves. This approach is often referred to as sap testing, and uses a variety of herbaceous indicator plants, including *Chenopodium quinoa*, various tobacco species, and cucumber. A significant number of berry viruses, for example RLMV and RpLV, are not known to be mechanically transmissible, necessitating other testing approaches, such as grafting. Here, a scion (stem and leaf section) from the plant under test is physically attached, through the close contact of cut surfaces that links the vascular tissues, to a different plant that develops symptoms when infected with the virus. For graft testing of RLMV and some other raspberry viruses the recipient (indicator) plant is often black raspberry (*Rubus occidentalis*) cvs. Munger or Cumberland, and the transfer of virus infection is revealed by necrosis of the apical stem and leaves.

Some viruses, such as RpLV or blackberry yellow vein associated virus, do not cause observable symptoms following grafting to indicator plants. To detect these viruses it is necessary to use tests that are designed specifically for each individual virus. For viruses that accumulate to high levels in infected plants, including RBDV, an antibody-based test, such as enzyme-linked immunosorbent assay (ELISA) or dot/tissue-blot, can be effective. For many low-titer berry viruses purification of virus particles for use in antibody production is difficult or impossible and it is more common than not

that the recombinant virus proteins expressed in bacteria are not folded correctly and are not immunogenic, producing low-quality antisera which cannot be used for routine testing.

Polymerase chain reaction (PCR) provides the most sensitive of the regularly used virus detection tests. For the development of a PCR test it is necessary to have prior information of the nucleic acid sequence of the virus and, if the virus genome is RNA, a reverse transcription step is necessary.

Although many diseases of berry fruit crops have been described and are listed in international publications [4–7], the causative agents are often not known. This uncertainty has even extended into the EPPO and PHPS guidelines for berry pathogen testing where, for example, the 2013 EPPO A1 list of pathogens recommended for regulation as quarantine pests includes raspberry leaf curl virus, and classifies this as a nepovirus. Raspberry leaf curl virus is not recognized by the International Committee on the Taxonomy of Viruses (ICTV) but ICTV does list Raspberry Scottish leaf curl virus as a pseudonym for the nepovirus raspberry ringspot virus (RpRSV). However, earlier work on raspberry leaf curl disease [4] suggests this disease to be aphid transmitted whereas nepoviruses generally, and RpRSV specifically, are transmitted by nematodes. Generally, individual plants carrying these original diseases have not been curated by growers or researchers, and it is not possible now to determine which viruses were the causative agents of these diseases. However, the retention of these historic disease and/or virus names in regulatory guidelines is a major issue for commercial fruit breeders and growers, greatly complicating the process of releasing new varieties into the marketplace.

The recent introduction of next generation sequencing (NGS) technologies has greatly improved the discovery and characterization of viruses and will eventually lead to the design of detection tests for all viruses that infect agriculturally and horticulturally important crops. This information will then enable us to understand which viruses are responsible for which berry fruit diseases.

In this chapter we describe methods for the isolation of nucleic acid (RNA and DNA) from berry plants that is suitable for subsequent amplification by RT-PCR and PCR, respectively, to enable the detection of viruses infecting these plants. We also provide a comprehensive list of primer pairs that can be used for detection of many berry-infecting viruses.

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## 2 Materials

1. SE Extraction Buffer ([8]) (*see Note 1*): 0.14 M NaCl, 2 mM KCl, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O (pH 7.4), 0.05 % Tween 20, 2 % w/v polyvinylpyrrolidone 40, 0.2 % w/v ovalbumin, 0.5 % sodium azide.

2. Qiagen RNeasy kit: add 10 µl β-mercaptoethanol to 1 ml buffer RLT before use.
3. Qiagen RNeasy kit: buffer RPE is supplied as a concentrate and must have 4 vol of ethanol (96–100 %) added before use. Buffer RLT contains guanidine isothiocyanate but its composition is confidential to Qiagen. Buffer RW1 contains a guanidine salt and ethanol but its composition is confidential to Qiagen. The composition of buffer RPE is confidential to Qiagen.
4. Qiagen DNeasy kit: add ethanol (96–100 %) to concentrated buffers AW1 (40 ml added to 17 ml buffer) and AW2 (38 ml added to 19 ml buffer) before use. Buffer AW1 contains a guanidine salt and ethanol but its composition is confidential to Qiagen. Compositions of buffers AW2, AP1, and P3 are confidential to Qiagen.
5. RNA extraction Buffer ([9]: for 1 l mix 24.2 g Tris base (final 200 mM Tris-HCl, pH 8.5), 12.66 g lithium chloride (final 300 mM), 15 g lithium dodecyl sulfate (final 1.5 %), 2.92 g EDTA or 3.72 g EDTA·2H<sub>2</sub>O (final 10 mM), 10 g sodium deoxycholate (final 1 %), 10.0 ml NP-40 (final 1 %). Immediately before use add β-mercaptoethanol (final 1 %) (*see Note 2*)).
6. Wash Buffer ([9]: for 1 l mix 10 ml 1 M Tris-HCl pH 7.5 (final 10 mM Tris-HCl, pH 7.5), 1 ml 0.5 M EDTA (final 0.5 mM EDTA), 10 ml 5 M NaCl (final 50 mM NaCl), 500 ml 100 % ethanol (final 50 %), 479 ml sterile H<sub>2</sub>O).
7. Potassium acetate solution, pH 6.5: for 1 l, mix 600 ml 5 M CH<sub>3</sub>COOK (490.7 g per liter), 285 ml dH<sub>2</sub>O, and 115 ml glacial acetic acid).
8. Silica preparation: Add 60 g silica particles (Sigma S5631) to 500 ml distilled H<sub>2</sub>O. Mix well and allow to settle for 24 h. Remove the upper 470 ml and discard. Add 500 ml distilled H<sub>2</sub>O with pH adjusted to pH 2 using HCl and allow to sit for 5 h or overnight. Remove the upper 440 ml and discard. Check to see that the slurry has a pH of 2. Autoclave and store in a dark bottle at room temperature or aliquot into 2.0 ml microcentrifuge tubes for storage at 4 °C for several months.
9. 0.01 M sodium citrate: dissolve 2.94 g of sodium citrate in 800 ml of RNase-free water. Bring pH to 7 with concentrated hydrochloric acid (10 M). Bring volume to 1 l with water.
10. Ethanol (96–100 %).
11. Isopropanol (propan-2-ol).

### 3 Methods

#### 3.1 Extraction of RNA

This method is as described by Thompson et al. [8] and combines initial extraction of plant tissue in their prescribed SE buffer, followed by RNA purification using the Qiagen RNeasy spin-column procedure.

1. Collect leaves to be tested and either tear off with gloved hands or cut with scissors to obtain 50–100 mg of leaf (*see Notes 3–6*). Clean the scissors between different samples by rinsing with water and drying with paper towel to prevent cross-contamination of samples.
2. Freeze leaf with liquid nitrogen in a clean mortar and grind to a powder using a pestle (*see Note 7*).
3. Transfer the powder to a 1.5 ml microcentrifuge tube, add 450 µl of SE buffer, and vortex to mix for 30 s.
4. Transfer 100 µl of the tissue homogenate to a new 1.5 ml microcentrifuge tube and add 450 µl RLT buffer (containing 1 % mercaptoethanol). Vortex for 30 s to mix thoroughly.
5. The extraction is continued as stated in the Qiagen RNeasy Plant Mini Kit guide book.
6. Thus, transfer the homogenized lysate to a QIAshredder spin column (purple color) and centrifuge at maximum speed (16,000 $\times g$ ) for 2 min (*see Note 8*).
7. The flow-through liquid contains the RNA and is retained in the collection tube. Transfer this liquid, without disturbing the cell debris pellet, to a new microcentrifuge tube and measure the volume with a pipette.
8. Add 0.5 vol of absolute (96–100 %) ethanol to the flow-through lysate and mix by pipetting.
9. Transfer the lysate/ethanol mixture to an RNeasy spin column (pink color) and centrifuge for 15 s at more than 8,000 $\times g$  (top speed for benchtop microcentrifuges is suitable). The RNA is captured onto the filter in the spin column.
10. Discard the flow-through and add 700 µl of buffer RW1 to the column. Respin at >8,000 $\times g$  for 15 s.
11. Discard the flow-through and add 500 µl buffer RPE to the spin column. Respin at >8,000 $\times g$  for 15 s.
12. Discard the flow-through and add another 500 µl buffer RPE to the spin column. Respin at >8,000 $\times g$  for 2 min to remove all traces of buffer solution from the column (*see Note 9*).
13. Place the spin column into a new 1.5 ml microcentrifuge tube. Add 30–50 µl of RNase-free water to the column. Leave at room temperature for 1 min to release the RNA from the filter,

then centrifuge at maximum speed for 1 min to collect the RNA solution in the microcentrifuge tube (*see Note 10*).

### **3.2 Extraction of RNA (Tzanetakis Laboratory)**

This method uses a silica-binding approach without a commercial kit, based on the method of Rott and Jelkmann [9].

1. Collect plant tissue (each sample to be extracted is about 50 mg).
2. Add 1 %  $\beta$ -mercaptoethanol to RNA Extraction buffer just before use. Grind tissue in a roller press (e.g., Wenig and Koch, Hannover, Germany) using two sequential aliquots of RNA Extraction Buffer (500  $\mu$ l + 500  $\mu$ l = total 1 ml), and collect homogenate in 2 ml tubes.
3. Remove 600  $\mu$ l supernatant of the above step and place into 2 ml tubes containing 600  $\mu$ l of CH<sub>3</sub>COOK solution. Invert tubes to mix.
4. Centrifuge at 15,000  $\times g$  for 10 min to pellet and remove cell debris.
5. Remove 750  $\mu$ l of supernatant and place into a 1.5 ml tube containing 750  $\mu$ l of 100 % isopropanol. Incubate at -20 °C for 30 min to precipitate RNA.
6. Centrifuge at 15,000  $\times g$  for 20 min.
7. Remove the supernatant and resuspend the nucleic acid pellet by vortexing (at least 2 min) in 500  $\mu$ l wash buffer. Add 20  $\mu$ l silica/milk glass preparation for nucleic acid binding and mix by vortexing for 10–15 s.
8. Pulse-operate the centrifuge until it achieves 10,000  $\times g$ .
9. Remove supernatant; add 500  $\mu$ l wash buffer and vortex until the pellet is disaggregated (10–15 s). Centrifuge at 15,000  $\times g$  for 30 s to pellet the silica.
10. Let the pellet dry either by standing at room temperature for 10–15 min or by using a vacuum dessicator, then resuspend it in 150  $\mu$ l 0.01 M citrate solution and incubate for 5 min at room temperature. Centrifuge at 15,000  $\times g$  for 1 min to pellet any residual silica. Transfer 100  $\mu$ l of the RNA solution to a new 1.5 ml tube (make sure that you do not pick up any of the silica which binds proteins and will inhibit all downstream reactions) and store at -80 °C.
11. Proceed to the reverse transcription (RT) reaction and use 2–5  $\mu$ l of the purified RNA in each 50  $\mu$ l volume RT reaction.

### **3.3 Extraction of Total DNA**

This method is suitable for DNA extraction from both Rubus and herbaceous material and uses a commercial kit (Qiagen DNeasy Plant Mini Kit—Cat. 69104).

A minority of the known berry fruit viruses have a DNA genome (RYNV, GVBaV, BRRSV, SVBV). Little, if any, work has

been done to accurately determine whether any particular DNA extraction method is best for a particular virus in a particular crop plant. The successful use of commercial DNA extraction kits according to the manufacturers' instructions has been reported (e.g., NucleoSpin plant II kit [Macherey-Nagel], ref. [10]) (*see Note 11*). In the MacFarlane laboratory for detection of RYNV and GVBaV we routinely use the Qiagen DNeasy Plant Mini Kit (cat. 69104) without additional modification.

1. Collect leaves to be tested and either tear off with gloved hands or cut with scissors to obtain 50–100 mg of leaf (*see Note 12*). Clean the scissors between different samples by rinsing with water and drying with paper towel to prevent cross-contamination of samples.
2. Freeze leaf with liquid nitrogen in a clean mortar and grind to a powder using a pestle.
3. Transfer the powder to a 1.5 ml microcentrifuge tube and add 400 µl of buffer AP1 (lysis buffer) and 4 µl RNase A (supplied in the kit). Vortex to mix for 30 s and incubate at 65 °C for 10 min. Mix by inversion several times during the incubation.
4. Add 130 µl buffer P3 (neutralization buffer), mix by repeated inversion and incubate on ice for 5 min.
5. Centrifuge for 5 min at maximum speed (16,000×*g*; depending on the microcentrifuge model) to pellet cell debris.
6. Transfer the supernatant (lysate) into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge at 16,000×*g* for 2 min (this step further removes cell debris and precipitates).
7. Collect flow-through into a new tube, without disturbing any pellet if present, and add 1.5 vol of buffer AW1. Mix solution by pipetting.
8. Transfer 650 µl of the mixture into a DNeasy Mini spin column in a 2 ml collection tube. Centrifuge for 1 min at about 6,000×*g*, discard the flow-through, and repeat with the remainder of the mixture.
9. Transfer the column to a new tube. Add 500 µl of buffer AW2 and centrifuge for 1 min at about 6,000×*g*. Discard the flow-through.
10. Add another 500 µl of buffer AW2. Centrifuge at 20,000×*g* for 2 min to ensure all traces of buffer are removed from the column.
11. Transfer the column to a new tube and add 100 µl of elution buffer AE. Incubate at room temperature for 5 min, then centrifuge at 6,000×*g* for 1 min to collect the eluted DNA.
12. Proceed to the PCR amplification using 1–5 µl of DNA in a 50 µl reaction.

### 3.4 Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

#### 3.4.1 Single-Strand cDNA Synthesis

These methods have been detailed previously in many different publications, as well as by the manufacturers of these reagents. No particular suppliers of RT and PCR reagents are specifically recommended for berry fruit virus detection, and we expect all commercially produced reagents to be suitable for virus detection. A generic RT-PCR process is described below:

1. Thaw purified plant RNA samples and reagents on ice.
2. In a sterile 1.5 ml microcentrifuge tube mix the RNA (1–5 µl) random hexamer primers (1 µl of 100 pmol/µl) (*see Note 13*), dNTPs (1 µl of a 10 mM mixture of dGTP, dCTP, dATP, and dTTP), and sterile distilled water to a volume of 12 µl.
3. Heat the mixture to 65 °C for 5 min to denature the RNA, then return the tube to ice. Collect tube contents by a brief centrifugation.
4. Add 4 µl 5× First-strand synthesis buffer, 2 µl 0.1 M dithiothreitol, 1 µl RNase inhibitor (e.g., RNaseOUT, Invitrogen), mix by pipetting, and incubate at 25 °C for 2 min.
5. Add 1 µl MuMLV reverse transcriptase (e.g., Superscript II, Invitrogen), mix by pipetting, and incubate at 42 °C for 1 h (*see Note 14*). Proceed to PCR step.

#### 3.4.2 PCR

1. In a 0.2 ml PCR tube in an ice bucket combine 10 µl 5× Reaction Buffer (*see Note 15*), 1 µl 10 mM dNTPs, 2–5 µl cDNA reaction mixture, 1 µl of each of two virus-specific oligonucleotides primers (20 pmol/µl), 0.25–0.5 µl Taq polymerase (according to the manufacturer's recommendation) (*see Note 16*), and sterile distilled water to a final total of 50 µl.
2. Mix by pipetting and transfer to a pre-programmed PCR machine for the reaction to proceed (*see Note 17*).

As an alternative to carrying out separate and sequential RT and PCR reactions, it is possible to use a formulation containing reagents for both reactions in a single tube (e.g., Illustra Ready-To-Go RT-PCR beads, GE Healthcare), and these have been successfully used in the MacFarlane laboratory. Because of the usual low titer achieved by berry fruit viruses in crop plants we routinely use PCR reactions having 40 cycles of amplification. Wherever possible it is best practice to include DNA/RNA samples from known infected and also uninfected plants in each set of reactions, however, obtaining and maintaining plants containing known reference isolates of berry fruit viruses can be difficult.

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## 4 Notes

1. The RNA extraction buffer of Thompson et al. [8] was designed to overcome long-standing problems encountered when isolating viruses or nucleic acids from strawberry. These problems were thought to result from secondary metabolites including tannins, polyphenols, and polysaccharides present in the leaf extracts. This study compared four different RNA extraction methods and found that use of SE buffer (*see* Subheading 2) in the extraction process gave reproducibly good results. We (at the James Hutton Institute, Dundee) routinely use this buffer for RNA extraction from all berry fruit leaves (strawberry, raspberry, blackcurrant), followed by purification using the Qiagen RNeasy kit.

Interestingly, Thompson et al. [8] were able to detect the DNA virus Strawberry vein banding virus (SVBV) when nucleic acid was extracted using SE buffer and the Qiagen RNeasy kit. SVBV could be detected by direct PCR (amplifying the genomic DNA) and by RT-PCR (amplifying, in addition, the viral RNA transcripts). They commented that including the reverse transcription step resulted in a fourfold dilution of the extracted nucleic acid, which reduced the concentration of potential inhibitors in the extract and increased the reproducibility of the amplification reaction.

2. Sodium dodecyl sulfate (SDS) can be used in place of lithium dodecyl sulfate.
3. Successful RT-PCR and PCR detection of, respectively, RNA and DNA plant viruses is influenced by (a) the titer of virus in the infected plant, (b) the amount of RNA or DNA extracted from the plant, (c) the “cleanliness” of the extracted nucleic acid (removal of inhibitory substances from the plant extract).
4. Many berry fruit-infecting viruses are “low” titer, requiring sensitive methods of detection. In most cases, the potential effects of sampling time (winter, spring, summer or autumn) on virus titer and ease of detection are not known. As with all tests, PCR/RT-PCR amplification detects viruses with titers down to a threshold that is different for each virus, however, quantitative information on berry fruit virus detection is almost nonexistent. Failure to detect a virus in a nucleic acid sample is not absolute proof that the virus is not present in that sample or in the plant from which the sample was taken.
5. It is known that symptom severity generally is increased if more than one virus is present in the plant. Whether the titer of particular viruses is increased, decreased, or unaffected by coinfection with other viruses is mostly not known.

6. Berry fruit virus testing usually is done in young leaves at or near full expansion size. These leaves are “soft,” allowing easier extraction of nucleic acids. Older leaves become tougher and eventually start to senesce, both of which reduce the ease of extracting nucleic acids. The distribution and relative titer of virus in different parts of the plant have not been studied for most berry fruit viruses, although sporadic distribution (present in some stems and leaves but not in others) has occasionally been reported (e.g., blackcurrant reversion virus). Some berry fruit viruses have been detected in roots but extraction of nucleic acids from roots is less efficient/produces lower yields than from leaves.
7. As an alternative to grinding in liquid nitrogen it is possible to homogenize samples using a bead beater with steel or tungsten beads (e.g., Qiagen TissueLyser).
8. Actual relative centrifugal force depends on the particular machine being used and is not critical for any of these steps. The MacFarlane lab uses an Eppendorf 5415R microcentrifuge where the maximum speed is 13,200 rpm (16,100 $\times g$ ). For all steps in these protocols the duration of centrifugation could be increased to compensate for lower actual speeds of other centrifuge models.
9. Repeated centrifugations at maximum speed can be useful to ensure that all traces of wash buffer (containing ethanol), which can interfere with downstream reactions, are removed from the sample.
10. Store eluted RNA samples at –20 °C if they are to be used for reverse-transcription PCR within 2 weeks. For longer periods of storage, –80 °C is preferable.
11. A recent publication (Methods in Molecular Biology 938. Phytoplasma Methods and Protocols, M. Dickinson and J. Hodgetts (Eds) 2013, Springer, New York) contains many methods for automated and manual purification of DNA suitable for PCR amplification of berry fruit viruses.
12. Leaf samples can be easily collected by closing the lid of a microcentrifuge tube over the leaf to be sampled. Four or five leaf discs collected together give sufficient material for nucleic acid extract using Qiagen kits. This method avoids the need for repeated cleaning of scissors etc between the collections of different samples.
13. Virus-specific primers (Table 1) (1 µl of a 100 pmol/µl solution) can be added to the reverse transcription reaction either in place of the random hexamer primers or in combination with them. The best combination should be determined empirically.

**Table 1**  
**Primer pairs suitable for detection of berry fruit viruses**

Virus name	Acronym	Crop	Genus	RNA or DNA virus	Published (RT)-PCR test: may be described in plants other than berry fruit	Reference
Alfalfa mosaic virus	AMV	Ribes	Alfamovirus	RNA	(AMV-F) CCATCATGAGTTCTTCACAAAG (AMV-R) TCGTCACGTCATCAGTGAAC CP primers, 351 bp	[11]
Apple mosaic virus	ApMV	Rubus, Fragaria, Sambucus, Ribes	Ilavirus	RNA	(Sense) ATCCGAGTGAACAGTCTATCCTCTAA (Antisense) GTAAACTCACTCCGTATCACGTACAA 262 bp	[12]
Arabis mosaic virus	ArMV	Fragaria, Ribes, Rubus, Vaccinium, Sambucus	Nepovirus	RNA	(C1642) TTGGCCCCAGATATAAGCGTAAAAAT (H1124) CAGGGATTGGGAGTTCGT 519 bp	[13]
Beet pseudoyellows virus	BPYV	Fragaria, Ribes	Crinivirus	RNA	BP CPm F (TTICATATTAAGGATGCGCAGA) BP CPm R (TGAAAAGATGTCRCTAATGATA) 334 bp	[14]
Black raspberry cryptic virus	BrCV	Rubus	Alphacryptovirus	dsRNA (not yet approved)	(BRcryptidetFa) CGGCGTGAAGGCCACAGT (BRcryptidetRa517) GCCGTCTGGTGCCT	Tzanetakis unpublished
Black raspberry necrosis virus	BRNV	Rubus	Secoviridae unassigned genus	RNA	(2197) TAGATGAGTGCCTCAAGTTGGTCCAC (2196) CCGATACAAACGGCCCTCGTCCAAAG 790 bp	[15]

Blackberry chlorotic ringspot virus	BCRV	Rubus	Ilarvirus (not yet approved)	RNA	(BCRV1836F) ACCTGCTGATCAGCTWTCAGAGAA (BCRV2237R) TAGAACATCGACCCAAAGGT qPCR (BCRV3.298F) AGGTTGAATATGGCTTTGACCC qPCR (BCRV3.298R) AAGCAGGCRCATCGCCCTTATAC qPCR (BCRV3.298 probe) 6FAM/6FAM/Zen/ TTCGATGGAG CT/3IABRFQ	[16]
Blackberry virus E	BVE	Rubus	Flexivirus	RNA	(BVE-F) CTTACCCACAACGGACTCCCTCC (BVE-R) GCATGGCGAGCATGTTTC 394 bp	[17]
Blackberry virus S	BIVS	Rubus	Marafivirus (not yet approved)	RNA	BIVS-CPF AATGTCACCTCCCAGGTGG BIVS-CPR ATGCGGCTCACGTCAGAGGG, 434 bp	[18]
Blackberry virus X	BIVX	Rubus	Alphaflexiviridae unassigned genus	RNA	CACCTAGCAGCCCTTGA, BVXF TGGTTTGACCAGCGAT, BVXR 510 bp	Tzanetakis unpublished
Blackberry virus Y	BIVY	Rubus	Brambyvirus	RNA	(BVY312F) CTGTGGGGAGATTGGAGAA (BVY695R) TCATTCATGGGTGTC 383 bp	[19]
Blackberry yellow vein associated virus	BYVaV	Rubus	Crinivirus	RNA	(BYVaVF) TTGAAAGGAAACTTCACGGA (BYVaVR) TAAGTTCATACGTTCCCTGCG (BYVaV2.867 probe 6) FAM/TIGAAAAGA/Zen/TGGGTYGGHGTGGACA/IABKfQ (BYVaV2.867F) ATAGAAGCCAGGTTAARACCTG (BYVaV2.867 R) CACRTYGTACCTCTAAAGCTCG	[20]
Blackcurrant reversion virus	BRV	Ribes	Nepovirus	RNA	(BRV1-10F) AGGCCTTCTCGCACAAACATCT (BRV1-10R) CAAGGGAGGGTGTCAAGTACA 327 bp	[21]

(continued)

**Table 1**  
**(continued)**

Virus name	Acronym	Crop	Genus	RNA or DNA virus	Published (RT)-PCR test: may be described in plants other than berry fruit	Reference
Blueberry latent spherical virus	BLSV	Vaccinium	Nepovirus	RNA	(Sense) GCTACCTCTTAAGAAAGGAGAT (Antisense) CCCCGGTTTCATCTCCGG, 609 bp	[22]
Blueberry latent virus	BBLV	Vaccinium	Amalgavirus	RNA	(BBLVdetF) CTGAGGGGGTGAAGCATATTAG (BBLVmidR) CCGTCTGTATGCTCCTAACAA	[23]
Blueberry leaf mottle virus	BLMoV	Vaccinium	Nepovirus	RNA	No test reported	[24]
Blueberry mosaic viroid-like RNA	BluMVD- RNA	Vaccinium	Unassigned viroid	RNA	No report	
Blueberry mosaic virus	BlMV	Vaccinium	Ophiovirus	RNA	BlMV MPdegF CCWGTATCAAGCATAGTYACAAG BlMV MPdegR AAGAAGGTTRGTGATTGAGA	[25]
Blueberry necrotic ring blotch virus	BNRBV	Vaccinium	New genus	RNA	(F) GGTTTCGACACCTCGGCATG (R) CCAGCTGCCCTTGAGACTTRTC 560 bp	[26]
Blueberry red ringspot virus	BRRSV	Vaccinium	Soymovirus	DNA	(RRSV3) ATCAGTCCCAGAAAAAGAAAGTA (RRSV4) TCCGAAAAATAAGATAGTGTCAAGC 549 bp	[27]
Blueberry scorch virus	BIScV	Vaccinium, Sambucus	Carlavirus	RNA	This virus is generally detected by ELISA but (F) GAAAGAAGCACCGGGCTCAATC (R) GGAGATCTGGCCATTGCTC 380 bp	[28]

Blueberry shock virus	BLSHV	Vaccinium	Ilarvirus	RNA	Degenerate ilarvirus primers: (Ilar1F5) GCNGGGWTTGGGDAARWCNAC (Ilar2R9) GGTGTRTGHGGRAAYTT ~380 bp	[29]
Blueberry virus A shoestring virus	BBSSV	Vaccinium	Closterovirus?	RNA?	No report	
Cherry leafroll virus	CLRV	Rubus	Nepovirus	RNA	(F) TGGCGACCGTGTAAACGGCA (R) GTCGGAAAGATTACGTAAAAGG 416 bp	[30]
Cherry rasp leaf virus	CRLV	Rubus Vaccinium Sambucus	Cheravirus	RNA	(JQ3D33FF) GCCAGTTCTCCAGTGAACC (JQ3D33FR) CAGITGAAACGGATTAA 429 bp	[31]
Cucumber mosaic virus	CMV	Ribes, Rubus, Fragaria, Sambucus	Cucumovirus	RNA	((CPTALL-5') YASYTITDRGGTTCAATTCC (CPTALL-3') GACTGACCATTAGCCCG 407 bp	[32]
Fragaria chiloensis cryptic virus	FCiLV	Fragaria	Deltapartitivirus?	RNA	(FCCV1F) AAGTCCTGAGCACTGCCAT (FCCV1R) TGAATACAAGTAACGGGAATTGA 152 bp	[33]
Fragaria chiloensis latent virus	FCiLV	Fragaria	Ilarvirus	RNA	(FCpolF) ACCACTTCACCCAGATCG (FCpolR) CAAGCCAACTCACCATGAC 152 bp	[34]
Gooseberry vein banding associated virus	GVBaV	Ribes	Badnavirus	DNA	(GVB1-forward) ACATCAAAGGGAAAGGACAAC (GVB1-reverse) TCTAAAAGCATCCACTACCAC 407 bp	[35]
Impatiens necrotic spot virus	INSV	Rubus	Tospovirus	RNA	(INSVF) GATCTGTCCTGGGATGTTIC (INSVR) GTCTCCCTCTGGTCTATAATCAT 460 bp	[36]

(continued)

**Table 1**  
**(continued)**

Virus name	Acronym	Crop	Genus	RNA or DNA virus	Published (RT)-PCR test: may be described in plants other than berry fruit	Reference
Peach rosette mosaic virus	PRMV	Vaccinium	Nepovirus	RNA	None published, antibody ELISA test available	[37]
Raspberry bushy dwarf virus	RBDV	Rubus	Idaeovirus	RNA	(U2) TTCAATCCTCAAATCTCAGCAAC (L3) CGTCGACGGCACGCCACCACA 245 bp	[38]
Raspberry latent virus	RpLV	Rubus	Reoviridae unassigned genus	dsRNA	(S3-F) GGCTGGGTACTGATCTTGG (S3-R) GCTAAATCCCCGGCCATC 268 bp	[39]
Raspberry leaf blotch virus	RLBV	Rubus	Emaravirus	RNA	(1287) ATCCAGTAGTGAAACTCC (1095) CACCATCAGGAACCTGTAAATGTTT 560 bp	[40]
Raspberry leaf mottle virus	RLMV	Rubus	Closterovirus	RNA	(991) CGAAACTTYYTACGGGAAAC (992) CCTTTGAATTCTTAACATCGT 470 bp	[41]
Raspberry leaf spot virus						
Raspberry mottle virus						
Raspberry ringspot virus	RpRV	Rubus, Fragaria, Ribes	Nepovirus	RNA	(RpRSVf1) TGTGTTCTGGGTTTGATGCT (RpRSVR1) GAGTGCATAGGGCTGTT 385 bp	[42]
Raspberry vein chlorosis virus	RVCV	Rubus	Rhabdovirus	RNA	(1531) CAGTAGAGGGGAGGGCTCCCT (1532) GTCCCACGTAAGGTCTGGGA 440 bp	[43]

Rubus canadensis	RuCV-1 virus-1	Rubus	Betaflexiviridae unassigned genus	RNA (RuCV-1F) CAAGGAGCGACATGGGGCG (RuCVRR) TGCCAGCACTGCAAACAGGACC 359 bp
Sowbane mosaic virus	SoMV (RuCMV)	Rubus, Ribes	Sobemovirus	RNA (1082) AGTCCTGGGTGTCATCTTG (1083) AAGGCCTTCTCAACGGTCTCA 326 bp
Rubus yellow net virus	RYNV	Rubus	Badnavirus	DNA (RYN1 -forward) TCCAAAACCTCCCAGACCTAAAC (RYN1 -reverse) ATATCGAAAAGGCCAC 350 bp
Strawberry chlorotic fleck virus	SCFV	Fragaria	Closterovirus	RNA (SCFaV CPhdetF) CGTGGGTGATCGCTAC (SCFaV CPhdetR) ATACGACGCCCTCTGT 392 bp
Strawberry crinkle virus	SCV	Fragaria	Cytorhabdovirus	RNA (SCD1FW) ACTGTAATGTCACCAAGAAAG (SCD1Rv) TTCTGACACTAGTAGATCTCC 573 bp
Strawberry latent ringspot virus	SLRSV	Fragaria	unassigned	RNA (SLRSV F) CCTCTCCAACCTGCTAGACT (SLRSV R) AAGGCGCATGAAGGTGTAACT 497 bp
Strawberry mild yellow edge virus	SMYEV	Fragaria	Potexvirus	RNA Nested PCR (A+C = 833 bp, then B+C = 406 bp) (Primer A) GCCAACAGCAAGAATCCTAT (Primer B) GATACTCGTCTACGAAGGCT (Primer C) TGCACTCTGTTGACCFTC 460 bp
Strawberry mottle virus	SmoV	Fragaria	Sadwavirus	RNA (Smdetnrc4a) TAAGGCGACCAACGACTGTGACAAG (Sm2nrcrb) ATTGGTTCACGTCCTAGTCAC 460 bp
Strawberry necrotic shock virus	SNSV	Fragaria	Ilarvirus (not yet approved)	RNA (SNSV CpbegF) GAGTATTCTGTAGTGAATTCTTGGA (SNSV CpendR) ATTATCTTAATGTGAGGCAACTCGT 824 bp

(continued)

**Table 1**  
**(continued)**

Virus name	Acronym	Crop	Genus	RNA or DNA virus	Published (RT)-PCR test: may be described in plants other than berry fruit	Reference
Strawberry pallidosis-associated virus	SpaV	Fragaria	Crinivirus	RNA	(SPL F) TGCTTAATGATGGAGACCTCG (SPL R) GGTGTCTAACTTGTCTGTTCC 517 bp	[52]
Strawberry pseudo mild yellow edge virus	SPMYEV	Fragaria	Carlavirus?	RNA	Antibody ELISA test available	
Strawberry vein banding virus	SVBV	Fragaria	Caulimovirus	DNA	(SVBVdeta) AGTAAGACTGTGGTAATGCCA (SVBVdetb) TTCTCTCCATGTAAGGCTTTGA 422 bp	[8]
Tobacco necrosis virus-D	TNV	Fragaria	Necrovirus	RNA	(GP1INT5') GTGTCAGTCATAACATACC (GP1INT3') GCCTATTGTGCTGTACCAAC 257 bp	[53]
Tobacco rattle virus	TRV	Rubus, Ribes	Tobravirus	RNA	(Primer A) CAGTCTATACACAGAACAGA (Primer B) GACGTGTGTACTCAAGGGTT 463 bp	[54]
Tobacco ringspot virus	TRSV	Rubus, Vaccinium, sambucus	Nepovirus	RNA	(MF05-21-R) CAATACGGTAAGTGCACACCCCCG (MF05-22-F) CAGGGGGCTGAGTGGGGCTC 320 bp	[55]
Tobacco streak virus	TSV	Vaccinium, Fragaria Rubus?	Ilovirus	RNA	(TSV CP F) ACGAGTATTAAAGTGGATGAAATTCT (TSV CP R) ACTTACAATAACGTCGAGGTGTTG 872 bp	[51]
Elderberry latent virus	EILV	Sambucus	Carmovirus	RNA	Antibody ELISA test available	

Tomato bushy stunt virus	TBSV	Sambucus	Tombusvirus	RNA	Antibody ELISA test available
Tomato black ring virus	TBRV	Fragaria, Rubus, Ribes, Sambucus	Nepovirus	RNA	(NEPOB-F) TCTGGGTTGGCYTTRACRGT (NEPOB-R) CTTRTCACTVCCATCRGTAA Degenerate primers for nepovirus subgroup B detection, including TBRV
Strawberry leaf curl virus	StLCV	Fragaria	Begomovirus	DNA	No test described
Grapevine Syrah virus 1				RNA	(BIVS-CPF) AATGTCACCTCCCAGGTCGG (BIVS-CPR) ATGGGGCTCACCGTCAAGAGGG (GSV-1-CPF) TCCCCAGCTTCAGGGTGAAATT (GSV-1R) GCATTGCTGCGCATGGAGG Marafì degenerate: (TymZ-F) GGSCCMGTSAAARAARTAYCA (TymZ-R) GCCAGRTTGTARTCRGRGTTG

14. Some reverse transcriptase enzymes have been engineered to work at temperatures higher than 42 °C, and may be useful for some virus/primer combinations.
15. Reaction buffers supplied with some commercial Taq or other DNA polymerases may already contain MgCl<sub>2</sub>. As a general rule, a final concentration of 1.5 mM allows efficient, specific amplification of most DNA/cDNA templates. However, sometimes it is necessary to adjust the concentration of MgCl<sub>2</sub> in the PCR reaction, and some PCR kits supply a separate MgCl<sub>2</sub> solution to allow the user to do this.
16. Many companies supply Taq polymerase or other DNA polymerase enzymes for PCR amplification. These may differ in their speed of synthesis, accuracy of DNA copying and cost but, in theory, all should be suitable for virus detection purposes.
17. To prevent mis-priming during the early stages of PCR, the PCR reaction is usually assembled on ice (where the Taq polymerase is not active) and the reaction transferred to the PCR machine only when it has reached working temperature (a so-called hot-start reaction). Some PCR enzymes are supplied premixed with an inhibitor, such as an antibody that binds to the polymerase, so that the reaction can be safely assembled at room temperature and the polymerase becomes active only after an initial incubation at high temperature where the inhibitor itself is disabled.

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# Chapter 18

## Metagenomics Approaches Based on Virion-Associated Nucleic Acids (VANA): An Innovative Tool for Assessing Without A Priori Viral Diversity of Plants

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Emmanuel Jacquot, and Philippe Roumagnac

### Abstract

This chapter describes an efficient approach that combines quality and yield extraction of viral nucleic acids from plants containing high levels of secondary metabolites and a sequence-independent amplification procedure for both the inventory of known plant viruses and the discovery of unknown ones. This approach turns out to be a useful tool for assessing the virome (the genome of all the viruses that inhabit a particular organism) of plants of interest. We here show that this approach enables the identification of a novel *Potyvirus* member within a single plant already known to be infected by two other *Potyvirus* species.

**Key words** Plant virus, Virus discovery, Diagnostic, *Dioscorea* spp., Viral particles, Random amplification, Cloning

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### 1 Introduction

The extraction of pure, intact, and high-quality nucleic acids from leaves of several plants, including tropical tuber crops, banana, salt marsh plant species, etc., is complicated due to high levels of secondary metabolites, phenolic compounds, highly viscous polysaccharides, and nucleic acid-degrading endonucleases [1]. These compounds can cause shearing of nucleic acids during extraction, such as degradation of RNA/DNA due to nucleases or inhibition of enzymatic activity due to polysaccharides. In addition, the oxidized form of polyphenols covalently binds to RNA/DNA producing a brown gelatinous material, which hampers reliable extraction of nucleic acids [1]. Methodological studies for setting up more efficient means of extracting nucleic acids of both higher quality and yield have led to the development of several protocols for isolating nucleic acids from plants containing high levels of secondary metabolites [1]. We here focus on yams (*Dioscorea* spp.),

which belong to this group of “tricky” plants and could be seen as a case study.

Yams (*Dioscorea* spp.), which produce starchy tubers with high nutritional value, are one of the most important food commodities in the tropics and subtropics. These plants are vegetatively propagated, thus promoting the accumulation of phytoviruses. To date, several virus species of the genera *Aureusvirus*, *Badnavirus*, *Carlavirus*, *Comovirus*, *Cucumovirus*, *Fabavirus*, *Macluravirus*, *Potexvirus*, and *Potyvirus* have been reported and characterized in *Dioscorea* spp. [2]. However, the diversity of viral species infecting yams remains largely unexplored. This incomplete inventory of plant viral diversity of yam is hindering effective quarantine and sanitation efforts.

The safety of regulated plant material exchanges presently relies heavily on “sequence-dependent” amplification techniques such as polymerase chain reaction (PCR) or nucleic acid hybridization, which are only suited to the detection and characterization of specific, well-characterized pathogens. By contrast, “sequence-independent” amplification approaches can potentially provide an ideal platform for identifying almost all known and unknown microbes present in any particular host organism. These “metagenomics” applications have already enabled the identification of novel pathogens through the rapid and comprehensive characterization of microbial strains and isolates within environmental and host tissue samples [3].

We describe here approaches that led to the analyses of the virome (the collection of all the viruses) of a yam plant maintained for a number of years at the CIRAD Yam Quarantine Station in Montpellier, France. This plant (*Dioscorea trifida* accession MP2) was known to be infected with two potyviruses: *Yam mild mosaic virus* (YMMV) and *Yam mosaic virus* (YMV).

Here we describe protocols of (1) rapid and reliable purification of viral particles and (2) virion-associated nucleic acids (VANA) extraction, specifically suited for extracting viral RNA/DNA from plants which are rich in polysaccharides and secondary metabolites. We also describe the “sequence-independent” amplification of the extracted nucleic acids, the cloning and sequencing steps and finally the data analysis.

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## 2 Materials

### 2.1 Purification of Viral Particles

1. Sterile pestle and mortar.
2. Liquid nitrogen.
3. Carborundum (Sigma-Aldrich).
4. Single channel pipettes (0.5–10 µL/10–200 µL/100–1,000 µL).

5. Extraction buffer: 0.5 M sodium–potassium phosphate buffer (58 g K<sub>2</sub>HPO<sub>4</sub>, 20 g NaH<sub>2</sub>PO<sub>4</sub>, 1 L deionized water), pH 7.4, 4 % PVP-40, 4 % Urea, 0.5 % 2-mercaptoethanol, 0.02 % sodium azide.
6. Miracloth filter.
7. Funnel.
8. Conical tubes (50 mL) (Falcon).
9. Triton® X-100 (Sigma-Aldrich).
10. Orbital shaker (Bioblock).
11. Centrifuge (Sorvall RC 6+ with rotor F-28/50).
12. Ultracentrifuge polycarbonate bottles (26.3 mL) (Beckman Coulter).
13. Pasteur pipette.
14. Sucrose (Sigma-Aldrich).
15. Ultracentrifuge (Beckman Coulter Optima LE-80 K with rotor 50.2 TI).
16. Deionized water.
17. RQ1 DNase buffer (Promega).
18. Microtubes (1.5 mL) (Eppendorf)
19. DNase I (Promega) et RNase A (Qiagen).

## **2.2 Viral Nucleic Acid Extraction**

1. RNeasy Plant Mini Kit (Qiagen).
2. Microtubes (1.5 mL) (Eppendorf)
3. Single channel pipettes (0.5–10 µL/10–200 µL/100–1,000 µL).
4. Molecular grade water.
5. Centrifuge (Beckman Coulter Microfuge 18).

## **2.3 Random RT-PCR Amplification of Purified Viral Nucleic Acids**

1. TransPlex® Whole Transcriptome Amplification Kit (Sigma-Aldrich).
2. Single channel pipettes (0.5–10 µL/10–200 µL/100–1,000 µL).
3. Microtubes for thermocyclers (200 µL).
4. Thermocycler.
5. Agarose.
6. TAE buffer (pH 8.4): 4.84 g Tris, 1.142 mL acetic acid, 18.612 g EDTA-Na<sub>2</sub>·2H<sub>2</sub>O, deionized water to prepare 1 L.
7. Horizontal electrophoresis equipment.
8. Ethidium bromide.
9. 10× loading buffer (Invitrogen).

10. 1 kb DNA ladder (Invitrogen).
11. UV transilluminator.
12. Scalpels.
13. Microtubes (2 mL) (Eppendorf).
14. QIAquick Gel Extraction Kit (Qiagen).

#### **2.4 Cloning and Sequencing**

1. Single channel pipettes (0.5–10 µL/10–200 µL/100–1,000 µL).
2. Microtubes (1.5 and 2 mL) (Eppendorf).
3. pGEM®-T Easy Vector System including JM109 High Efficiency Competent Cells (Promega).
4. 42 °C water bath.
5. LB medium: 10 g Bacto®-tryptone, 5 g Bacto®-yeast extract, 5 g NaCl, deionized water to prepare 1 L. Adjust pH to 7.0 with NaOH. Add 15 g agar to prepare solid LB medium.
6. Shaking incubator Minitron (Infors AG).
7. 37 °C incubator.
8. Ampicillin.
9. IPTG.
10. X-Gal.
11. Plastic petri dishes (10 cm in diameter).
12. Petri dish spreaders.
13. Sterile toothpicks.
14. Microtubes for thermocyclers (200 µL).
15. GoTaq® Hot Start Colorless Master Mix (Promega).
16. Primers:
  - SP6 (5'-ATTTAGGTGACACTATAG-3').
  - T7P (5'-TAATACGACTCACTATAGGG-3').
17. Molecular grade water.
18. Agarose.
19. TAE buffer (pH 8.4): 4.8 g Tris, 1.1 mL acetic acid, 18.6 g EDTA-Na<sub>2</sub>·2H<sub>2</sub>O, deionized water to prepare 1 L.
20. Horizontal electrophoresis equipment.
21. Ethidium bromide.
22. 10× loading buffer (Invitrogen).
23. 1 kb DNA ladder (Invitrogen).
24. UV transilluminator.

### 3 Methods

#### 3.1 Purification of Viral Particles

(Modified from ref. [4])

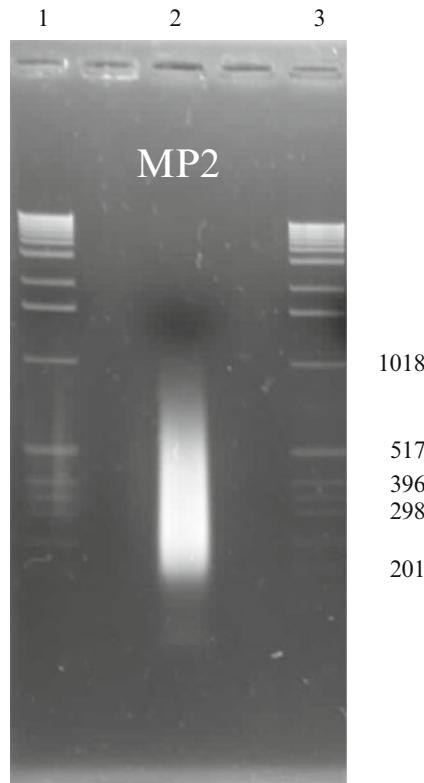
1. Grind 5 g of leaf material in liquid nitrogen in the presence of about 100 mg of carborundum using a pestle and a mortar.
2. Add 15 mL of sodium-potassium phosphate extraction buffer and homogenize.
3. Transfer and filter the homogenized plant extracts in a Miracloth filter placed on a funnel and a 50 mL conical tube.
4. Add 330  $\mu$ L of Triton® X-100 to the filtrate and mix during 15 min with an orbital shaker.
5. Centrifuge at 14,000  $\times \text{g}$  for 10 min at 4 °C.
6. Transfer the supernatant in a 26.3 mL ultracentrifuge polycarbonate bottles.
7. Add 6 mL of a 30 % sucrose solution diluted in 0.2× sodium-potassium phosphate extraction buffer, at the bottom of the tube with a Pasteur pipette.
8. Centrifuge at 148,000  $\times \text{g}$  for 1 h at 4 °C.
9. Discard the supernatant by pipetting.
10. Wash once the tube carefully with few milliliters of deionized water.
11. Add 150  $\mu$ L of 1× RQ1 DNase buffer and let the resulting pellet resuspend overnight at 4 °C.
12. Transfer the viral particles suspension by pipetting in a 1.5 mL Eppendorf tube.
13. Add 15  $\mu$ L of RQ1 DNase (1 U/ $\mu$ L) and 1.5  $\mu$ L of RNase A (7 U/ $\mu$ L), and incubate at 37 °C for 2 h.

#### 3.2 Viral Nucleic Acid Extraction

1. Extract RNA from the total volume of viral particle suspension obtained at the previous step (approximately 166.5  $\mu$ L) with the RNeasy Plant Mini Kit according to the manufacturer's protocol.
2. Elute RNA in a 1.5 mL microtube by adding 50  $\mu$ L of molecular grade water to the center of the RNeasy Mini Spin column and by centrifugation at 8,000  $\times \text{g}$  for 1 min at room temperature.

#### 3.3 Random RT-PCR Amplification of Purified Viral Nucleic Acids

1. Add 16.6  $\mu$ L of the purified viral nucleic acids to 2.5  $\mu$ L Library Synthesis solution (TransPlex® Whole Transcriptome Amplification (WTA) Kit) and amplify the viral nucleic acids by Random RT-PCR amplification with the WTA Kit according to the manufacturer's protocol.
2. Load 50  $\mu$ L of final PCR product, previously mixed with 0.1 volume of 10× loading buffer and 8  $\mu$ L of 1 kb DNA ladder, on 1.2 % agarose gel in 1× TAE buffer.



**Fig. 1** Agarose gel analysis of the random RT-PCR amplifications of the purified viral nucleic acids of the yam plant MP2. *Lane 1* and *3*: 1 kb ladder size marker (Invitrogen); *lane 2*: amplifications (smear) of the purified viral nucleic acids of plant MP2

3. Migrate during 30 min with a voltage of 100 V.
4. Stain the gel for 20 min in a 0.5 µg/mL ethidium bromide solution and wash the stained gel in water for 20 min.
5. Visualize nucleic acids under ultraviolet light of the UV transiluminator and check for the presence of a smear corresponding to PCR product sizes ranging from 100 to 1,000 bp (Fig. 1).
6. Cut the gel containing PCR products ranging in size from 500 to 1,000 bp with a sterile razor blade, transfer the excised gel cube in a 2 mL microtube, and purify the PCR products with the QIAquick Gel Extraction Kit according to the manufacturer's protocol.
7. Elute purified PCR products (amplicons) in a 1.5 mL microtube by adding 50 µL of molecular grade water to the center of the QIAquick column and by centrifugation at 17,900 × g for 1 min at room temperature.

### 3.4 Cloning and Sequencing

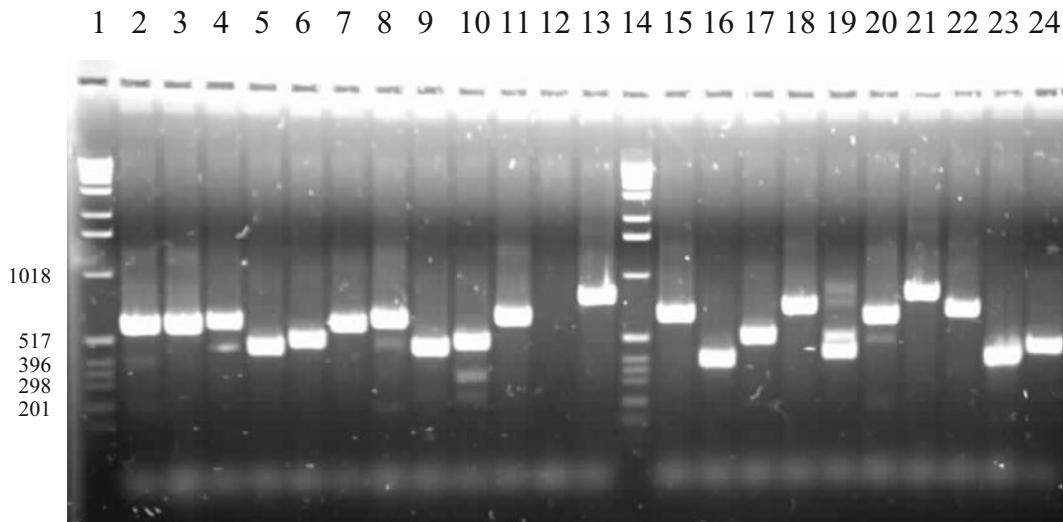
1. Add 3 µL of purified random RT-PCR amplicons in a 1.5 mL microtube containing 7 µL of ligation mixture from the Cloning pGEM®-T Easy System kit. This mixture consists of

5  $\mu$ L 2 $\times$  Rapid Ligation Buffer, 1  $\mu$ L pGEM<sup>®</sup>-T Easy Vector (50 ng/ $\mu$ L), and 1  $\mu$ L T4 DNA Ligase (3 U/ $\mu$ L).

2. Incubate the reaction overnight at 4 °C.
3. In a 2 mL microtube, add 2  $\mu$ L of the ligated fraction and 50  $\mu$ L of JM109 competent cells.
4. Incubate the tube on wet-ice for 20 min.
5. Heat-shock the cells for 45 s in a water bath at exactly 42 °C.
6. Immediately return the tube to wet-ice for 2 min.
7. Add 950  $\mu$ L of liquid LB medium and incubate for 1.5 h at 37 °C with shaking (~200 rpm).
8. Prepare the selective medium by adding into 10 cm plastic petri dish, 25 mL solid LB medium, 2.5 mg ampicillin, 2 mg X-Gal, and 3 mg IPTG.
9. Plate 100  $\mu$ L of the transformed JM109 culture onto the selective medium.
10. Incubate the plates overnight at 37 °C.
11. Add 25  $\mu$ L of PCR mix (12.5  $\mu$ L GoTaq Hot Start Colorless Master Mix, 0.5  $\mu$ L SP6 primer (10  $\mu$ M), 0.5  $\mu$ L T7P primer (10  $\mu$ M), 11.5  $\mu$ L molecular grade water) into single 200  $\mu$ L PCR tubes.
12. Select and collect single white colonies using sterile toothpicks, and discard blue colonies.
13. Soak toothpick (and transformed bacteria) into PCR tubes (one toothpick (i.e., a single colony) per PCR tube).
14. Close the tubes, put them into the thermocycler and run PCR using the following conditions: an initial denaturation step at 95 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min.
15. Load 10  $\mu$ L of final PCR product, previously mixed with 0.1 volume of 10 $\times$  loading buffer and 8  $\mu$ L of 1 kb DNA ladder, on 1.2 % agarose gel in 1 $\times$  TAE buffer.
16. Migrate during 20 min with a voltage of 100 V.
17. Stain the gel for 20 min in a 0.5  $\mu$ g/mL ethidium bromide solution and wash the stained gel in water for 20 min.
18. Visualize nucleic acids under ultraviolet light of the UV transilluminator (Fig. 2).
19. Select amplicons with the size larger than 250 bp and sequence them (Sanger method using the universal SP6 and T7P primers).

### **3.5 Analysis of Sequence Data**

1. Raw sequences are visualized using Chromas 2.33 (Technelysium).
2. The quality of chromatograms is checked and parts of the sequences harboring double peaks or ambiguous peaks are



**Fig. 2** Screening of white transformed JM109 *E. coli* colonies (expected to host recombinant pGEM®-T Easy Vector) using SP6/T7P-PCR. *Lane 1* and *14*: 1 kb ladder size marker; *lane 2–13* and *15–24*: amplifications of the recombinant plasmids containing random RT-PCR products of purified viral nucleic acids of plant MP2

removed. Then, the remaining cleaned sequences are trimmed in order to remove the pGEM®-T Easy cloning vector sequences.

3. Sequence data of all the amplified fragments are assembled using the CAP3 sequence assembly program (for example, see Institut Pasteur Biology IT Center Mobyle website: <http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::cap3>).
4. Sequence similarity searches are performed on assembled and non-assembled cleaned sequences using the BlastN and BlastX methods implemented in the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) by comparing the nucleotide and nucleotide-translated queries against nucleotide collection (nr/nt) and nonredundant protein sequences (nr) databases, respectively (Fig. 3).
5. Best hits of the Blast results are filtered on the taxonomy and the e-values. Only hits matching for viruses and e-values less than  $10^{-3}$  are retained.

#### 4 Notes

1. Only a fraction of the nucleotidic sequences (6 out of 80 sequences, 7.5 %) obtained using this approach corresponded to viral sequences stored in the NCBI databases. The remaining sequences corresponded to plants (19/80), fungi (1/80), and unknown organisms (42/80).

polyprotein [Pea seed-borne mosaic virus]

Sequence ID: [emb|CAC86256.1](#) Length: 137 Number of Matches: 1

Range 1: 49 to 111 GenPept Graphics						Next Match	Previous Match
Score	Expect	Method	Identities	Positives	Gaps	Frame	
65.5 bits(158)	3e-11	Compositional matrix adjust.	28/64(44%)	46/64(71%)	1/64(1%)	+2	
Query 2		GMGTTKQRRFVSYGFDVDEYDELRFMDPITGITYDRPIYSVDALEIEQSISDDRQQILLES				181	
		GMG K R+FV+ YGFD EY +RF+DP+TG+TYDR	++++++I	DDR +	+E+		
Sbjct 49		GMGVKTRKFVNYYGFDPEYSIVRFVDPLTGLTYDRHTME-HMMMDVQEAGDDRNEAIEN				107	
Query 182	EQFE	193					
	++ +						
Sbjct 108	DELD	111					

**Fig. 3** The nucleotide-protein Blast (BlastX) result from NCBI for one nucleotide sequence obtained from the yam plant MP2. This BlastX result indicates that the yam plant MP2 is putatively infected by an unknown *Potyvirus* according to the 82 % amino acid identity species demarcation threshold set by the *Potyvirus* study group of the International Committee on Taxonomy of Viruses [2]. Noteworthy, the nucleotide Blast (BlastN) result from NCBI for this nucleotide sequence did not provide any significant hit

2. Expected sizes of the PCR products (500–1,000 bp) isolated from the gel (Subheading 3.4, step 6) were selected in order to get at once the longest sequences with the Sanger sequencing method. However, the actual sizes of the cloned fragments were ranging from 89 to 663 bp, suggesting that resolution of small fragments in agarose gel remains incomplete during standard migration processes. The average size of the cloned fragments can be increased by repeating once the migration/gel cleaning step.
3. The Sanger clone-based sequencing approach used in this study has proven to be a powerful and reliable tool for assessing the viromes of a yam plant. Interestingly, the PCR fragments produced by the VANA-based approach can potentially be used by Next-Generation Sequencing (NGS) technologies. Combining VANA-based approach and NGS technologies is likely to be the next step for identifying almost all known and unknown microbes present in any particular host organism.

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# Chapter 19

## Detection of *Potato spindle tuber viroid* and Other Related Viroids by a DIG Labelled RNA Probe

Wendy A. Monger and Colin Jeffries

### Abstract

Viroids can cause diseases of considerable economic importance; in Europe the main concern is with pospiviroids that may affect the tomato and potato industries. Methods for detection are required that are both sensitive and robust. The detection method described here is a probe hybridization method with a commercially available digoxigenin (DIG) labelled full-length *Potato spindle tuber viroid* (PSTVd) RNA probe. This method detects PSTVd and all other known pospiviroids.

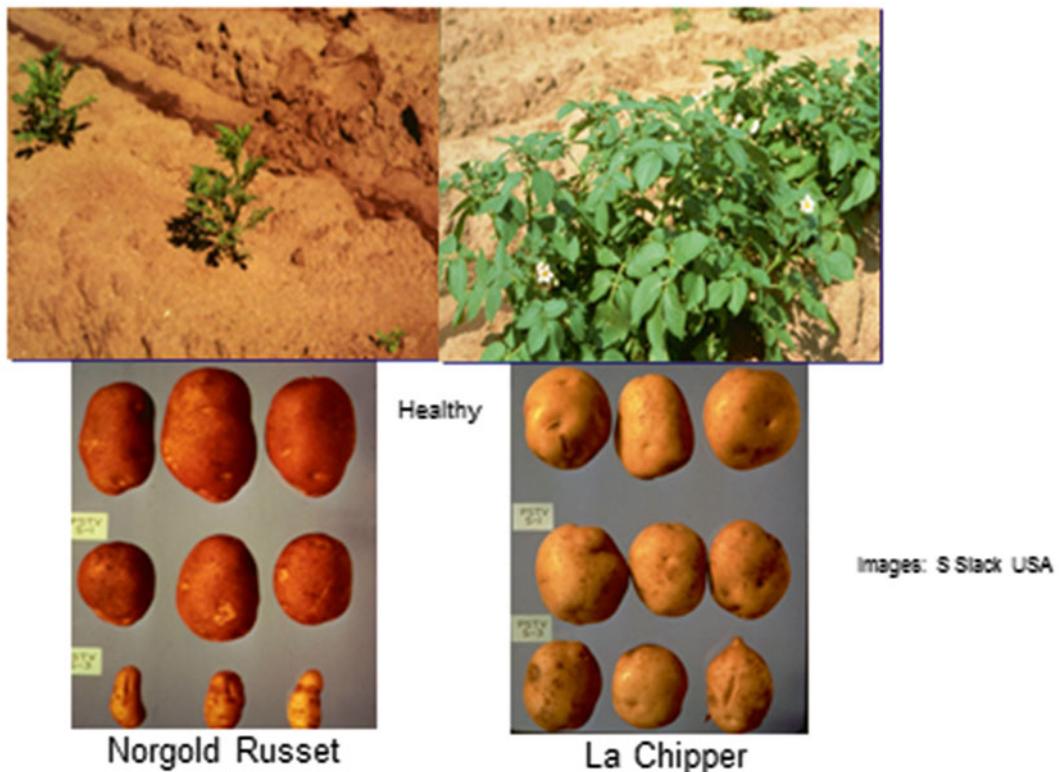
**Key words** Potato, Tomato, *Potato spindle tuber viroid* (PSTVd), *Pospiviroid*, RNA extraction, Digoxigenin RNA probe (DIG-probe)

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### 1 Introduction

Viroids are the smallest known pathogen of plants, consisting of a single strand circular RNA molecule 341–364 nucleotides in size that form strong secondary structures through covalent bonding between nucleotides. Although viroids can produce symptoms that are often mistaken for viruses, they differ from viruses in many ways, the main one being that their genome does not code for any proteins. All biological functions like replication and movement require utilizing host proteins; this is achieved by the secondary structure of viroids interacting with host cell factors. To date there are 32 approved viroid species [1], classified into two families, the *Avsunviroidae* (that replicate in the chloroplasts) and the *Pospiviroidae* (that replicate in the nucleus).

Within the family *Pospiviroidae* is the genus *Pospiviroid* that contains the species *Potato spindle tuber viroid* (PSTVd). PSTVd is the only viroid known to infect cultivated species of potato naturally; symptoms of infection are variable depending on the potato cultivar, environment, and viroid strain [2]. Plants may appear smaller, more upright with small leaves and tubers elongated or misshapen but may also have very few symptoms (Fig. 1). The



**Fig. 1** Two potato cultivars Norgold Russet and La Chipper infected with PSTVd. Potato plants are in the 3rd year of infection. Tubers (from *top* to *bottom*) are healthy, 1st year of infection, 3rd year of infection

*Pospiviroid*, *Mexican papita viroid* (MPVd) has been found infecting the wild potato species *Solanum cardiophyllum* [3]. Other members of the genus include *Chrysanthemum stunt viroid* (CSVd), *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd), *Iresine viroid* (IrVd), *Pepper chat fruit viroid* (PCFVd), *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd) and *Tomato planta macho viroid* (TPMVd). All pospiviroids have been shown to be transmitted to potato except for IrVd-1 [4, 5]; reported symptoms are similar to those for PSTVd [6–8]. Pospiviroids that have been found to naturally infect tomato plants are PSTVd, TCDVd, CLVd, CEVd [9], TASVd, TPMVd, and MPVd [10]. CSVd [11, 12] and PCFVd [13] can be transmitted to tomato experimentally. Symptoms can be severe with chlorosis, deformed plants, bunchy tops, and small fruit. Other host plants of the pospiviroids include ornamentals such as petunia that do not usually show symptoms [5].

PSTVd is highly infectious and is easily transmitted mechanically by machinery and by contact. When in a dried form either as naked RNA or in dried tissue or sap it will remain infectious indefinitely. PSTVd can be transmitted in true potato seed via

infected pollen or ovules [14, 15]. Experimental acquisition and transmission of PSTVd by *Myzus persicae* from plants coinfected by *Potato leafroll virus* has been reported [16–18]. However, only TPMVd has been shown to be efficiently transmitted by aphids without the presence of an assisting virus [19]. Most viroid outbreaks in tomato are thought to occur from infected seed. Seed transmission rates are low, but one infected plant can quickly spread the disease to neighboring plants in the confines of commercial glasshouses. Infection of tomato from neighboring symptomless ornamental host plants has also been confirmed [20].

PSTVd is a quarantine pest in the European Union, and statutory testing is required on potato material received for post-entry quarantine, material for nuclear stock production, and breeding material not eligible for the issue of a plant passport. For a comprehensive review of pospiviroids in Europe see the European Food Safety Authority [21].

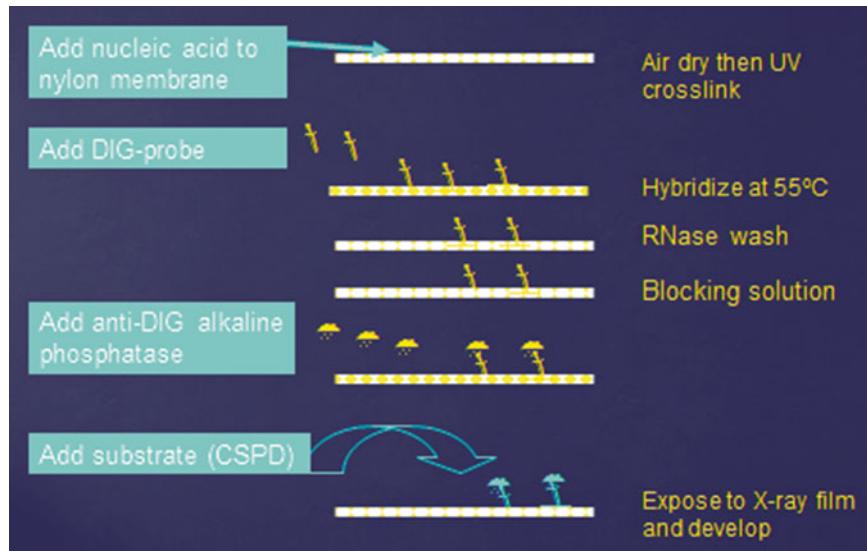
### **1.1 Methods of Viroid Identification**

The lack of a protein shell means that antibody based testing cannot be used to detect viroids. A comprehensive list of detection methods can be found in EPPO standard PM7/33 [22] which is currently under review, and in an International Plant Protection Standard ISPM 25, Annex 7 [23]. There are currently three main types of detection method, all usually requiring RNA extraction from plant material for maximum sensitivity.

*Return-PAGE.* This requires running a polyacrylamide gel under denaturing conditions to separate the small circular viroid RNAs from other plant RNA. Since viroid detection is independent of the viroid sequence Return-PAGE has an advantage over other methods in detecting non targeted or new viroids. However, it is time-consuming and less suitable for large-scale diagnosis. Also it is less sensitive than hybridization and PCR-based methods described in PM7/33 [24].

*End-point RT-PCR* [9] and *Real-time RT-PCR* [25, 26]. The speed of these methods and their capacity for high-throughput testing gives them a cost-effective advantage. They are highly sensitive and have become the most commonly used methods for detecting viroids. The disadvantage is their reliance on sequence similarity between their targets and primer–probe combination. Viroids are pathogenic RNAs of plants, replicated by a plant DNA-dependent RNA polymerase II lacking proofreading activity, resulting in nucleotide misincorporation and high rates of nucleotide variability between viroid isolates. Genome variability means that PCR-based methods might occasionally fail to detect some isolates.

*Hybridization.* This has advantages over Return-PAGE since it is more sensitive, and over PCR-based methods because use of a



**Fig. 2** Digoxigenin (DIG) probe labelling system

labelled full-length viroid probe is unlikely to miss the detection of divergent isolates [24]. However, it is more time-consuming than PCR-based methods and requires a greater level of staff competency. Although any established plant RNA extraction method could be used to generate the RNA for hybridization, we use a method that preferentially extracts low-molecular-weight RNA using a two-step PEG (polyethylene glycol) fractionation. This yields a RNA sample with a greater proportion of small RNA's, thus increasing the sensitivity of the assay used for viroid detection.

The detection method described here uses the digoxigenin (DIG) probe labelling system (Agdia Incorporated, USA) that enables non-radioactive labelling of nucleotides (Fig. 2). In our laboratory it is an ISO 17025 accredited test method. For detection, nucleic acid extract is spotted onto a membrane. The DIG RNA probe (DIG-probe) is added and hybridizes with the viroid target. An anti-digoxigenin antibody conjugated to alkaline phosphatase binds to the hybridized probe. The antibody-probe hybrids are visualized by the addition of a chemiluminescent substrate that emits light in the presence of alkaline phosphatase which, is then recorded on X-ray film. The probe is a full-length PSTVd monomer, and it is not specific for PSTVd and hybridizes with other pospiviroids depending on sequence similarity and the hybridization temperature used. The following pospiviroids have been detected in our laboratory using the DIG-probe method described: CSVd, CEVd, CLVd, IrVd, MPVd, PCFVd, PSTVd, TASVd, TCDVd, and TPMVd. Other workers have reported similar results when using the DIG-PSTVd probe, e.g., Singh et al. [9] has detected TCDVd and Agdia, (pers. comm.) has detected CSVd and CEVd.

## 2 Materials

### 2.1 Small RNA Extraction Method

1. *PEG 6000*: 0.02 g aliquots (powder form) in 1.5 mL microfuge tubes, two tubes per sample.
2. *Lithium chloride extraction buffer*: LiCl 14.8 g; glycine 2.25 g. Dissolve in 80 mL of distilled water adjust to pH 8.8 with HCl or NaOH. Make up to 100 mL.
3. *Phenol-chloroform mix*: phenol crystals 200 g; chloroform 200 mL; octan-1-ol 4 mL; hydroxyquinoline 0.4 g. Dissolve the phenol with the chloroform then add the other reagents. Store in a fridge, use only in a fume hood.
4. 10 % SDS.
5. 50:50 methanol–ether wash solution.
6. Sand (Sigma).

### 2.2 Sample Loading onto the Membrane

1. Biodyne B nylon membrane (VWR).

### 2.3 DIG Labelled RNA Probe

1. DIG Easy-Hyb Buffer (Roche).
2. DIG-labelled PSTVd probe (Agdia).
3. RNase A solution (Sigma); aliquot into 20 µL volumes. Before use denature the RNase by heating one aliquot to 100 °C for 15 min.
4. 10× maleic acid buffer: maleic acid 116.1 g; NaCl 88.4 g; NaOH pellets 30.0 g. Dissolve in 800 mL of distilled water, adjust pH to 7.5 with NaOH, and make up to 1,000 mL. Use at a 1× dilution.
5. *Blocking solution*: Blocking reagent (Roche) 4 g; 1× maleic acid buffer 40 mL. Microwave for 10 s prior to autoclaving solution.
6. Anti-DIG-alkaline phosphatase (Roche).
7. CSPD (chemiluminescence substrate) (Roche).
8. 10× detection buffer: TRIS 12.1 g; NaCl 5.8 g; dissolve in 90 mL of distilled water and adjust pH 9.5 with HCl. Use at a 1× dilution.
9. 20× SSC: NaCl 173.3 g; sodium citrate 88.2 g. Dissolve in 800 mL distilled water, adjust pH 7.0 with NaOH and make up to 1,000 mL.
10. *Wash buffer 1*: 20× SSC 100 mL; 10 % SDS 10 mL; make to 1,000 mL with distilled water.
11. *Wash buffer 2*: 20× SSC 5 mL; 10 % SDS 10 mL; make to 1,000 mL with distilled water.
12. Biomax MS film (Sigma).

## **2.4 Developing the Autoradiograph**

1. Developer (Sigma).
2. Water.
3. Fixer (Sigma).

## **3 Methods**

The probe hybridization method is used routinely in our laboratory for potato microplants, leaves, and tuber eyes, but is also used for testing other crops species and ornamental plants.

### **3.1 Sampling of Plant Material**

The amount of viroid in a plant can be affected by environmental conditions such as temperature and light levels, therefore potato microplants or potato plants for testing are grown at a temperature of 18 °C or higher, and with at least a 14-h photoperiod. Additionally potato microplants can have low levels of viroid after subculturing, therefore 4–6 weeks old microplants with stems of about 5 cm length and with well-formed leaves are tested, and are tested individually. Leaves of potato plants (and also tomato) are sampled near the top of the plant (where viroid levels are highest), bulked in samples of 10 and punched with a 10 mm diameter cork borer to give discs for testing. Tubers can be bulked in samples of 5, if held at 25 °C for 2 weeks prior to sampling; otherwise they are tested individually or in pairs. The total weight of a sample should be approximately 200 mg. We use positive controls of 2 and 4 mg of infected material made up to 200 mg with healthy material. The 2 mg positive controls (standards) are prepared annually. Positive infected sap is diluted with healthy sap and aliquoted to give the equivalent of 2 mg infected leaf and 198 mg healthy leaf per vial, freeze dried and stored at 4 °C. In practice these controls have remained stable over at least a year and are used to monitor the performance of the assay. The 4 mg positive control is freshly prepared by adding 4 mg of leaf tissue from an infected plant to 196 mg of healthy leaf. When tested this sample may show variability from assay to assay.

### **3.2 Small RNA Extraction Method**

Refer to **Notes 1–6** before starting this section. Unless otherwise stated centrifugation is done using a bench top refrigerated microfuge set at maximum speed and 4 °C.

1. Using a pestle and mortar grind the sample with a few grains of sand, 20 µL of 10 % SDS and 180 µL of LiCl extraction buffer.
2. Add 400 µL of phenol–chloroform mix, stir and pour slurry into a microfuge tube.
3. Centrifuge for 20 min.

4. Remove 190 µL of the top aqueous phase, taking care not to draw up any of the phenol phase, and add to one of the PEG containing microfuge tubes. Vortex the tube until the PEG has dissolved.
5. Centrifuge for 20 min.
6. Remove 190 µL of the supernatant, do not disturb pellet, and add to the second PEG containing microfuge tube; vortex to dissolve.
7. Centrifuge for 20 min.
8. A pellet should be visible. Remove supernatant with a fine tip plastic pastette and discard.
9. Add approx. 500 µL of methanol–ether wash to each tube.
10. Centrifuge for 2 min.
11. Without disturbing the pellet draw off the wash solution with a fine tip plastic pastette.
12. Leave pellets to dry for at least 2 h. Dried pellets can be held at room temperature until required.
13. Immediately before use, resuspend each pellet in 20 µL of sterile purified water and vortex.

### **3.3 Sample Loading onto the Membrane**

Refer to **Notes 7** and **8** before starting this section.

1. Mark out the Biodyne B membrane into 5 mm squares using a sharp pencil and clean ruler.
2. For each sample apply two spots (each 3 µL) to the membrane. Samples are numbered 1–4 in Fig. 3.
3. After spotting store the RNA samples at –20 °C.
4. Two different positive controls are used (S and X, 2 and 4 mg infected leaf respectively). X is double spotted the same way as the samples and S is spotted in a diagonal pattern so the developed autoradiograph can be correctly orientated (Fig. 3).

1	2	3	4
1	2	3	4
	X	S	
	X	PP1	S

**Fig. 3** Layout of the membrane. 1–4 are the samples for test, S and X are positive controls (2 and 4 mg infected leaf respectively), PP1 is the unique test number for this membrane

5. Negative controls are not routinely used since the majority of samples that we test are negative, and these serve as the negative control. It is also relatively straight forward to collect new material for test should samples give a positive result. Negatives must be included if positive samples are more likely and retesting of samples is not an option.
6. The membrane is numbered in pencil in an empty square with the test reference number (PPI in Fig. 3) since more than one filter may be processed at any one time.
7. Leave the membrane to air dry for a minimum of 30 min. Cut the sample area of the membrane out.

### **3.4 Preparation and Hybridization with DIG Labelled RNA Probe**

Additional information on the use of this probe can be found from the Agdia website <https://orders.agdia.com/Documents/m178.pdf>.

#### *3.4.1 Preparation of the PSTVd DIG Probe*

Prepare the DIG probe according to the instructions supplied.

- Briefly centrifuge to ensure the content is pelleted at the bottom of the tube.
- Resuspend first in 200 µL DIG Easy-Hyb Buffer by vortexing and then make up to the 8 mL volume stated for one tube. Small membranes require one tube of probe and larger membranes two.

A probe can be reused at least 4 times. Store at -70 °C in a plastic tube. To reuse defrost and denature by standing in 80 °C water for at least 15 min.

#### *3.4.2 Hybridization*

Refer to **Notes 8** and **9** before starting this section.

We perform the hybridization step and most washing steps in glass dishes. Small membranes are put in a plastic weigh boat inside the glass dish for the hybridization step, this uses less probe. Volumes used for the washing steps are designed for our dishes and may require scaling up or down depending on the user's equipment.

Throughout the hybridization and washing steps the membrane must not dry out.

1. Fix the samples by cross-linking on a UV transilluminator for 2 min 30 s.
2. In a dish cover the membrane with enough DIG Easy Hyb Buffer to wash the membrane, then put on an orbital shaker at room temperature for 15–30 min.
3. Put the resuspended or denatured probe into a dish and place the membrane face down onto it.
4. Seal the dish with two layers of Saran™ Wrap and hybridize overnight in a preheated incubator at 55 ± 5 °C with shaking.

5. Remove the dish and turn the incubator to 80 °C.
6. On an orbital shaker wash the membrane in a dish containing Wash buffer 1 for 5 min at room temperature followed by a 15 min wash in fresh Wash buffer 1 (approx. 40 mL) containing 1 µL (of 10 mg per mL) RNase A per 10 mL of buffer.
7. Wash the membrane twice for 15 min each with shaking in the pre heated 80 °C incubator using lidded containers containing Wash buffer 2 (approximately 100 mL each) that has been pre-heated to 65 °C using a microwave.
8. Wash the membrane on an orbital shaker at room temperature in 1× maleic acid buffer for 5 min.
9. Transfer the membrane to 1× maleic acid buffer containing 1 mL Blocking solution for every 10 mL of buffer (approx. 40 mL total). Incubate at room temperature on an orbital shaker for 30 min.
10. Centrifuge the anti-DIG-alkaline phosphatase solution maximum speed in a benchtop centrifuge for 30 s before use.
11. Add anti-DIG-alkaline phosphatase at a dilution of 1:10,000 (e.g., 4 µL in 40 mL) to the blocking solution and membrane; take care to mix the solution away from the membrane.
12. Incubate for 30 min at room temperature on the orbital shaker.
13. Wash the membrane twice for 15 min each at room temperature on the orbital shaker with 1× maleic acid buffer (approx. 100 mL each).
14. Wash the membrane for 5 min at room temperature on the orbital shaker with 1× detection buffer (approx. 40 mL).
15. Dilute CSPD (chemiluminescence substrate) 1:100 in 1× detection buffer (volumes normally 200–800 µL per membrane).
16. Place the wet membrane, sample side up, on Saran™ Wrap (the wrap should be of sufficient size to be able to fold over the top of the membrane). Pipette diluted CSPD over the membrane, tilt the wrap to ensure the membrane is completely covered.
17. Fold the wrap on top of the membrane and using a paper towel thoroughly squeeze out air bubbles and liquid.
18. In a dark room set up an autoradiograph cassette. Attach the Saran™ wrapped membrane to a card and expose to X-ray film.
19. Incubate the cassette at 37 °C for 2–3 h.

### **3.5 Developing the Autoradiograph**

1. In a dark room set up three trays, one with developer, one with water and one with fixer.
2. Open cassette in the dark and place the X-ray film in the developer for 90 s.
3. Wash the film in the water for 20 s.

4. Place the film in the fixer for 60 s.
5. Wash the film in the water tray again, and then under a running tap to remove any chemical traces.
6. Once dry hold up to the light and look for spots.

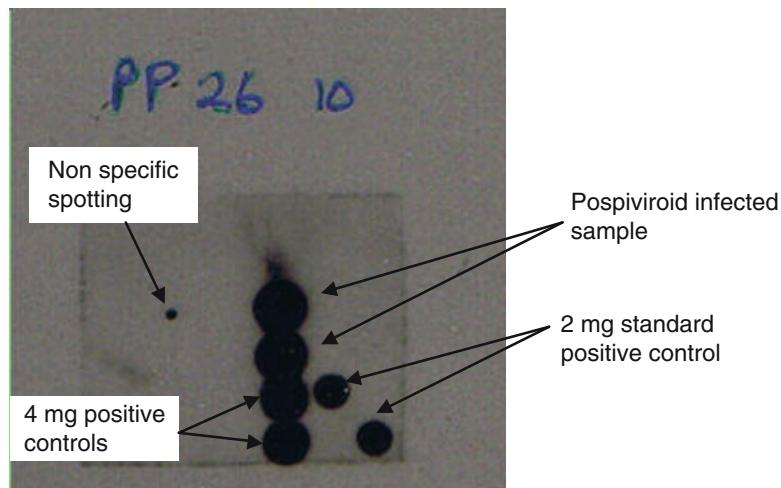
Keep the working solutions of developer and fixer in air tight containers to be reused, change 3 monthly or when the developer becomes dark in color.

### **3.6 Interpretation of Results**

This is usually straightforward. The positive controls can be orientated and any positive samples should be clear from the double spot on the membrane (Fig. 4). There is always variation between membranes but the positives should always be visible. If the positive controls are not as expected, the test must be repeated. We store the image of the control spots from each test on an Excel spreadsheet so as to monitor test performance (trend analysis).

A positive result could mean that any of the pospiviroids are present in the sample.

The extracted RNA can be further used for RT-PCR or real-time RT-PCR to confirm detection or in the case of RT-PCR to generate a PCR product for sequencing to identify the viroid species. The positive control sequence should be known so as to ensure no cross contamination from the positive control has occurred.



**Fig. 4** Autoradiograph showing positive controls, an infected sample, and non-specific spotting. The test reference number does not show on an autoradiograph so it is written on (PP26 10)

### 3.7 Troubleshooting

- *The whole autoradiograph has a high level of background.* The probable cause is light getting into the dark room or the cassette during incubation. Take extra light excluding precautions.
- *The area of the autoradiograph where the membrane is has a high background.* The RNase step should reduce nonspecific probe binding, so check the RNase-treatment step. The overnight hybridization incubation step may have gone below the recommended  $55 \pm 5$  °C, therefore use a min/max thermometer to check this step.
- *Nonspecific spotting on the membrane.* This can be caused by the anti-DIG-alkaline phosphatase solution crystals forming but the centrifuging step before use should prevent this. It is probably caused by CSPD not being properly squeezed out of the Saran™ Wrap before exposing the x-ray film.
- *The positive spots are weak or absent.* The positive control may be weak therefore always use a second control on the membrane. The overnight hybridization step may have been over  $55 \pm 5$  °C therefore use a min/max thermometer to check this step. The probe may not have been made or denatured properly therefore use a different probe. The lithium chloride extraction buffer may be at the wrong pH therefore make fresh buffer.

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## 4 Notes

1. This method uses powdered PEG 6000 a chemical often used in virus purification but can also be used first at a low concentration to remove proteins and high molecular weight nucleic acid and at a higher concentration to precipitate smaller RNA's like viroids. The PEG comes in flake form and needs to be in powder form, use either a pestle and mortar or a coffee grinder to produce a fine powder. Two microfuge tubes containing 0.02 g of PEG (0.0198–0.022 g) are required per sample.
2. A PEG scoop can be made to enable rapid dispensing of the powdered PEG into microfuge tubes. To make a PEG scoop weigh out 0.02 g of powdered PEG into a 1.5 mL microfuge tube. Mark the level of the PEG and cut off the tip of the microfuge tube at the mark. Insert the point of a disposable scalpel into the plastic wall; this provides a handle for the scoop. Weigh the first few tubes produced by the scoop each time it is used.
3. To avoid contamination, lids of microfuge tubes can be opened using pieces of laboratory paper towel.

4. If there is not enough aqueous top phase, (**step 4** of Subheading 3.2; this can happen with drier plant material) add LiCl buffer to make up the volume.
5. The PEG can be difficult to dissolve by vortexing; it goes into solution much faster if a pipette tip is in the tube while vortexing with the lids open.
6. All PEG must be dissolved or it may interfere with detection.
7. The maximum width and length of the membrane used will depend on the container sizes available for hybridization and washing steps.
8. Always wear gloves when handling the membrane.
9. Once dry the samples are stable on the membrane which can be held at room temperature.

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# Chapter 20

## Microarray Platform for the Detection of a Range of Plant Viruses and Viroids

Ian Adams, Catherine Harrison, Jenny Tomlinson, and Neil Boonham

### Abstract

Diagnostic microarrays are a useful tool for the simultaneous detection of multiple targets. In this chapter we describe the use of a simple tube-based microarray platform for the detection of plant infecting viruses and viroids.

**Key words** Microarray, Plant virus, Viroid, Diagnostic

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### 1 Introduction

The use of microarrays for plant virus detection offers the potential to assay for hundreds of virus species simultaneously in a single procedure.

Microarrays were first introduced as a development of dot blot macroarrays for the measurement of differential gene expression in *Arabidopsis* [1]. The initial arrays used cloned and PCR-amplified DNA as probes printed onto glass slides and sample nucleic acid was labeled with fluorescent dye allowing hybridization to be visualized. Later arrays were manufactured by printing pre-synthesized oligonucleotides onto the slides, or by synthesizing the probes *in situ*, and used fluorescence, colorimetric, or electric currents to detect hybridization [2].

Microarrays were first used for viral pathogen detection in 2002 by Wang et al. [3] who identified a range of human viruses in patients with upper respiratory tract infections using a 70-mer oligonucleotide array. Plant virus microarrays were first developed by Boonham et al. [4] and Lee et al. [5] who developed PCR product-based arrays for detection of a number of potato viruses and a cucurbit virus array [6]. Bystricka et al. [7] then went on to develop an oligonucleotide array which made array manufacture easier and cheaper. Since then, glass slide oligonucleotide arrays have been

developed for genotyping of plum pox virus [8] and detection of grapevine viruses [9], a range of plant viruses [10], viroid genera [11], and plant virus genera [12].

Glass slide-based arrays, although successful in detecting virus infection, have failed to be adopted as a routine diagnostic. This is largely due to the complicated manipulations required to successfully perform a glass slide-based microarray assay. Several solutions have been proposed to the problem of carrying out routine plant virus diagnostic microarrays. Tiberini and Barba [13] developed a CombiMatrix (Irvine, USA) array which allowed multiple samples to be hybridized simultaneously and can be used with a simplified microchip circuit-based detection system. Luminex xMAP beads (Life Tech, USA) have also been used to detect plant viruses [14]. This is a bead-based array system in which differently-labeled beads are mixed to allow multiple viruses to be detected. Another alternative, and the focus of this chapter, is the Clondiag Array Tube.

Array Tubes consist of a small, 200–400 feature oligonucleotide-based glass array mounted in the bottom of an micro-centrifuge tube. The tube format simplifies processing of the arrays, with the significant advantage that they can be used by anyone with experience of PCR in a standard molecular biology laboratory. In addition, the use of nonfluorescent, colorimetric detection allows Array Tubes to be scanned using a simple and inexpensive scanner. Array Tubes have been developed for bacteria [15], fungi [16], human viruses [17] and plant viruses [18] and have been used to deliver diagnostic services in a routine setting e.g. Identibac [19].

The Array Tube protocol consists of seven steps: RNA extraction; target amplification and clean-up; array hybridization; array washing; signal development; array scanning and results interpretation. These steps are detailed in the protocol below.

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## 2 Materials

1. RNA extracted from plant (*see Note 1*).
2. Array Tubes (Alere, Jena, Germany) (*see Note 2*).
3. Specific primer mix (any supplier of oligonucleotides) (*see Note 3*).
4. Go Taq polymerase and PCR buffer (*see Note 4*).
5. dNTPS and Biotin dCTP (*see Note 5*).
6. Molecular-grade water.
7. Pipettors—separate sets required for setting up reactions, adding DNA to reactions and post-PCR steps.
8. Filter tips for pipettors.
9. Vortexer.

10. Microcentrifuge, Benchtop centrifuge.
11. Standard PCR thermocycler.
12. QIAquick PCR Purification kit (Qiagen).
13. Nexterion hybridization buffer (Schott).
14. 20× Sodium chloride sodium citrate (SSC): 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0.
15. Wash buffer I (2× SSC, 0.01 % Triton X-100).
16. Wash buffer II (2× SSC).
17. Wash buffer III (0.2× SSC).
18. Phosphate buffered saline (PBS).
19. Blocking solution (PBS plus 0.02 % milk powder, 0.002 % Triton X-100).
20. Anti-biotin HRP-linked antibody (New England Biolabs) (*see Note 6*).
21. Conjugation solution (blocking solution plus 1 % antibody).
22. PBS containing 0.1 % Tween20.
23. seramunGrün chip substrate (Seramun Diagnostica, Germany) (*see Note 7*).
24. Thermal shaker or shaking incubator.
25. 0.6 ml and 2 ml tubes.
26. ATR03 Array Scanner (Alere) (*see Note 8*).
27. IconoClust Scanning software (Alere) (*see Note 9*).

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### 3 Methods

#### 3.1 Target Amplification and Clean-Up (*See Note 10*)

1. Set up a master mix for the reactions containing the components detailed in Table 1. Make up enough for the number of reactions to be run plus at least four extra reactions to allow for pipetting inaccuracies.
2. Vortex briefly, then pipette 24 µl aliquots of master mix into the wells of a 96-well PCR plate or individual 0.2 ml reaction tubes.
3. Add 1 µl of sample RNA extract (or water for No Template Control reactions), to give a total reaction volume of 25 µl. Do not perform this step in the cabinet used for setting up master mix.
4. Transfer the plate or tubes to a suitable thermal cycling instrument, and run the following cycling conditions: 94 °C for 2 min, followed by 40–45 three-step cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s and a final extension step of 72 °C for 5 min (*see Note 11*).

**Table 1**  
**Reagents required to make up amplification master mix**

Component	Starting concentration	Volume per reaction ( $\mu\text{l}$ )	Final concentration
5× PCR buffer		10	$\times 1$
MgCl <sub>2</sub>	25 mM	7	3.5 mM
Nucleotide mix (see Note 4)	4 mM	4	0.32 mM
Primer mix	See Note 3	4	See Note 3
Go Taq polymerase	5 U/ $\mu\text{l}$	0.25	1.25 U/reaction
Molecular-grade water		To a final reaction volume of 49 $\mu\text{l}$	

This is a potential stopping point as the sample can be stored at -20 °C until required.

### **3.2 Sample Clean-Up Using QIAquick PCR Purification Columns (See Note 12)**

1. Add 5 volumes (125  $\mu\text{l}$ ) buffer PB to PCR product. Add to column and spin at 14,500  $\times g$  for 30 s. Discard flowthrough.
2. Add 750  $\mu\text{l}$  buffer PE, spin for 30 s.
3. Discard flow through and spin column for 1 min to dry the membrane.
4. Transfer the column to a fresh tube and add 20  $\mu\text{l}$  molecular-grade water, spin at 14,500  $\times g$ , and repeat elution with 20  $\mu\text{l}$  water.
5. Transfer sample to a 0.6 ml tube and add 70  $\mu\text{l}$  of Nexterion hybridization buffer.

### **3.3 Array Hybridization**

1. Condition the array tubes by adding 500  $\mu\text{l}$  of molecular-grade water to the array tube and incubate in a thermal shaker at 55 °C, 500 rpm for 5 min.
2. Remove the water and add 500  $\mu\text{l}$  of Nexterion hybridization buffer to the array tube and incubate in a thermal shaker at 55 °C, 500 rpm for 5 min.
3. Preheat sample at 95 °C for 4 min then cool to 55 °C
4. Remove the Nexterion hybridization buffer from the array and add 100  $\mu\text{l}$  of the sample directly to tube.
5. Incubate in a thermal shaker for 1 h at 55 °C at 500 rpm.

### **3.4 Array Washing (See Note 13)**

1. Remove the buffer containing the sample from the array and add 500  $\mu\text{l}$  of Wash Buffer I. Incubate at 20 °C, 500 rpm for 5 min.

2. Remove the buffer from the array and add 500 µl of Wash Buffer II. Incubate at 20 °C, 500 rpm for 5 min.
3. Remove the buffer from the array and add 500 µl of Wash Buffer III. Incubate at 20 °C, 500 rpm for 5 min.
4. Remove the buffer from the array and add 100 µl of Blocking Solution. Incubate at 20 °C for 15 min without shaking.
5. Remove the buffer from the array and add 100 µl of Conjugation Solution. Incubate at 20 °C for 15 min without shaking.
6. Remove the buffer from the array and add 500 µl of PBS containing 0.1 % Tween20. Incubate at 20 °C, 500 rpm for 5 min. Repeat this wash a total of three times.

### **3.5 Signal Development and Scanning**

1. Remove buffer from array and add 100 µl SeramunGrün chip substrate.
2. Clean external glass of the array tube using 70 % ethanol and lens tissues.
3. After 15–30 min, run the Iconoclust software using the specific layout file (\*.icpck) provided by Alere for the array. Place the array in the ATR03 scanner and scan (*see Note 14*).

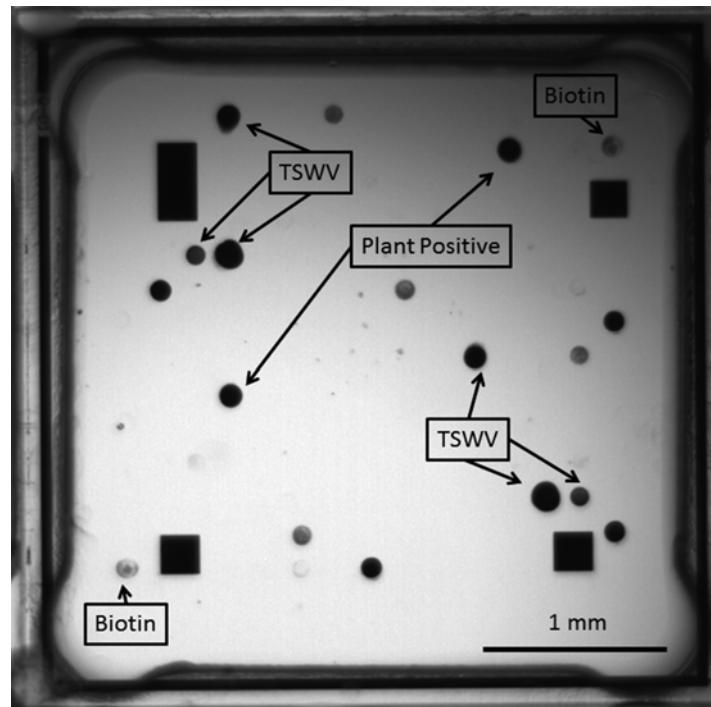
### **3.6 Interpretation of Results**

Figure 1 shows a typical array with the detection of potato viruses. The Iconoclust software outputs the data in a tab delimited text file which can be imported into excel for analysis. The data produced includes spot intensity (signal 0–1), confidence and validity for each spot. The validity (true/false) and confidence (0–1) are measures of whether the signal is based on measurement of a specific signal or if it is due to artifacts or background. We have multiple probes in duplicate for each target virus and only probes with validity = “true” and a confidence > 0.75 are used in determining the presence of a virus. An ANOVA statistical analysis with a Dunnets post-hoc test against the negative control is carried out on the duplicate data from all the probes for each virus target. Only viruses with signal significantly higher than the negative control are considered to have been detected. Figure 2 shows a plot of the average signal obtained from probes for each virus obtained from the array shown in Fig. 1. TSWV gave an average signal of  $1.5 \pm 0.10$  and along with the plant positive control and spotted biotin were the only targets with signal significantly above the negative control.

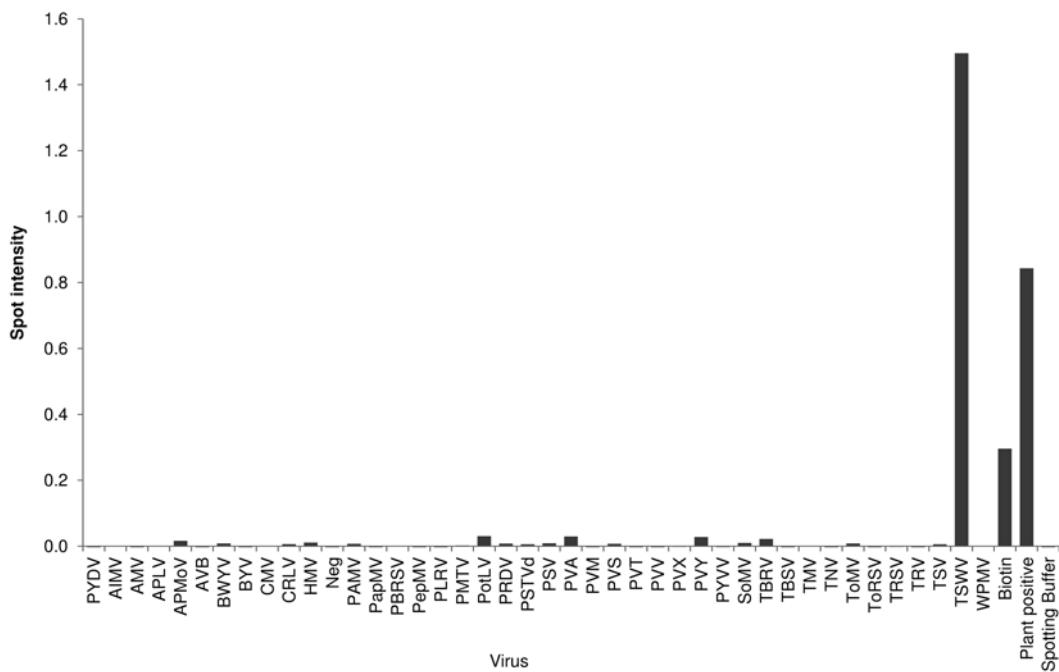
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## **4 Notes**

1. PCR quality RNA is required for this procedure. We typically use RNA extracted using the Qiagen RNeasy kit (Qiagen) or magnetic bead-based extraction [20], but any protocol typically used for PCR or real-time PCR detection of plant viruses should be adequate.



**Fig. 1** Example array showing detection of Tomato spotted wilt virus (TSWV) and various controls on a potato sample



**Fig. 2** Example plot of data obtained from array shown in Fig. 1



**Fig. 3** An array tube being placed in the ATR03 scanner



**Fig. 4** Bottom of an array tube. The oligonucleotide spots can be seen on left

2. Array Tubes are custom synthesized by Alere to a user's specification. Figure 3 shows an array tube and the ATR03 reader. Figure 4 shows the oligonucleotide spots on an array tube. Principles of array probe design are the same as for the design of PCR and real-time PCR primers. Probes are designed using alignments of sequence from target and nontarget organisms, and the specificity will come from locating primers and probes at the positions of mismatched bases. Target nucleic acid should be relatively short as long targets are more likely to contain secondary structure which can hinder hybridization to the probes and shorter PCR products are amplified more efficiently. Probe melting temperatures are typically designed to be between 60 and 65 °C. Short probes (20–30 bp) can be

designed to give a higher degree of specificity; however, longer probes (50–70 bp) can be used to achieve greater sensitivity, tolerate mismatched bases (especially useful for RNA virus detection) and are therefore best suited for plant virus detection. For further increases in specificity, mismatches should be located in the center of the probe and the probe close to the forward primer with a longer overhang at the 5' end of the PCR product [15]. Control probes are also included on the array. We routinely include probes for plant RNA probes based on Cytochrome oxidase I or RbcL genes. A negative probe is also included and biotin control spots are typically included on all arrays. During the validation and testing process probes are checked to determine if they detect their desired target, don't detect their desired target or hybridizing to incorrect targets. Incorrectly hybridizing probes are removed from analysis and not included in future iterations of the array. A number of incorrectly hybridizing probes can be seen as the unlabelled spots in Fig. 1.

3. Array Tubes require target nucleic acid to be amplified and biotin labeled; there are several possible approaches to achieve this. Random anchored amplification did not give the level of sensitivity required [21] so our preferred method is to multiplex specific PCR resulting in specific amplification products for each array probe. We use an asymmetric PCR method that uses unequal concentrations of primers (typically 1:10 or 1:20) to generate partially single-stranded DNA. The use of asymmetric PCR was found to allow the multiplexing of many primer pairs and the detection of multiple virus targets at differing concentrations in the same sample. Asymmetric PCR also reduces the competition between the target and nontarget strands and the probe. Reducing the concentration of the limiting primer also reduces its melting temperature below the reaction annealing temperature. Primers must therefore be designed with this in mind. The limited primer is designed with a melting temperature 2 °C higher than the nonlimited primer. For Clondiag arrays a ratio of 12.5:1 reverse to forward primer was found to give the optimum balance of sensitivity and ability to multiplex. A mix containing 2 µM of each reverse primer and 0.16 µM of each forward primer will give this ratio and is what should be used in master mix in Table 1.
4. We have found that Go Taq works well but other polymerases should work equally well.
5. Biotin-11-dUTP or biotin-11-dCTP is used to replace some of the dTTP or dCTP in the dNTP mix used for PCR. The nucleotide mix used in the master mix in Table 1 contains 25 % dTTP, dATP, dGTP, 16.25 % dCTP, 8.5 % Biotin dCTP.

6. The original Alere protocols used HRP-linked streptavidin to detect the biotin-labeled sample DNA hybridized to the array. We have found that streptavidin can be unstable and can give weak signals, however, and HRP-linked anti-biotin antibody performs much better.
7. An alternative substrate which gives similar results is True Blue Peroxidase Substrate from KPL.
8. The ATR03 scanner can be obtained from Alere.
9. IIconoClust software comes with the ATR03 scanner and an \*.icpck layout file for this software is provided with custom Array Tubes.
10. We routinely carry out PCR mastermix set-up in a flow cabinet. This is not essential but standard procedures for avoiding PCR contamination should be used.
11. This is a typical PCR set-up and can be optimized for specific primer sets.
12. We use a QIAquick clean-up and the reagents mention here (PB, PE) are from this kit. Other PCR clean-ups could be used.
13. While carrying out the washing steps it is important not to let the array dry out. Also never centrifuge the array tubes as the array will detach from the tube.
14. Use of the IIconclust software is described in the manual which accompanies the software and ATR03 scanner. Scan time varies somewhat with array probe set and peroxidase substrate. Serum Green (the preferred substrate) typically gives optimum signal at 30–45 min and then starts to fade. Other substrates may take longer.

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# Chapter 21

## Multiplex Detection of Plant Pathogens Through the Luminex Magplex Bead System

**René A.A. van der Vlugt, Henry van Raaij, Marjanne de Weerdt, and Jan H.W. Bergervoet**

### Abstract

Here we describe a versatile multiplex method for both the serological and molecular detection of plant pathogens. The Luminex MagPlex bead system uses small paramagnetic microspheres (“beads”), either coated with specific antibodies or oligonucleotides, which capture respectively viruses and/or bacteria or PCR products obtained from their genetic material.

The Luminex MagPlex bead system allows true multiplex detection of up to 500 targets in a single sample on a routine basis. The liquid suspension nature of the method significantly improves (1) assay speed, (2) detection limits and (3) dynamic range. It can also considerably reduce labor and consumables costs.

**Keywords** Luminex, Virus, Bacteria, Serological detection, Molecular detection, Beads, Hybridization, Multiplex, Plant pathogens

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### 1 Introduction

Adequate control of plant viruses requires the use of virus-free starting and planting material. For many years ELISA was the standard routine method used. However, over the recent years molecular detection techniques have quickly become more popular (for a recent review *see* ref. 1). Especially Real-time PCR assays, often referred to as TaqMan assays have become a standard because of their ease of use and reliability. Also the multiplex capabilities of TaqMan are often mentioned as an additional advantage. Multiplex testing, i.e. testing for more than one target in a single sample, can indeed offer significant cost and time benefits because it can decrease overall handling time and reduce the amount of consumables and reagents needed, especially when testing larger numbers of samples.

The multiplex capabilities of TaqMan are limited to four targets because of the different, partially overlapping fluorescent signals resulting from multiple reporter and quencher dyes used for detection. This limits the number of assays, which can be accurately detected in a

single tube. Therefore, true multiplex testing requires a number of parallel TaqMan assays which create additional cost and reduce the time benefits.

The Luminex method uses a generic fluorochrome for detection in combination with specific capturing of targets on an extensive array of polystyrene, paramagnetic beads. It offers true multiplex capabilities and allows the simultaneous detection of up to 500 targets either based on serological or molecular technologies.

The serology-based Luminex xMAP technology, as well as the molecular-based xTAG technology, both described below, provides a quick and state-of-the-art platform which utilizes multiplex detection while retaining the standard 96-well format and workflow.

### 1.1 Luminex xMAP Serological Assay

Current Double Antibody Sandwich (DAS)-ELISA assays usually employ specific antisera, which allow the detection of a specific pathogen. To detect other pathogens, generally separate assays are required. The xMAP technology developed by Luminex (Austin, TX, USA) is based on a universal bead immune assay [2, 3] and expands the multiplex immunological detection capability of ELISA and allows simultaneous detection of a large number of pathogens.

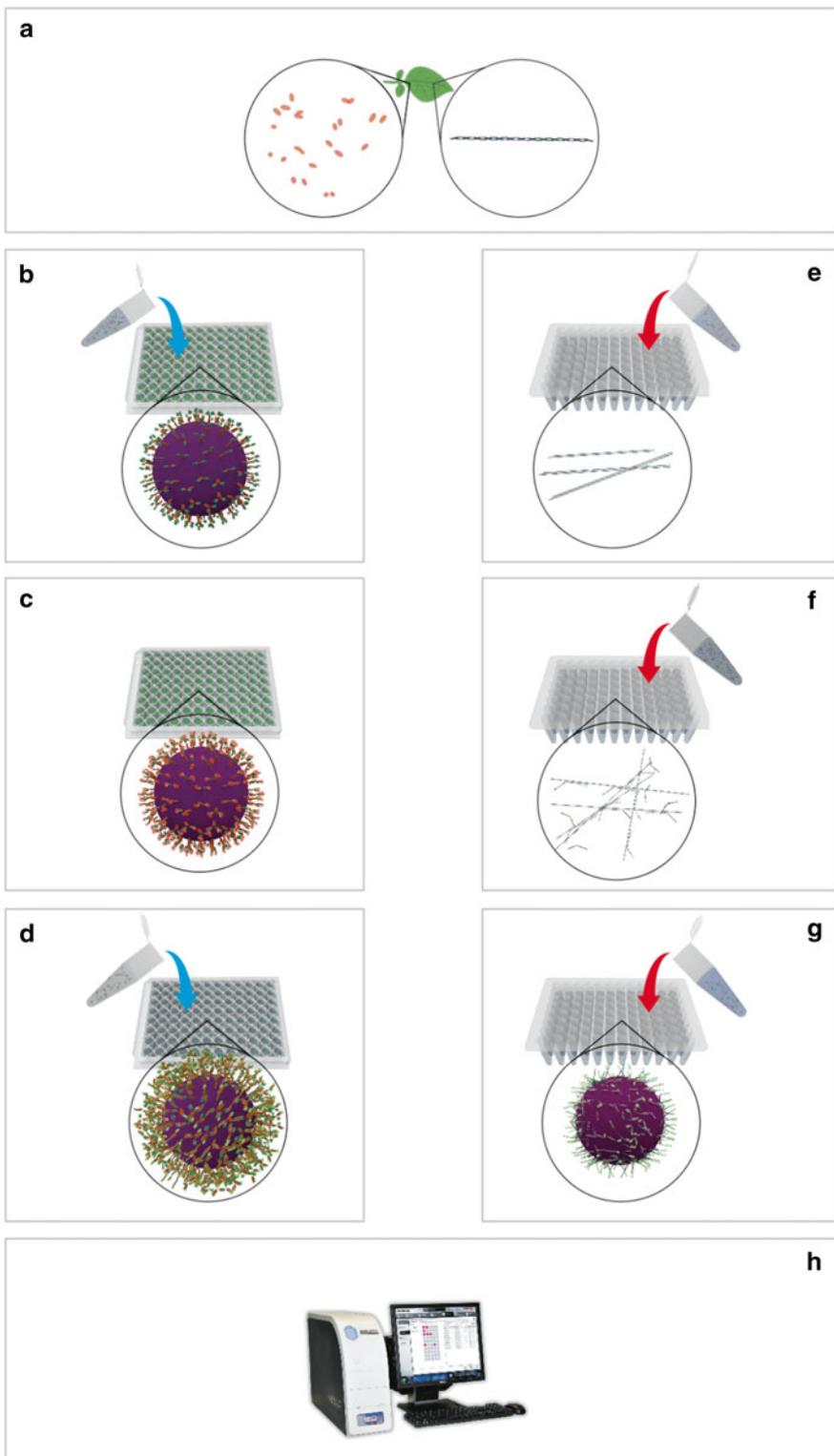
The system is based on the use of polystyrene beads ( $6.5\text{ }\mu\text{M}$ ) internally stained with two fluorochromes creating an array of maximum theoretical capacity of up to 500 different individual bead addresses. These beads are also paramagnetic, which permits highly stringent washing procedures using a magnet. This significantly reduces background problems in complex or difficult matrices.

Beads of a particular bead address (i.e. a unique combination of internal dyes) are covalently coupled with a pathogen-specific antibody. Different beads are then combined to create custom “bead sets” for the detection of specific combinations of plant pathogens. The procedure of the bead-based immune assay is identical to the standardized (DAS)-ELISA and can be seamlessly integrated in existing workflows.

Plant samples, prepared in the standard ELISA buffer (Fig. 1a), are transferred to a standard 96-well microtitre plate. The antibody-coated bead set is added (Fig. 1b) and the pathogens in the sample

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**Fig.1** (continued) pathogens. After a second washing step the Streptavidin-R-Phycoerythrin reporter is added and binds to the biotinylated secondary antibody (d). The complex comprising the individual beads and their levels of fluorescence is then measured on the Luminex hardware (h). For the xTAG assay the DNA (or RNA) is amplified after extraction from plant material (a) using the outer primers (e). TSPE primers are added to the purified amplicons and subsequently elongated if the target exists (f). After appropriate washing, beads and Streptavidin-R-Phycoerythrin reporter are added and the elongated TSPEs anneal to the complementary xTAG beads (g). Identical to the xMAP assay this complex is then measured on the Luminex hardware (h)



**Fig.1** Overview of the Luminex xMAP (a–d, h) and xTAG technology (a, e–h). Sample material to be used for the xMAP assay is transferred to a microtitre plate (b). To each well a mixture of different bead addresses each coupled with its own specific antibody is added after which the antibodies capture the plant pathogens (c). After washing the biotinylated secondary antibodies are added which subsequently bind to the captured plant

are captured by the different antibodies attached to the individual beads (Fig. 1c). After a short wash step, using a magnet to capture the beads at the bottom of the plate, the beads are subsequently incubated with the appropriate mixture of biotinylated secondary antibodies. Following the magnetic wash step a fluorescent reporter (Streptavidin-R-Phycoerithrin) is added (Fig. 1d). After a short incubation, during which the fluorescent reporter binds to the biotinylated secondary antibodies, the samples are analyzed. The different beads and the attached fluorescent reporter are detected using either a laser-based small flow cytometer or a LED-based image analyzer (Fig. 1h). A user-defined number of individual beads of each specific address are interrogated by the analyzer for both their internal color (the “bead-address”) and amount of the fluorescent reporter attached to the secondary antibody. Presence of a particular pathogen results in a positive signal for the fluorochrome in combination with the virus-specific bead address. Similar to a standard DAS-ELISA the amount of virus or pathogen present in the sample is correlated with the median level of fluorescence measured on the virus or pathogen-specific beads (Median Fluorescent Intensity=MFI).

Typical DAS-ELISA protocols may take 1–2 days while the Luminex xMAP procedure generally takes no more than 3–4 h, including sample preparation. The use of pre-defined bead mixtures, stable for a prolonged period of time, avoids pre-coating of ELISA plates while improved fluid-dynamics allows much shorter incubation times [4]. A further advantage is improved detection limits and a much higher dynamic range [5] as well as considerable savings in costs, through significant reductions in labor and consumables (e.g. plates and reagents).

## 1.2 Luminex xTAG Molecular Assay

As discussed above the issue of “cross-talk” between fluorescent signals limits the multiplex capabilities of current real-time PCR technologies. One platform that offers a much higher degree of multiplex nucleic acid detection is the Luminex MagPlex bead system. This system uses the same 6.5  $\mu\text{M}$  paramagnetic internally labeled polystyrene beads as the xMAP system discussed above but these beads are pre-coupled with a highly specific anti-TAG oligonucleotide sequence. This allows, when combined, up to 500 different nucleic acid sequence targets to be tested simultaneously in a single xTAG assay.

After nucleic acid extraction from plant material (Fig. 1a), the xTAG assay involves a first multiplexed PCR step (for DNA), or a Reverse Transcriptase-PCR step (RT-PCR; for RNA), employing one or more specific primer sets for each of the targets (Fig. 1e). If desired generic primer sets for e.g. a particular genus can be included in this step as well as internal controls for DNA/RNA extraction. Following purification of the (RT)-PCR products using standard methodology, the next step is a multiplexed asymmetric

PCR step termed Target-Specific Primer Extension (TSPE). In this linear amplification step, one or more primers specific to each of amplification products from the first (RT)-PCR step are extended in the presence of biotinylated CTP (Fig. 1f). The 5'-end of each TSPE primer contains a unique additional 24 nt sequence, the anti-TAG, complementary to the TAG sequence coupled to a specific bead address. Following the TSPE reaction, all TSPE products are hybridized to the xTAG bead mixture (xTAG/anti-xTAG hybridization) (Fig. 1g). Following a short washing step employing magnetic capturing of the beads/beads, a fluorescent reporter molecule (Streptavidin-R-Phycoerithrin) is used to detect the incorporated biotin molecules. The bead-TSPE product complexes are detected on the Luminex instruments similarly as was described above for the serological xMAP method (Fig. 1h).

This system not only offers much higher multiplex possibilities than current TaqMan methods but also allows more levels of specificity to be incorporated. The specificity will be determined at two levels: At level 1, the specificity of the primers or their generic nature in the initial reverse transcriptase PCR step determines whether a specific target sequences (or group, or particular genus) is amplified. Alternatively more than one locus from a particular target organism can be amplified generating more amplicons, which can be detected in the subsequent TSPE reactions. At level 2, the use of multiple TSPE primers directed against several targets amplified in the first step adds another level of specificity to the assay. Different strategies can be applied; either different TSPE primers are directed against the same amplicon or more than one amplicon per target can be detected each by its own TSPE primer. Following these strategies (or a combination of both) means that always a particular and pre-defined pattern of TSPE positive signals is required for identification of a target or targets. The potentially high level of multiplexing can also be used to improve the reliability of the test. Including a plant-specific internal control can function as RNA extraction control, while using both genus-specific and species-specific markers in one test identifies positive samples by a minimum of two positive signals. The usefulness of this approach is demonstrated by two recently developed multiplex xTAG assays; one for all nine known pospi-viroids, the other assay for the simultaneous detection of *Tomato yellow leaf curl virus* and biotype distinction of its whitefly vector *Bemisia tabaci* [6, 7].

### 1.3 Data Measurement and Analysis

Samples in the microtitre plates are analyzed on the Luminex instruments (similar hardware is offered by Luminex, BioRad, Merck Millipore and Life Technologies). The results of the measurements are also stored in a standard CSV-file, which can be used for further analysis.

## 2 Materials

### 2.1 xMAP Multiplex Serological Assay

1. Tissue grinder. Different systems may be used in accordance with the end-users preferences. As long as a thorough maceration of plant tissue is accomplished.
2. Standard extraction buffer (Table 1).
3. Stock solution Phosphate Buffered Saline (PBS) [10×] (Table 2).
4. PBS-Tween (Table 3).
5. Blocking agent (Table 4).
6. Bead Working Buffer (Table 5) and Bead Stock solution.
7. 2nd biotinylated antibodies (SAS).
8. Streptavidin-R-Phycoerythrin (Prozyme or Moss, USA).

**Table 1**  
**Composition of standard extraction buffer**

Name	Concentration
PBS [10×]	100 ml [1×]
Egg ovalbumin (grade II)	0.2 % (w/v)
PVP-40	2 % (w/v)
Tween-20	0.05 % (v/v)
NaN <sub>3</sub>	0.05 % (w/v)
Make up to 700 ml with double-distilled water (BiDist), adjust pH to 7.3 and make up to 1,000 ml, store at 4 °C	

**Table 2**  
**Composition of Phosphate Buffered Saline (PBS) [10×]**

Name	Formula	Mol. Weight (g/mol)	Concentration
Sodium chloride	NaCl	58.44	1.37 M
Potassium chloride	KCl	74.55	26.8 mM
Potassium dihydrogen phosphate	KH <sub>2</sub> PO <sub>4</sub>	136.09	14.7 mM
di-Sodium hydrogen phosphate	Na <sub>2</sub> HPO <sub>4</sub>	142.00	100 mM
Make up to 700 ml with double-distilled water (BiDist), adjust pH to 7.3 and make up to 1,000 ml, store at 4 °C			

**Table 3**  
**Composition of PBS-Tween**

Name	Concentration
PBS [10×]	10 ml [1×]
Tween-20	0.1 % (v/v)
Make up to 100 ml with double-distilled water (BiDist), store at 4 °C for maximum of 1 week	

**Table 4**  
**Composition of blocking agent**

Name	Concentration
PBS [10×]	10 ml [1×]
Tween-20	0.1 % (v/v)
Milk powder (Blotting grade Blocker nonfat dry milk, Biorad)	5 % (w/v)
Make up to 100 ml with double-distilled water (BiDist), store at 4 °C for maximum of 1 week	

**Table 5**  
**Composition of Bead Working Buffer [2×]**

Name	Concentration
PBS [10×]	20 ml [2×]
Tween-20	0.05 % (v/v)
Milk powder (Blotting grade Blocker nonfat dry milk, Biorad)	5 % (w/v)
Make up to 100 ml with double-distilled water (BiDist), store at 4 °C for maximum of 1 week	

9. Wash buffer (Table 6).
10. Positive controls.
11. 96-wells low binding microtitre plate.
12. 96-wells microtitre plate format magnetic stand (Luminex Product No. 120.27).
13. Hardware: Luminex Magpix or Luminex LX200 or Luminex Flexmap-3D or similar hardware as supplied by BioRad, Merck Millipore or LifeTechnologies.
14. Software: Luminex Exponent version 4.2.

**Table 6**  
**Composition of wash buffer**

Name	Concentration
PBS [10×]	10 ml [1×]
Tween-20	0.05 % (v/v)
Milk powder (Blotting grade Blocker nonfat dry milk, Biorad)	1.25 % (w/v)
Make up to 100 ml with double-distilled water (BiDist), store at 4 °C for maximum of 1 week	

## 2.2 xTAG Multiplex Molecular Assay

1. Tissue grinder (*see* Subheading 2.1).
2. RNA purification kit: Access-RT-PCR system (Promega, Cat. no. A1250) or equivalent.
3. Purification of PCR products with Sephadex-G50 (96-wells plate).
4. Platinum Tsp DNA polymerase.
5. TSPE 10× Buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl).
6. 50 mM MgCl<sub>2</sub> (Invitrogen, Cat. No. 11448-024) or equivalent.
7. Biotin-14-dCTP (Invitrogen, Cat. No. 19518-018).
8. 20× dNTP—mix (Mix of dATP, dGTP, dTTP: 100 µM each).
9. Streptavidin-R-Phycoerythrin (Prozyme or Moss, USA).
10. 2× Tm Hybridization Buffer (*see* Table 10).
11. 96-wells V-bottom PCR plate and cover.
12. 96-wells format magnetic stand (Luminex Product No. 120.27).
13. Pipettors, tips, microcentrifuge tubes, etc.
14. Centrifuge to accommodate 96-wells microtitre plates.
15. Samples.
16. Hardware: Luminex Magpix or Luminex Lx200 or Luminex Flexmap-3D or similar hardware as supplied by BioRad, Merck Millipore or LifeTechnologies.
17. Software: Luminex Exponent version 4.2.

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## 3 Methods

### 3.1 xMAP Multiplex Serological Assay

#### 3.1.1 Sample Preparation

1. Grind plant or leaf samples in PBS-Tween in a 1/10 (w/v) ratio (*see* Note 1) using your preferred method (*see* Note 2).
2. Store cold until use.

### 3.1.2 xMAP Assay

All amounts mentioned below are for one 96-wells microtitre plate assay. When using different numbers of wells or plates all amounts should be adjusted accordingly.

1. Preparation of the Bead Solution: just before use (*see Note 3*) vortex the Bead Stock solution vigorously for 30 s (*see Note 4*). Very briefly spin the tube before opening and then mix the content by pipetting up and down several (e.g. 5) times.
2. To prepare the final Bead Solution add 200 µL of Bead Stock solution to 4.8 ml Bead Working Buffer (*see Table 5*) and mix by vortexing. *Attention:* Do not spin after last mixing!
3. Add 50 µL of the Bead Solution to all wells needed. (Samples + neg. control + pos. control(s)).
4. Add 50 µL of sample per well, as well as positive and negative controls.
5. Cover the ELISA plate with aluminum foil (*see Note 5*).
6. Shake the ELISA plate for 20 min (use appropriate shaker) and avoid sample cross-over (*see Note 6*).
7. In the meantime prepare the Secondary Antibody Solution: Spin the SAS labeled tube shortly before opening. Add 200 µL SAS stock solution to 9.8 ml PBS-Tween. For each well you need 100 µL freshly prepared Secondary Antibody Solution.
8. Remove the aluminum foil from the ELISA plate.
9. Wash the ELISA plate (*see Note 7*) according to **steps 10–16**.
10. Place the plate on the magnetic plate stand (*see Notes 8 and 9*).
11. Wait for at least 1 min to allow the beads to settle.
12. Flick plate to remove supernatant from the well (microtitre plate should be kept on the magnetic stand!).
13. Add 100 µL Bead Wash Buffer to each well (microtitre plate should be kept on the magnetic stand!).
14. Wait 30 s.
15. Flick plate to remove Bead Wash Buffer from the well (microtitre plate should be kept on the magnetic stand!).
16. Remove the plate from the magnetic stand.
17. Add 100 µL of the previously prepared Secondary Antibody Solution (*see step 7*) to each well.
18. Cover the ELISA plate with aluminum foil.
19. Shake the ELISA plate for 30 min (use appropriate shaker) and avoid sample cross-over.
20. During the last 10 min of this SAS incubation prepare fresh Streptavidin-R-Phycoerythrin solution. Spin Streptavidin-R-Phycoerythrin tube shortly before opening. Add 1 µL Streptavidin-R-Phycoerythrin to 1 ml Wash Buffer. You need

75 µL of fresh prepared Streptavidin-R-Phycoerythrin solution per well. Keep tube in dark.

21. Remove the aluminum foil from the ELISA plate.
22. Wash the ELISA plate as described in **steps 10–16** (*see above*).
23. Remove the ELISA plate from the magnetic stand. To each well add 75 µL of the Streptavidin-R-Phycoerythrin solution made in **step 20**.
24. Cover the plate with aluminum foil.
25. Shake for 15 min (use appropriate shaker, and avoid sample cross-over, *see Note 6*).
26. Remove the aluminum foil from the ELISA plate.
27. Wash the ELISA plate as described in **steps 10–16** (*see above*).
28. Add 100 µL Wash Buffer to each well.
29. Shake for 5 min (use appropriate shaker, and avoid sample cross-over, *see Note 6*).
30. Proceed to assay measurement (*see Note 10*).

### **3.1.3 xMAP Assay Measurement**

1. Launch Exponent Software and use appropriate protocol (*see Note 11*).

2. Select correct number of samples
3. Run measurement

## **3.2 xTAG Multiplex Molecular Assay**

### **3.2.1 Sample Preparation**

1. Transfer plant or leaf samples (fresh, frozen or dried), to a bead-beater tube with stainless steel beads, freeze in liquid N<sub>2</sub> and beat to fine powder. Alternatively other methods may be used for grinding plant or leaf material prior to DNA or RNA extraction (*see Note 2*).
2. Extract DNA or RNA using a commercially available kit according to the manufacturers instructions.
3. Store isolated DNA or RNA in the freezer (minimally at -20 °C but preferably at -80 °C) until use.

### **3.2.2 xTAG Assay**

All amounts mentioned below are for one 96-wells plate assay. When using different numbers of wells or plates all amounts should be adjusted accordingly.

The protocol below describes an xTAG assay for the detection of RNA. In case detection of DNA is required the initial multiplexed PCR amplification step should be adjusted accordingly.

1. Perform a multiplex one-tube RT-PCR on each RNA sample (*see Note 12*). Prepare (RT)-PCR reaction mixture (*see Note 13*) (*see Table 7*) and run (RT)-PCR programme (*see Note 14*), (*see Table 8*).
2. Remove primers from the initial (RT)-PCR reactions. For a large number of reactions this can be conveniently done

**Table 7**  
**RT-PCR reaction mix for the initial multiplex target amplification step (Promega Access (RT)-PCR system)**

Reaction components RT-PCR	Stock concentration	Volume ( $\mu$ L) in each reaction (final volume 25 $\mu$ L)	Final concentration
AMV/Tfl buffer	5 $\times$	5.0	1 $\times$
dNTP's	10 mM	0.5	0.2 mM
MgSO <sub>4</sub>	25 mM	1.0	1.0 mM
Outer primers (Fw + Rv)	4 $\mu$ M	2.5	400 nM
AMV reverse transcriptase	5 U/ $\mu$ L	0.5	2.5 U
TfL Taq Polymerase	5 U/ $\mu$ L	0.5	2.5 U
RNA		1.0	
MQ	Make volume up to 25 $\mu$ L		

**Table 8**  
**(RT)-PCR cycling conditions for the initial multiplex target amplification step (see Note 14)**

Step	Temperature	Duration
1	45 °C	45 min
2	94 °C	2 min
3	94 °C	30 s
4	55 °C	30 s
5	68 °C	1 min
6	Repeat steps cycles 3–5: 40 times	
7	68 °C	5 min
8	4 °C	Forever

using Sephadex 96-wells Plate (see Note 15) using following steps 3–11.

3. Carefully remove the seal from the Sephadex plate.
4. Add 300  $\mu$ L sterile water to every well you want to use (see Note 16).
5. Cover the plate with accompanying lid, and incubate for a minimum of 2 h at room temperature, or overnight at 4 °C in a sealed plastic bag.

**Table 9**  
**Reaction mix for the Template-Specific Primer Extension (TSPE) reaction**

Reaction components TSPE reaction mix	Stock concentration	Volume ( $\mu\text{L}$ ) in each reaction (final volume 15 $\mu\text{L}$ )	Final concentration
Tsp buffer	10×	2	1×
MgCl <sub>2</sub>	50 mM	0.5	1.25 mM
TAG-TSPE primer mix	20×	1	25 nM each
Tsp DNA polymerase	5 U/ $\mu\text{L}$	0.15	0.75 U
dNTP mix (-dCTP)	100 $\mu\text{M}$ each	1	5 $\mu\text{M}$ each
Biotin dCTP	400 $\mu\text{M}$	0.25	5 $\mu\text{M}$
BiDist water		10.1	

6. Place the plate on top of a regular 96-wells microtitre plate.
7. Spin down combined plates at  $750 \times g$  for 90 s.
8. Remove the lower microtitre plate, and replace with a new (labeled) plate.
9. Add the PCR-products to the center of the wells of the Sephadex plate prepared in the previous steps.
10. Spin at  $750 \times g$  for 90 s and remove the Sephadex-plate.
11. Cover the lower microtitre plate (with your cleaned PCR products) with foil to avoid evaporation.
12. Perform the multiplex Template-Specific Primer Extension (TSPE) reactions on the purified multiplex (RT)-PCR reactions following **steps 13–16** (see Note 17).
13. Prepare 15  $\mu\text{L}$  of stock TSPE reaction mix (see Table 9) for every RT-PCR product to be tested.
14. Aliquot 15  $\mu\text{L}$  from this stock per reaction in a PCR tube.
15. Add 5  $\mu\text{L}$  of cleaned up PCR product from **step 11** to each tube.
16. Run the TSPE reaction in a PCR-machine according to the protocol in Table 10.
17. Prepare the Magplex bead working mixture (25  $\mu\text{L}$  per reaction) containing 2.5  $\mu\text{L}$  concentrated Magplex bead mixture and 22.5  $\mu\text{L}$  2× Tm Hybridization Buffer (Table 11).
18. Hybridize the Magplex beads to the TSPE products (see **steps 19–24**).
19. Add 5  $\mu\text{L}$  of each TSPE reaction to appropriate wells in the microtitre plate.
20. Adjust the total volume to 25  $\mu\text{L}$  by adding 20  $\mu\text{L}$  of BiDist to each sample well.

**Table 10**  
**Template-Specific Primer Extension (TSPE) reaction cycling conditions**

Step	Temperature	Duration
1	96 °C	2 min
2	94 °C	20 s
3	58 °C	30 s
4	72 °C	30 s
5	Repeat steps cycles 2–4: 35 times	
6	4 °C	Forever

**Table 11**  
**Tm Hybridization Buffer [2×]**

Name	Concentration
NaCl	0.4 M
Tris	0.2 M
Triton X-100	0.16 %

**Table 12**  
**Tm Hybridization Buffer [1×]**

Name	Concentration
NaCl	0.2 M
Tris	0.1 M
Triton X-100	0.08 %

21. Pipet 25 µL of BiDist to each background well.
22. Add 25 µL of the Magplex bead mixture to each (used) well.
23. Cover the plate to prevent evaporation and denature at 96 °C for 90 s.
24. Hybridize at 37 °C for at least 30 min in thermal cycler (*see Note 18*).
25. Before starting the wash procedure prepare fresh Streptavidin-R-Phycoerythrin solution. Spin Streptavidin-R-Phycoerythrin tube shortly before opening. Add 1 µL Streptavidin-R-Phycoerythrin to 1 ml 1× Tm Hybridization Buffer (Table 12).

You need 75 µL of fresh prepared Streptavidin-R-Phycoerythrin solution per well. Keep tube in dark (*see Note 19*).

26. Dilute the concentrated solution 1,000 times in 1× Tm Hybridization Buffer (Table 12).
27. Wash the microtitre plate through the following steps 28–38.
28. Place the plate on the magnetic plate stand (*see Notes 20 and 21*).
29. Wait for at least 1 min to allow the beads to settle at the bottom of the plate.
30. Remove supernatant from the wells; flick plate; the microtitre plate should be left on the magnetic stand!
31. Resuspend the pelleted Magplex beads by adding 75 µL 1× Tm Hybridization Buffer (Table 12) to each well, the microtitre plate should be left on the magnetic stand!
32. Wait 30 s.
33. Flick plate to remove supernatant from the well the microtitre plate should be left on the magnetic stand!
34. Resuspend the pelleted Magplex beads by adding 75 µL 1× Tm Hybridization Buffer (Table 12) to each well the microtitre plate should be left on the magnetic stand.
35. Wait 30 s.
36. Flick plate to remove supernatant from the well the microtitre plate should be left on the magnetic stand!
37. This gives a total of 2 washes.
38. Remove the plate from the magnetic stand.
39. Resuspend the beads in each well by adding 75 µL of the previously prepared 1× Tm Hybridization Buffer (Table 12) containing 2–8 µg/ml Streptavidin-R-Phycoerythrin (*see step 25*).
40. Incubate for 15 min at room temperature. Therefore place the plate on a shaker and cover it with aluminum foil (*see Note 19*) and avoid cross-over.
41. Pellet the Magplex beads by placing the plate on a magnetic separator and allow separation to occur for 60 s. Remove the supernatant with the plate on the magnetic stand.
42. Resuspend the pelleted Magplex beads in 75 µL of 1× Tm Hybridization Buffer (Table 12).
43. Proceed to assay measurement (*see Note 22*).

### 3.2.3 xTAG Assay Measurement

1. Launch Exponent Software and use appropriate protocol (*see Note 23*).
2. Indicate correct number of samples.
3. Run measurement.

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## 4 Notes

1. Generally a 1:10 (w/v) ratio of plant material/PBS-Tween is recommended but other ratios may be appropriate depending on the plant material under investigation or your preferences.
2. Different systems can be used for tissue grinding like plastic bags with nylon netting inside, mortar and pestle, Pollähne press (MEKU Erich Pollähne GmbH, Germany). Make sure to avoid cross-contamination between samples.
3. Allow all reagents to equilibrate to room temperature before use in the assay.
4. Upon storage beads tend to settle at the bottom of the tube and should therefore be thoroughly re-suspended just before use.
5. Cover the assay plate containing beads with aluminum foil during all incubation steps (the beads are light sensitive and must be protected from strong light at all times to avoid photo bleaching).
6. The plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells.
7. If the samples contain a high amount of debris the washing step can be repeated.
8. Make sure to clip the plate securely onto the magnetic plate stand using the clips attached to the stand.
9. With respect to the use of the magnetic ELISA plate holder there are several important notes to be made:
  - (a) The magnetic plate holder is very powerful; Keep away from card with magnate strips (e.g. bank and credit cards).
  - (b) Use the magnetic plate holder only during the wash steps
  - (c) Store the magnetic plate holder in a dark and dry place far away from the paramagnetic beads
  - (d) The 96 well flat-bottom microtitre plate should fit well within the magnetic stand and securely fastened using the attached clips
  - (e) Important: the beads are located at the bottom of the plate
  - (f) All wash steps are performed with the 96-well plate attached to the magnetic plate stand.
  - (g) Pipette tips are occasionally bent; avoid contact with the bead pellets.
10. If the plate cannot be measured immediately after the assay is finished, seal the plate, cover with aluminum foil and store the plate at 2–8 °C for up to 24 h. Prior to measurement, agitate the plate on the plate shaker at room temperature for 10 min.

11. If no protocol exists in the Exponent software, create a new protocol according to the instruction manual.
12. All downstream and upstream primers for all target sequences should be present in the initial (RT)-PCR reaction. In rare cases of primer interference separate (RT)-PCR reactions can be performed which, after purification of the (RT)-PCR products (**step 3**) can be pooled for the further procedure.
13. Allow 10 % extra of every reaction component to compensate for pipetting inaccuracies.
14. Depending on primer Tm, the annealing temperature of the (RT)-PCR may vary.
15. Alternatively purification of initial (RT)-PCR reactions can also be performed using any other commercially available PCR-clean up kit.
16. Also only a portion of the Sephadex plate can be used for purification of the initial (RT)-PCR reactions. The rest of the plate can later be used in additional purifications. To this end the plate can be stored at room temperature.
17. Alternatively instead of using the 10× Tsp reaction buffer + the MgCl<sub>2</sub> also the 10× PCR buffer from Roche (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>) can be used.
18. Cover the assay plate containing beads with aluminum foil during all incubation steps (The beads are light sensitive and must be protected from strong light at all times to avoid photo bleaching).
19. The Streptavidin-R-Phycoerythrin solution is light sensitive and must be protected from strong light at all times to avoid photo bleaching. Cover the assay plate containing beads with aluminum foil during all incubation steps.
20. Make sure to clip the plate securely onto the magnetic plate stand using the clips attached to the stand.
21. With respect to the use of the magnetic ELISA plate holder there are several important notes to be made:
  - (a) The magnetic plate holder is very powerful; Keep away from card with magnate strips (e.g. bank and credit cards).
  - (b) Use the magnetic plate holder only during the wash steps
  - (c) Store the magnetic plate holder in a dark and dry place far away from the paramagnetic beads
  - (d) The 96 well flat-bottom microtitre plate should fit well within the magnetic stand and securely fastened using the attached clips
  - (e) Important: the beads are located at the bottom of the plate

- (f) All wash steps are performed with the 96-well plate attached to the magnetic plate stand.
  - (g) Pipette tips are occasionally bent; avoid contact with the bead pellets
22. If the plate cannot be measured immediately after the assay is finished, seal the plate, cover with aluminum foil and store the plate at 2–8 °C for up to 24 h. Prior to measurement, agitate the plate on the plate shaker at room temperature for 10 min.
  23. If no protocol exists in the Exponent software, create a new protocol according to the instruction manual.

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# Chapter 22

## Next-Generation Sequencing of Elite Berry Germplasm and Data Analysis Using a Bioinformatics Pipeline for Virus Detection and Discovery

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### Abstract

Berry crops (members of the genera *Fragaria*, *Ribes*, *Rubus*, *Sambucus*, and *Vaccinium*) are known hosts for more than 70 viruses and new ones are identified continually. In modern berry cultivars, viruses tend to be asymptomatic in single infections and symptoms only develop after plants accumulate multiple viruses. Most certification programs are based on visual observations. Infected, asymptomatic material may be propagated in the nursery system and shipped to farms where plants acquire additional viruses and develop symptoms. This practice may result in disease epidemics with great impact to producers and the natural ecosystem alike. In this chapter we present work that allows for the detection of known and discovery of new viruses in elite germplasm, having the potential to greatly reduce virus dispersal associated with movement of propagation material.

**Key words** Virus detection, Virus discovery, DOP-PCR, Next-generation sequencing, VirFind

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### 1 Introduction

Berry crops (blackberry, blueberry, cranberry, currants, elderberry, raspberry, and strawberry) are perceived as healthy fruits or “super-foods” and have increased in popularity in recent years. As a consequence, they are now planted widely and in very diverse environments ranging from subtropic to the subarctic regions in addition to temperate climates where these plants have their centers of origin. The change in growing environments, the constant movement of germplasm and the expansion of virus vectors to new areas have led to a significant increase in the number of viruses that infect these crops [1–4]. These viruses are transmitted by a plethora of vectors, from aphids and nematodes to mites and fungi, and most are asymptomatic in single infections in modern cultivars while several do not cause visual symptoms on indicator plants. The situation makes virus disease control a daunting undertaking;

as several regulatory agencies function on the basis that berry viruses cause visual symptoms. This has led to the movement of infected material through the propagation pipelines and distribution internationally given that a phytosanitary certificate may only require visual plant inspection.

On the other hand, the sheer number of new viruses identified since the turn of the century has not allowed for the in-depth study of virus biology and epidemiology. This, in turn, has led to the development of detection tests that are based on one or a few isolates, making some of the tests potentially unreliable as the population structure of the virus is unknown. *Blackberry chlorotic ringspot virus* (BCRV) presents an excellent example of how knowledge of the population structure of a virus can dramatically improve detection reliability. The first BCRV isolate sequenced has as low as 68 % nucleotide sequence identity to the more than 30 isolates that have been studied since then [5]. Given the lack of reliable antibody detection, the virus is routinely tested for using reverse transcription-PCR. Oligonucleotide primers based on the first sequenced isolate may have missed the majority of the isolates circulating in commercial fields, a problem that has been addressed after the development of detection protocols based on the sequences of a wide array of isolates from different hosts [6].

The problem of misidentification or false negatives is particularly important for generation 1 (G1) or the top tier plants in certification schemes, since these individuals serve as the source of all plants for any particular cultivar. A G1 plant needs to be free of all known pathogenic viruses (excluding cryptic agents such as endorna-, cryptic, or amalgaviruses) to ensure the best quality plants; allowing for growers to achieve better yielding and high-quality produce. The solution to the complex problem of virus detection can be addressed with the use of next-generation sequencing (NGS) and the application of complex bioinformatic analyses of the resulting data. NGS is a powerful tool that allows for detection of pathogens without any *a priori* knowledge of their genetic composition. In other words, NGS is a sequence-neutral tool, able to detect any isolate of a particular virus but also assist in the discovery of new agents. Users can either send their total nucleic acids to NGS sequencing service providers, or there are several protocols available for preparation of nucleic acids for NGS [7–10]. In the case of virus detection/discovery it is important to include the RNA fraction of nucleic acids as the vast majority of berry viruses have an RNA genome.

Although there are several molecular biology protocols fine-tuned for the particular application of the NGS-derived data, the same is not true for bioinformatics. Data analysis requires the expertise of a professional bioinformatician, introducing a bottleneck in the process. In the case of new viruses, knowledge of the genome structure or protein functions of a virus is essential;

meaningful data may be misidentified by someone who does not have an in-depth knowledge of virology. In the case of human virology, there are several bioinformatics tools developed specifically for virus detection [11–16]. Their common approaches are to map to the reference human genome, perform various sequence comparison steps using Blast, and categorize NGS reads into non-human, microbial, or viral integrated sequences. However, there was not a pipeline developed as a generic virus detection/discovery package, able to identify viruses regardless of the host organism.

In this chapter, we present a degenerate oligonucleotide primed (DOP) RT-PCR method, with two sets of primers each containing barcodes, to amplify enriched double-stranded RNA for NGS sequencing. The NGS outputs are then analyzed using VirFind, a novel online tool for virus detection and discovery. This combination of laboratory work and bioinformatics procedure has been used successfully to identify various known and unknown viruses of berry crops.

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## 2 Materials

Only use analytical-grade reagents and purified deionized water. Prepare and store all reagents and solutions at room temperature unless otherwise specified. Take extra caution when working with methylmercury hydroxide. All instruments in this section can be substituted with similar instruments of the same functions.

### **2.1 Components for Total Nucleic Acid Extraction of Nonacidic Plant Tissues**

1. 250 mL Nalgene™ centrifuge bottles (PPCO—polypropylene copolymer, with sealing cap, Thermo Scientific).
2. Sorvall RC 6+ centrifuge (Thermo Scientific).
3. Fiberlite F14-6 x 250y fixed angle rotor (Thermo Scientific).
4. Econo-Column chromatography columns, 2.5 × 20 cm (BioRad).
5. Corning conical-bottom tubes (50 mL/PP/Plug, Cole-Parmer).
6. Liquid nitrogen.
7. 95 % or absolute ethanol.
8. Cellulose C6288 (Sigma-Aldrich).
9. Commercially available stock solutions: 1 M HCl, 5 M NaCl, 0.5 M EDTA (ethylenediaminetetraacetic acid), β-mercaptoproethanol, 10 % SDS (sodium dodecyl sulfate), 1× Tris-EDTA (TE).
10. 1 M Tris-HCl, pH 7.5 or 8.5: Weigh 121.14 g of Tris-HCl powder and dissolve in 500 mL of water in a glass beaker (*see Note 1*). Adjust the pH with 1 M HCl while stirring, measuring the pH until it reaches 7.5 or 8.5. Bring final volume to 1 L.

11. 10×STE (Sodium Tris EDTA): Add to a 1 L graduated cylinder or a glass beaker 200 mL of 5 M NaCl, 200 mL of 1 M Tris–HCl pH 7.5, 20 mL of 0.5 M EDTA, and 580 mL of water to make up to 1 L. Prepare 1× and 2×STE from 10×stock solution.
12. STE-saturated phenol: Melt solid phenol in its glass bottle at 50 °C in a water bath. Add 20 % (v/v) 1×STE and stir on a magnetic shaker until the two phases become an emulsion. Allow the emulsion to separate and store at 4 °C.
13. Washing buffer: 18 % STE ethanol. Mix 100 mL of 10×STE and 190 mL of 95 % ethanol. Adjust to 1 L with water.
14. Glassmilk: Add 60 g of silica particle (Sigma S5631) to 500 mL of water, mix and allow to settle for 24 h. Discard the upper 470 mL of liquid. Add 500 mL of water with pH adjusted to 2 using HCl and allow to settle 5 or more hours. Discard the upper 440 mL and check to see that slurry pH = 2. Autoclave and store the mix in a dark bottle at room temperature, or aliquot into 2 mL Eppendorf tubes for storage at 4 °C for several months.

## **2.2 Components for Total Nucleic Acid Extraction of Acidic Plant Tissues**

1. Miraclot (Calbiochem).
2. RNA extraction buffer: Mix 200 mL of 1 M Tris–HCl, pH 8.5, 12.66 g of lithium chloride, 15 g of sodium dodecylsulphate, 2.92 g of EDTA, 10 g of sodium deoxycholate, and 10 mL of NP40S (Sigma-Aldrich). Adjust to 1 L using water. Add 1 % (v/v) β-mercaptoethanol just before use.
3. Potassium acetate buffer: Mix 600 mL of 5 M potassium acetate ( $\text{CH}_3\text{CO}_2\text{K}$ ), 285 mL of water, 115 mL of glacial acetic acid, and 98.15 g of solid potassium acetate.

## **2.3 Components for Double-Stranded RNA Enrichment**

1. Centrifuge 5430 (Eppendorf) with fixed-angle rotor F-35-6-30.
2. Centrifuge 5424 (Eppendorf) with fixed-angle rotor FA-45-24-11.
3. Denaturing agent: 0.04 M methylmercury hydroxide ( $\text{CH}_4\text{HgO}$ ). In a 1.5 mL Eppendorf tube, add 48 μL of water to 2 μL of 1 M  $\text{CH}_4\text{HgO}$  and mix well by pipetting up and down 4–5 times. To test methylmercury hydroxide activity, transfer 2 μL of this solution to another 1.5 mL Eppendorf tube and mix with 2 μL of 0.1 M DTT. If the mixture turns cloudy, the 0.04 M  $\text{CH}_4\text{HgO}$  solution is ready for use. Each 0.04 M  $\text{CH}_4\text{HgO}$  solution after preparation from fresh stock can be used for 30 days (see Note 2).
4. RNA wash buffer: Mix 10 mL of 1 M Tris–HCl, pH 7.5, 1 mL of 0.5 M EDTA, 10 mL of 5 M NaCl, 500 mL 100 % ethanol, and 479 mL of water.
5. T1 RNase (Sigma-Aldrich).

## **2.4 Components for Reverse Transcription, Degenerate Oligo-Primed PCR, and DNA Quality Control**

6. 2 M MgCl<sub>2</sub>.
7. DNaseI (Sigma-Aldrich D5025): Dilute in 1× STE to 1 U per µL and store at 4 °C.
1. RiboLock RNase Inhibitor (Thermo Scientific).
2. Maxima Reverse Transcriptase (Thermo Scientific).
3. RNase H (Thermo Scientific).
4. Primer sequences can be found in Table 1. Concentration of all primers used in this study is 20 nM.
5. NanoDrop™ 1000 spectrophotometer (Thermo Scientific).
6. Taq DNA polymerase (GenScript).
7. 10×Taq buffer (GenScript).
8. GeneJET PCR Purification Kit (Thermo Scientific).
9. GelRed (Biotium).
10. HyperLadder 100 bp (Bioline).

## **3 Methods**

All the steps in Subheading 3 are carried out at room temperature unless otherwise specified. Wear protective eye goggles, lab coat, and gloves, especially when working with methylmercury hydroxide, liquid nitrogen, and phenol.

### **3.1 Total Nucleic Acid Extraction for Nonacidic Plants**

1. Switch on the Sorvall RC 6+ centrifuge and install Fiberlite F14-6 x 250y Fixed Angle Rotor. Set the temperature at 4 °C and close the centrifuge lid to cool down centrifuge and rotor (*see Note 3*).
2. Using a set of prechilled mortar and pestle, grind 20 g of leaf tissue in liquid nitrogen.
3. Transfer the tissue powder to 250 mL Nalgene centrifuge bottles. Make sure that liquid nitrogen is completely evaporated; then add 40 mL of 2×STE, 1 mL of β-mercaptoethanol, 10 mL of 10 % SDS, and 30 mL of STE-saturated phenol. Close the cap tightly and shake the bottles at 200 rpm for at least 30 min.
4. Equilibrate the bottles using 2×STE (*see Note 4*), and centrifuge them at 4 °C for 10 min at 9,820 × *g* in the Sorvall centrifuge with Fiberlite rotor.
5. Gently transfer the upper aqueous phase to new 250 mL Nalgene centrifuge bottles without disturbing the pellet (*see Note 5*). Equilibrate the bottles and adjust the solution to 16.5 % (w/w) ethanol. Add 1 g of fibrous cellulose powder and shake the bottles at 200 rpm for at least 30 min.

**Table 1**  
**Primer sequences used in this study**

Primer name	Nucleotide sequences
BG4A-RT	5'-CATTGCTGGGTGCCTGGTAAANNNNNN-3'
BG4A-I1-PCR	5'-CGTGATCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I2-PCR	5'-ACATCGCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I3-PCR	5'-GCCTAACATTGCTGGGTGCCTGGTAAA-3'
BG4A-I4-PCR	5'-TGGTCACATTGCTGGGTGCCTGGTAAA-3'
BG4A-I5-PCR	5'-CACTGTCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I6-PCR	5'-ATTGGCCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I7-PCR	5'-GATCTGCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I8-PCR	5'-TCAAGTCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I9-PCR	5'-CTGATCCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I10-PCR	5'-AAGCTACATTGCTGGGTGCCTGGTAAA-3'
BG4A-I11-PCR	5'-GTAGCCCATTGCTGGGTGCCTGGTAAA-3'
BG4A-I12-PCR	5'-TACAAGCATTGCTGGGTGCCTGGTAAA-3'
KpnI-RT	5'-TGGTAGCTTTGATCANNNNNN-3'
KpnI-I1-PCR	5'-CGTGATAGAGTTGGTAGCTCTTGATC-3'
KpnI-I2-PCR	5'-ACATCGAGAGTTGGTAGCTCTTGATC-3'
KpnI-I3-PCR	5'-GCCTAAAGAGTTGGTAGCTCTTGATC-3'
KpnI-I4-PCR	5'-TGGTCAAGAGTTGGTAGCTCTTGATC-3'
KpnI-I5-PCR	5'-CACTGTAGAGTTGGTAGCTCTTGATC-3'
KpnI-I6-PCR	5'-ATTGGCAGAGTTGGTAGCTCTTGATC-3'
KpnI-I7-PCR	5'-GATCTGAGAGTTGGTAGCTCTTGATC-3'
KpnI-I8-PCR	5'-TCAAGTAGAGTTGGTAGCTCTTGATC-3'
KpnI-I9-PCR	5'-CTGATCAGAGTTGGTAGCTCTTGATC-3'
KpnI-I10-PCR	5'-AAGCTAACAGAGTTGGTAGCTCTTGATC-3'
KpnI-I11-PCR	5'-GTAGCCAGAGTTGGTAGCTCTTGATC-3'
KpnI-I12-PCR	5'-TACAAGAGAGTTGGTAGCTCTTGATC-3'

6. Balance the weight of the bottles using 18 % STE ethanol, and centrifuge them at 4 °C for 10 min at 9,820 × g in the Sorvall centrifuge with Fiberlite rotor.
7. Gently but immediately discard the supernatant and dissolve the pellet in 100 mL of 18 % STE ethanol (*see Note 6*).

Centrifuge for 10 min at  $9,820 \times g$  as above. Repeat this step at least twice if the cellulose is white, or more if the cellulose is darker.

8. Dissolve the pellet in 100 mL of 18 % STE ethanol and pour the mixture into the chromatography columns. Wash the cellulose in the column with 200 mL of 18 % STE ethanol and repeat this step at least twice if the cellulose is white, or more if the cellulose is darker (*see Note 7*).
9. Pipet into each column 10 mL of STE and vortex. Collect the liquid in 50-mL Corning conical-bottom tubes and pour it back to the column. Repeat this step twice.

### **3.2 Total Nucleic Acid Extraction for Acidic Plants**

1. Perform **steps 1** and **2** of the Subheading [3.1](#), but start only with 10 g of leaf tissue.
2. Transfer the tissue powder to 250 mL Nalgene centrifuge bottles. Make sure that liquid nitrogen is completely evaporated; then add 50 mL of RNA extraction buffer. Close the cap tightly and shake the bottles at 200 rpm for at least 15 min.
3. Add to each bottle 50 mL of potassium acetate buffer and invert the bottles several times.
4. Equilibrate the bottles using potassium acetate buffer, and centrifuge them at 4 °C for 10 min at 15,000  $\times g$  in the Sorvall centrifuge with Fiberlite rotor.
5. Collect the supernatant, and pass it through miracloth. Equilibrate the bottles using potassium acetate buffer before adding 0.8 volume isopropanol and centrifuge the bottles at 20,000  $\times g$  for 30 min.
6. Immediately discard the supernatant and dissolve the pellet in 100 mL of 18 % STE ethanol. Centrifuge for 10 min at  $9,820 \times g$  as above. Repeat this step at least twice if the cellulose is white, or more if the cellulose is darker.
7. Dissolve the pellets in 100 mL of 18 % STE ethanol and pour the mixture into the chromatography columns. Wash the cellulose with 200 mL of 18 % STE ethanol and repeat this step at least twice if the cellulose is white, or more if the cellulose is darker.
8. Pipet into each column 10 mL of STE and vortex. Collect the liquid in 50-mL Corning conical-bottom tubes and pour it back to the column. Repeat this step twice.

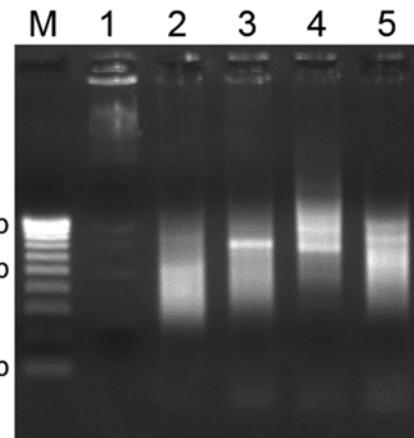
### **3.3 Double-Stranded RNA Enrichment**

1. To the tubes with 10 mL of STE containing total nucleic acid from Subheading [3.1](#) or Subheading [3.2](#), add 400 U of T1 RNase, 500 µL of 2 M MgCl<sub>2</sub>, and 25 units of DNaseI. Incubate the tubes at 37 °C for 1 h to digest DNA and single-stranded RNA.

2. Add 500  $\mu$ L of 0.5 M EDTA, pH 8, and adjust the solution to 50 % (w/w) ethanol. Add 20  $\mu$ L of glass milk and invert the tubes several times. Spin in Centrifuge 5430 with fixed-angle rotor F-35-6-30 at 1,000  $\times g$  for 30 s. Dispose of the liquid and wash the pellet with 30 mL of RNA wash buffer. Repeat this step twice.
3. Dissolve the glassmilk in 1 mL of RNA wash buffer. Transfer the liquid to 1.5 mL Eppendorf tube. Spin in Centrifuge 5424 with fixed-angle rotor FA-45-24-11 at 15,000  $\times g$  for 15 s. Discard the supernatant and dry the pellet under vacuum until completely dry.
4. Add 150  $\mu$ L of 1 $\times$  TE, heat to 70 °C for 5 min, vortex briefly, and centrifuge the Eppendorf tube at 15,000  $\times g$  as above for 1 min.
5. Gently collect 100  $\mu$ L of the aqueous phase containing enriched dsRNA without disturbing the pellet.
6. Run 5  $\mu$ L of the dsRNA preparation in a 0.8–2 % agarose gel to observe the dsRNA bands under UV.

### **3.4 Reverse Transcription, Degenerate Oligo-Primed PCR, and DNA Quality Control**

1. Methylmercury hydroxide denaturation: In a 1.5 mL Eppendorf tube, aliquot 4  $\mu$ L of dsRNA preparation and mix with 4  $\mu$ L of methylmercury hydroxide preparation (*see Note 2*). Incubate in fume hood for 10 min.
2. Reverse transcription: Set up a 25  $\mu$ L reaction as follows: 5  $\mu$ L of 5 $\times$  RT buffer, 1  $\mu$ L of dNTP (0.2 mM each), 1  $\mu$ L of 20  $\mu$ M BG4A-RT or KpnI-RT primer, 4  $\mu$ L of denatured dsRNA, 13.6  $\mu$ L of water, 6 U of RiboLock, and 50 U of Maxima™ reverse transcriptase. Also set up a water control reaction. Pipet up and down for 4–5 times and incubate the reactions at 25 °C for 10 min, 50 °C for 60 min, and then at 85 °C for 5 min to deactivate the reverse transcriptase.
3. Degrade the RNA strand in RNA–DNA hybrids: Add 1 unit of RNaseH to the above RT reaction. Incubated at 37 °C for 1 h. Inactivate the enzyme by heating at 65 °C for 10 min.
4. Purification of cDNA: Purify the cDNA using the GeneJET PCR Purification Kit following the manufacturer's recommendations except using 200  $\mu$ L binding buffer (8 $\times$  of the RT reaction) to increase the yield of single-stranded cDNA. Elute the cDNA using 50  $\mu$ L of water.
5. Degenerate Oligo-Primed (DOP) PCR: Set up a 100  $\mu$ L PCR reaction as follows: 10  $\mu$ L of 10 $\times$  Taq buffer, 4  $\mu$ L of 20 nM BG4A-PCR or KpnI-PCR primer depending on the RT primer used, with appropriate barcodes if multiplexing is needed (Table 1 presents two sets, each containing one RT and 12 PCR primers), 2  $\mu$ L of dNTPs (0.2 mM each), 2  $\mu$ L of cDNA,



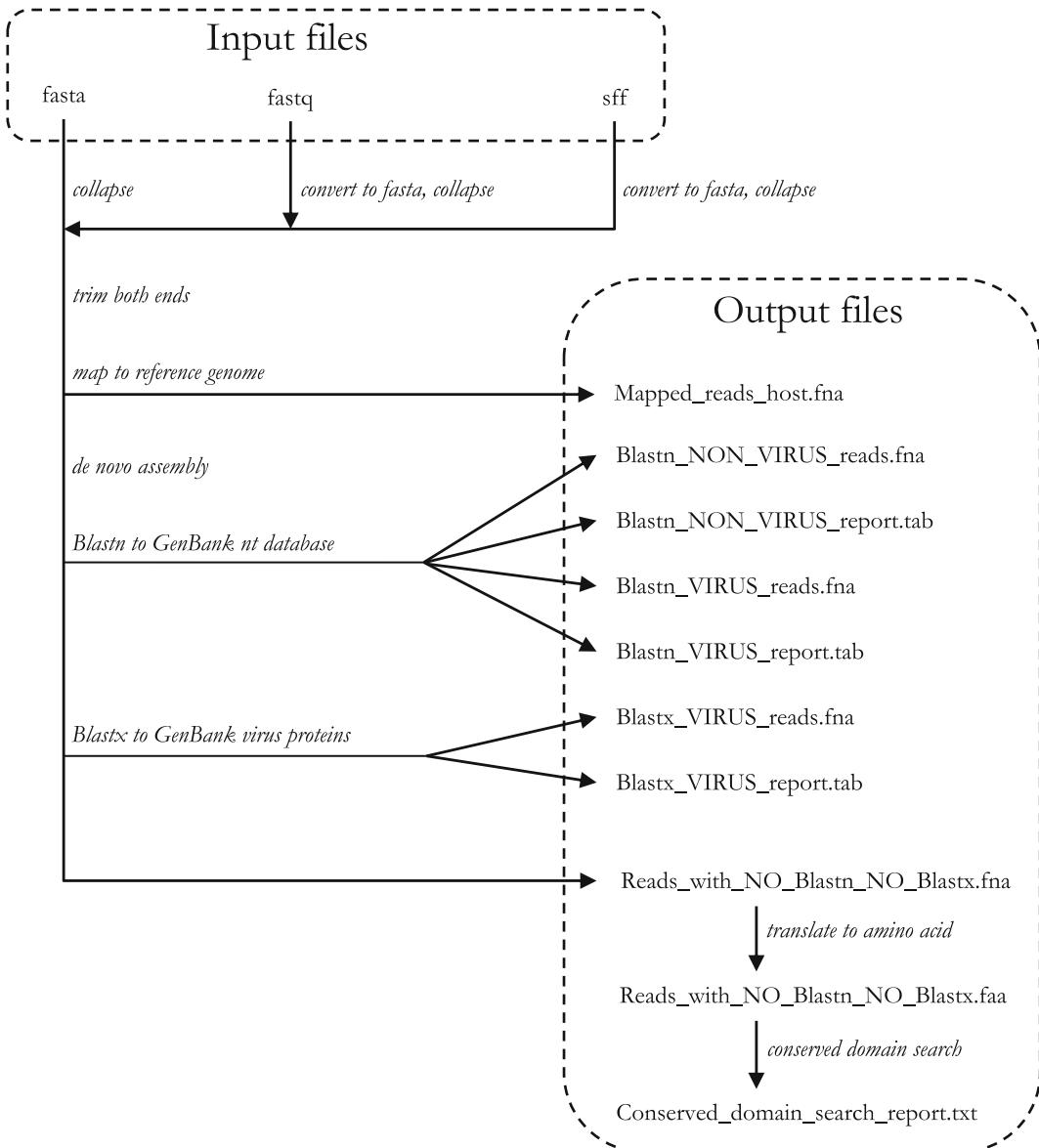
**Fig. 1** An example of agarose gel electrophoresis of five different Degenerate Oligo-Primed PCR products. Lane M is HyperLadder 100 bp. While lane 1 has DNA of short concrete bands together with high molecular weight smear and not recommended for further sequencing, lanes 2–5 have DNA smear around the 300–1,000 bp region and can be used for NGS

81.6  $\mu$ L of water, and 2 U of Taq DNA polymerase. The PCR program consists of 2 min denaturation at 94 °C followed by 35 cycles of 20 s at 94 °C, 20 s at 50 °C, and 30 s at 72 °C, concluding with 10 min extension at 72 °C.

6. Visualize PCR product after electrophoresis of 5  $\mu$ L in 2 % TBE-agarose gels stained with GelRed according to the manufacturer's recommendations. Also use HyperLadder 100 bp as molecular size marker. If there is a smearable PCR product between 300 and 1,000 bp region (Fig. 1), proceed to the next step.
7. Purification of PCR DNA: Purify the remaining PCR product using the GeneJET PCR Purification Kit following the manufacturer's recommendations. Elute the DNA using 50  $\mu$ L of water.
8. DNA Quality Control: Measure the DNA quality and quantity using a NanoDrop 1000 instrument according to manufacturer's recommendation. Usually for next-generation sequencing (NGS), a sample should contain at least 3  $\mu$ g of DNA with  $A_{260/280}$  reading higher than 1.7. After this step, the DNA is ready for NGS sequencing.

### 3.5 Next-Generation Sequencing and Data Analysis Using VirFind

1. Send the DNA preparation to an NGS sequencing service. Receive the raw NGS output files (sff format for 454, and fastq format for Illumina sequencing).
2. Go to <http://virfind.org>. Register for a new account.



**Fig. 2** A sketch of all the steps performed by VirFind to detect and discover viruses using Next-Generation Sequencing data. The bioinformatics tool can accept different NGS file formats, clean and assemble the sequences before generating various Blast and conserved domain search outputs for virus identification

3. Upload the raw NGS files to VirFind ftp server, and complete the sequence submission form to instruct how the pipeline will run.
4. VirFind will start the automated analysis of the raw NGS files (Fig. 2). Briefly, the file will be transferred to VirFind bioinformatics server, converted to fasta format, collapsed,  $n$  nucleotides trimmed ( $n$ =user's choice) from both ends, mapped to reference genome (user's choice) by Bowtie2. Mapped sequences will be output, while unmapped sequences will be subject to *de novo* sequence assembly by Velvet with

different kmers (overlapping nucleotides). Assembled contigs will be subject to Blastn against GenBank nt database with *e*-value of user's choice (default = 0.05), generating four different output files (Blastn\_VIRUS\_reads.fna, Blastn\_VIRUS\_report.tab, Blastn\_NON\_VIRUS\_reads.fna, Blastn\_NON\_VIRUS\_report.tab). Sequences not detected by Blastn will be subject to Blastx against all GenBank virus proteins with *e*-value of user's choice (default = 0.005), generating two different output files (Blastx\_VIRUS\_reads.fna, Blastx\_VIRUS\_report.tab). CAP3 assemblies will also be generated on top of Blastn and Blastx reads. Sequences not detected by Blastx will be output to Reads\_with\_NO\_Blastn\_NO\_Blastx.fna and Reads\_with\_NO\_Blastn\_NO\_Blastx.faa. Conserved domain search (user's choice) of the .faa file against GenBank CDD database will be performed with *e*-value of 0.05 output to Conserved\_domain\_search\_report.txt.

5. Users will use the information from VirFind tabular output files and Conserved\_domain\_search\_report.txt to decide whether there are viruses present in their sample. Blastn\_VIRUS\_reads.fna shows reads that show high similarity to GenBank sequences, while Blastx\_VIRUS\_reads.fna shows reads that share lower similarity to GenBank sequences. Reads\_with\_NO\_Blastn\_NO\_Blastx.fna shows reads that cannot be detected by Blastn and Blastx with the chosen *e*-values (*see Note 8*).

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#### 4 Notes

1. Having water at the bottom of the cylinder and stirring helps to dissolve Tris-HCl more quickly. If using a glass beaker, Tris-HCl can be dissolved faster if the water is warmed to about 37 °C. However, the downside is that care should be taken to bring the solution to room temperature before adjusting pH.
2. Wear double gloves and work in a fume hood with protective lab coat and eye goggles.
3. Cooling down the Sorvall RC 6+ centrifuge to 4 °C will take approximately 15–30 min. The centrifuge should be switched on with rotor installed, lid closed, and temperature selected prior to doing tissue grinding.
4. When doing equilibration, remember to weigh the centrifuge bottle's cap also.
5. Try to collect the colorless upper aqueous phase (containing RNA), but not to touch the solid phase at the bottom. It is better to leave the last few mL of aqueous phase behind than collecting any of the nonaqueous phase.

6. Sometimes the pellet will float or disintegrate into the aqueous phase. If this happens, centrifuge the bottles at higher speed and longer time. Remember to check the g force limit of the bottles.
7. Sometimes the chromatography columns will be clogged and liquid flow will be slow. Introduce air pressure to speed up the process, especially in the last wash to remove all 18 % STE ethanol from the cellulose before eluting the bound dsRNA.
8. This file may have host sequences, but might also contain sequences of new viruses that are significantly different from those deposited in the GenBank. Users will need some experience to determine whether a read reported by VirFind is a real virus sequence and if it is an isolate of a known species, or a completely new virus species belonging to such and such genus/subfamily/family/order. VirFind output also provides a Readme.txt file containing detailed instruction about how to look for known/novel virus sequences in the sequencing datasets.

## Acknowledgement

This study was supported by the USDA-APHIS-NCPN grants 10-11-8100-1572.

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# Chapter 23

## Metagenomic Next-Generation Sequencing of Viruses Infecting Grapevines

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### Abstract

Next-generation sequencing (NGS) technologies, for the first time, provide a truly “complete” representation of the viral (and other) pathogens present in a host organism. This is achieved in an unbiased way, and without any prior biological or molecular knowledge of these pathogen(s). During recent years a number of broad approaches, for most of the popular NGS platforms, have been developed. Here we describe such a protocol—one that accurately and reliably analyze viruses (and viroids) infecting grapevine. Our strategy relies on the synthesis of cDNA sequencing libraries from dsRNA, extracted from diseased grapevine tissues; the sequencing of these on an Illumina platform, and a streamlined bioinformatics pipeline to analyze the NGS data, yielding the virus composition (virome) of a specific grapevine tissue type, organ, entire plant, or even a vineyard.

**Key words** Deep sequencing, High-throughput sequencing, NGS, Virome, dsRNA, Virus discovery, Virus diagnostics

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### 1 Introduction

In addition to being globally recognized as economically the most important fruit crop, grapevine (*Vitis* spp.) also seems to be suffering from more virus diseases than any other perennial crop. Currently more than 70 intracellular infectious agents (65 viruses, 5 viroids, and 8 phytoplasmas) have been reported to infect grapevine [1]. Studies to quantify the economical losses due to virus and virus-like diseases are only recently starting to appear, and are mostly directed at regional scenarios, i.e. case studies of specific virus diseases in specific cultivars for a particular wine-producing region [2–4]. In one such study on Grapevine leafroll disease, yield losses of 27 % and quality penalties of 8 % (as a result of changes in pH, sugar, and acid levels) have been reported [4]. In 2012, these figures translated to financial losses of \$25,000 to \$40,000 per hectare over a 25-year life span of a Cabernet franc vineyard, which

certainly support claims that global collective losses to virus diseases in grapevine run into millions of dollars annually.

Most virus diseases in grapevine, and certainly the economically important ones, are caused by virus complexes. Of these, Grapevine leafroll disease (GLD) ranks as the most destructive, and number one in terms of global distribution and economical losses. GLD is also the most complex disease of grapevine, with 11 viruses reported to be associated with the disease [1]. Since 2011, and after complete viral genome sequences became readily available, a consolidation was proposed among the Grapevine leafroll-associated viruses (GLRaVs). The current taxonomy stipulates five GLRaV species (GLRaV-1, -2, -3, -4, and -7) in three genera (*Closterovirus*, *Ampelovirus*, and *Velarivirus*) [1]. Typical GLD symptoms (Fig. 1a, b) have been reported for single or mixed infections of these GLRaV species (and even other grapevine viruses). As a result, the unequivocal etiology of GLD is still evading scientists. However, GLRaV-3, the type species of the genus *Ampelovirus*, and by far the most ubiquitous member, is globally considered the “main etiological agent,” playing a pivotal role in disease development [5].

Grapevine fanleaf, a nematode-borne degeneration disease, is the oldest known virus disease of grapevines. The etiological agent, Grapevine fanleaf virus (GFLV; genus *Nepovirus*) is believed to have originated in ancient Persia, from where it spread via vegetative propagation material to other wine producing regions. The disease is still considered the most important grapevine disease in Europe and other wine-producing parts of the “Old World.” The disease is endemic to areas where its soil-borne nematode vector is present, and most wine cultivars are susceptible (Fig. 1c–e). Contrary to the other important grapevine virus diseases, GFLV has been firmly established as the only etiological agent of fanleaf degeneration [1].

The Rugose wood complex of trunk diseases can be divided into four types of disorders. These are Rupestris stem pitting, Kober stem grooving, LN33 stem grooving, and Corky bark. As is the case with GLD, the etiology of the Rugose wood disorders is complex and not yet fully elucidated. Four viruses have been found associated with the different types of Rugose wood disorders. These are Grapevine rupestris stem pitting-associated virus (GRSPaV, genus *Foveavirus*), and Grapevine viruses A, B, and D (GVA, GVB, GVD, genus *Vitivirus*) [1]. The complexity of these disorders is exacerbated by the fact that these viruses all seem to be latent (and hence symptomless) in ungrafted *Vitis vinifera*, but display symptoms (Fig. 1f) when grafted onto grapevine indicator hosts (*Vitis rupestris*, LN 33, and Kober 5BB). Various types of Rugose wood disorders occur world-wide, where they cause significant economical losses in wine grape cultivars [1].



**Fig. 1** Characteristic symptoms of the most important virus diseases in grapevine. Grapevine leafroll disease: interveinal reddening and downward rolling of leaf margins in red wine cultivars (a), and interveinal chlorosis and downward rolling of leaf margins in white cultivars (b). Grapevine fanleaf degeneration: leaf yellowing (c), fan-like spread of leaf veins (d) and vein banding of leaves (e). Rugose wood complex: typical pits or vertical grooves in the woody cylinder of the trunk (f). Shiraz disease: failure of lignification results in droopy, green canes in mature plants, and a general retardation of grow (g)

Minor virus diseases of grapevine include emerging diseases like Shiraz disease (Fig. 1g) and -decline, the Grapevine fleck complex, Grapevine vein necrosis disease and Red blotch disease, but all of these are of limited economical consequence because of narrow host range or geographical distribution. Virus-like diseases caused by viroid infection, include Grapevine yellow speckle, Hop stunt viroid, Australian grapevine viroid, and Citrus exocortis viroid [1].

Disease manifestation seems to be limited to symptom expression, with no significant economical implications reported yet.

The intracellular nature of plant viruses implies that control is largely limited to preventative measures. Crucially important to the success of any preventative strategy is accurate and reliable diagnostics. The evolution of plant virus diagnostics over the last few decades essentially produced three sustainable technologies that were readily adopted by testing laboratories: biological (hardwood) indexing, with the ability to diagnose a disease (as opposed to a specific pathogen), ELISA, which relies on the detection of pathogen-specific proteins [6], and PCR-based methods that detects pathogen nucleic acids.

Next-generation sequencing (NGS), while initially developed as a high-throughput sequencing technology, has rapidly been adapted for numerous applications in biological science, including virus diagnostics. Diagnostics based on ELISA and PCR are limited to viruses for which antibodies and virus-specific nucleotide sequences are available. In contrast, NGS is the only truly unbiased assay to study the virus populations (viromes) in specific tissue types, single plants or entire orchards and vineyards. The assembly and identification of viral genomes, both known and unknown, by NGS of a metagenomic nucleic acid sample, extracted from the host plant, have indeed become feasible and have led to major advances in elucidating virus disease etiology [7–10]. Even though the uptake of this powerful technology into routine diagnostic pipelines has been slow and largely limited to virus discovery [11, 12], NGS-based diagnostics is likely to radically change the face of plant virus detection in the near future [12]. As with any new technology, rigorous development is needed to take an assay from proof-of-concept to high-throughput industrial applications. This is particularly true for NGS, since it is fully reliant on bioinformatic analysis of the massive datasets generated by the technology. The importance and value of concomitant development of bioinformatic tools cannot be overstated.

Four major NGS methodologies have been developed in parallel over the last 10 years, each adopted and commercialized by the large platform manufacturers [13, 14]. Pyrosequencing, developed by 454 Life Sciences (Roche) was the first of the NGS platforms to be commercialized. This technology relies on the detection of pyrophosphate released upon nucleotide incorporation during the synthesis of a complementary DNA strand. Sequencing-by-synthesis technology, currently the most widely used NGS approach and developed by Solexa (Illumina), uses four fluorescently labeled, reversible terminator bases to massively parallel sequence bridge-amplified DNA clusters on a glass surface. Sequencing-by-ligation was developed and commercialized by Applied Biosystems (Life Technologies), and is based on DNA amplification by emulsion PCR, and the preferential ligation of matching sequences from a

pool of labeled oligonucleotides. Ion Torrent sequencing (Life Technologies) is based on standard sequencing-by-synthesis chemistry, but uses a novel, semiconductor-based system to detect hydrogen ions that are released when nucleotides are incorporated during DNA synthesis. Each of these platforms has their advantages and disadvantages, and all rely on in-house developed bioinformatics software to do various types of data analyses. Several freely available, as well as commercial software tools are available for NGS data analysis.

One application of NGS in plant virology is determining the entire viral population associated with a particular disease. NGS has the advantage that it can also identify (and relatively quantify) each virus that is associated with a given disease in a plant. Several virus metagenomic studies have been reported over the last 5 years [7–10]. While these all yielded metagenomic NGS data of viral populations associated with particular diseases, they differ in the starting material utilized (double-stranded RNA, total RNA, or small RNA), the sequencing platform used, and the approach used to assemble the viral genomes present in the infected plant samples.

Here we provide a detailed protocol for the metagenomic determination of the virome of a diseased grapevine plant. The protocol describes sample preparation, dsRNA extraction, NGS library construction and sequencing ( $2 \times 250$  paired-ended run on an Illumina MiSeq), and bioinformatic data analysis (Raw data clean-up, de novo assembly, and read-mapping).

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## 2 Materials

### 2.1 dsRNA Extractions

1. Porcelain mortar and pestle or mechanical tissue homogenizer with a 20 mm diameter saw tooth bit (Polytron® PT 2100, Kinematica).
2. Liquid nitrogen.
3. 10× STE stock solution: 50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 6.8.
4. 10 % Sodium dodecyl sulfate (SDS) in reverse osmosis (RO)-H<sub>2</sub>O.
5. Autoclaved RO-H<sub>2</sub>O.
6. 1× STE (1 in 10 dilution of 10× Stock solution in RO-H<sub>2</sub>O).
7. 1× STE(EtOH): 1× STE with 16 % EtOH (v/v) (*see Note 1*).
8. 3 M Sodium acetate (NaOAc) pH 5.5.
9. 40 mg/ml of Bentonite in RO-H<sub>2</sub>O.
10. 2-mercaptoethanol.

11. STE-saturated phenol or H<sub>2</sub>O-saturated phenol (*see Note 2*).
12. Cellulose (Sigma) or Cellulose powder MN2100 (Macherey-Nagel) (*see Note 3*).
13. Chromatography columns: two sizes, 1.5 cm×50 cm and 1 cm×20 cm (Biorad).
14. 50 ml Oak Ridge Centrifuge tubes (capacity 40 ml) (Nalgene).
15. Absolute ethanol (EtOH) 99 %.
16. 70 % EtOH.
17. DNase RQ1 and 10× DNase RQ1 buffer (Promega).
18. RNase T1 diluted to working stock of 10 U/μl with RO-H<sub>2</sub>O (Roche).
19. Phenol:Chloroform:Isoamylalcohol (25:24:1).
20. Chloroform:Isoamylalcohol (24:1).
21. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.
22. Benchtop centrifuge.
23. Electrophoresis: 1 % Agarose-TAE gel: 40 mM Tris–HCl, 20 mM Acetic acid, 1 mM EDTA.
24. DNA Molecular marker (GeneRuler 1 kb ladder, Thermo Scientific or equivalent).
25. 6× DNA loading dye (Thermo Scientific).

## 2.2 Library Construction and Sequencing

1. Reagents and materials required are listed in the TruSeq® RNA sample preparation kit v2 (Illumina).

## 2.3 Bioinformatics

1. Hardware: A UNIX-based operating system with at least 8 GB RAM (Apple) is recommended for use with most software. However, commercial software will run on a Windows computer with 4 GB RAM. Access to a High Performance Computer (HPC) is not essential for the basic analysis, but recommended (*see Note 4*).
2. Free software: FastQC, FASTX-Toolkit, Blast2GO (*see Note 5*).
3. Web-based resources: NCBI GenBank, NCBI-BLAST.
4. Commercial software: CLC genomics workbench (Examples of alternative commercial software: Geneious, or free software: Velvet, Bowtie) (*see Note 6*).

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## 3 Methods

The following methods outline the extraction of metagenomic dsRNA from grapevine bark scrapings for the construction of NGS sequencing libraries and the basic bioinformatic analysis of the data.

### 3.1 Sample Preparation

Mature lignified virus-infected cane material is selected (field-grown material is generally better than greenhouse material) for dsRNA extraction (*see Note 7*). The outer bark is removed first from the canes to expose the dark green phloem layer. The phloem layer is scraped off with a sharp knife by holding the blade perpendicular to the cane and moving it back and forth with downward pressure taking care not to scrape too deep into the lighter green xylem. Cane material can be stored at 4 °C before processing but once phloem scrapings have been made it must be stored at -80 °C until required for extraction.

### 3.2 dsRNA Extraction

Double-stranded RNA is extracted from grapevine phloem scrapings using a modified cellulose affinity purification method. The method is in principle a batch purification method with column wash steps adapted from Morris and Dodds [15].

The extraction is conducted at room temperature (22 °C) unless stated otherwise.

1. Prepare the extraction buffer fresh before use. Add 9 ml 10× STE to 36 ml H<sub>2</sub>O, 15 ml 10 % SDS, 25 ml Phenol (STE), 25 ml Chloroform, 1.2 ml Bentonite suspension, 1 ml 2-mercaptoethanol (*see Note 8*).
2. Grind 10 g grapevine phloem scrapings (plant material) in liquid nitrogen to a very fine powder.
3. Add extraction buffer to powdered material in a 250 ml bottle.
4. Shake at 150×*g* for 30 min on an orbital shaker.
5. Transfer to 50 ml Oakridge centrifuge tubes and centrifuge for 15 min at 10,000×*g*.
6. Carefully collect supernatant (S/N) by pipetting and add EtOH to final concentration of 16 % (v/v) (*see Note 9*).
7. Add 3 g of cellulose, and adjust volume to 300 ml with 1× STE(EtOH) in a 500 ml bottle.
8. Shake at 150×*g* on an orbital shaker for 45 min.
9. Load cellulose-containing extract on large column (1.5 cm × 50 cm) and let it run through to pack the cellulose.
10. Wash column with 100 ml 1× STE(EtOH).
11. Elute dsRNA with 40 ml 1× STE.
12. Collect the eluent and add EtOH to final concentration of 16 % (v/v).
13. Add 0.5 g cellulose to eluent in a 250 ml bottle.
14. Shake at 150×*g* on an orbital shaker for 30 min.
15. Load cellulose-containing eluent on small column (1 cm × 20 cm) and let it run through to pack the cellulose.
16. Wash column with 50 ml 1× STE(EtOH).

17. Elute dsRNA with 9 ml 1× STE into 50 ml Oakridge centrifuge tube.
18. Add 0.9 ml 3 M NaOAc and 27.6 ml absolute EtOH.
19. Store the 50 ml Oakridge centrifuge tube at -20 °C overnight.
20. Pellet dsRNA by centrifugation: 10,000×*g*, at 4 °C for 60 min.
21. Decant the S/N and add 25 ml 70 % EtOH.
22. Centrifuge at 10,000×*g* at 4 °C for 30 min.
23. Decant the S/N and centrifuge at 10,000×*g* at 4 °C for 2 min.
24. Aspirate the pellet and allow pellet to dry (approximately 20 min).
25. Add 50 µl (RO-H<sub>2</sub>O or TE buffer) to pellet and centrifuge at 10,000×*g* at 4 °C for 2 min (*see Note 10*).
26. To proceed with optional DNase/RNase treatments (**steps 27–41**): transfer dsRNA (RO-H<sub>2</sub>O) to 0.2 ml microcentrifuge tube. Alternatively, transfer dsRNA (TE) to 1.5 ml microcentrifuge tube and go directly to evaluation and final precipitation (**step 42**) (*see Note 11*).
27. Add the following to the 50 µl dsRNA in the 0.2 ml microcentrifuge tube: 12.5 µl 10× RQ1 DNase Buffer, 2.5 µl RQ1 DNase (2.5 Units), 5 µl of RNase T1 working stock (50 Units).
28. Incubate at 37 °C for 30 min in heating block or thermal cycler.
29. Transfer content to 1.5 ml microcentrifuge tube containing 500 µl Phenol:Chloroform:Isoamylalcohol (25:24:1).
30. Vortex thoroughly for 10 s.
31. Add 430 µl 1× STE, vortex thoroughly for 10 s and centrifuge at 13,200×*g* at 4 °C for 10 min.
32. Recover the aqueous phase (±500 µl) and transfer to a new 1.5 ml microcentrifuge tube containing 500 µl Chloroform: Isoamylalcohol (24:1).
33. Vortex thoroughly for 10 s and centrifuge at 13,200×*g* at 4 °C for 10 min.
34. Recover the aqueous phase (±500 µl) and transfer to a new 2 ml microcentrifuge tube and add 50 µl 3 M NaOAc and 1.25 ml absolute EtOH.
35. Precipitate dsRNA at -80 °C for 120 min.
36. Pellet dsRNA by centrifugation: 13,200×*g* at 4 °C for 60 min.
37. Decant S/N and add 500 µl 70 % EtOH.
38. Centrifuge at 13,200×*g* at 4 °C for 20 min.
39. Decant S/N and centrifuge at 13,200×*g* at 4 °C for 2 min.
40. Aspirate the pellet and allow to dry (approximately 20 min).

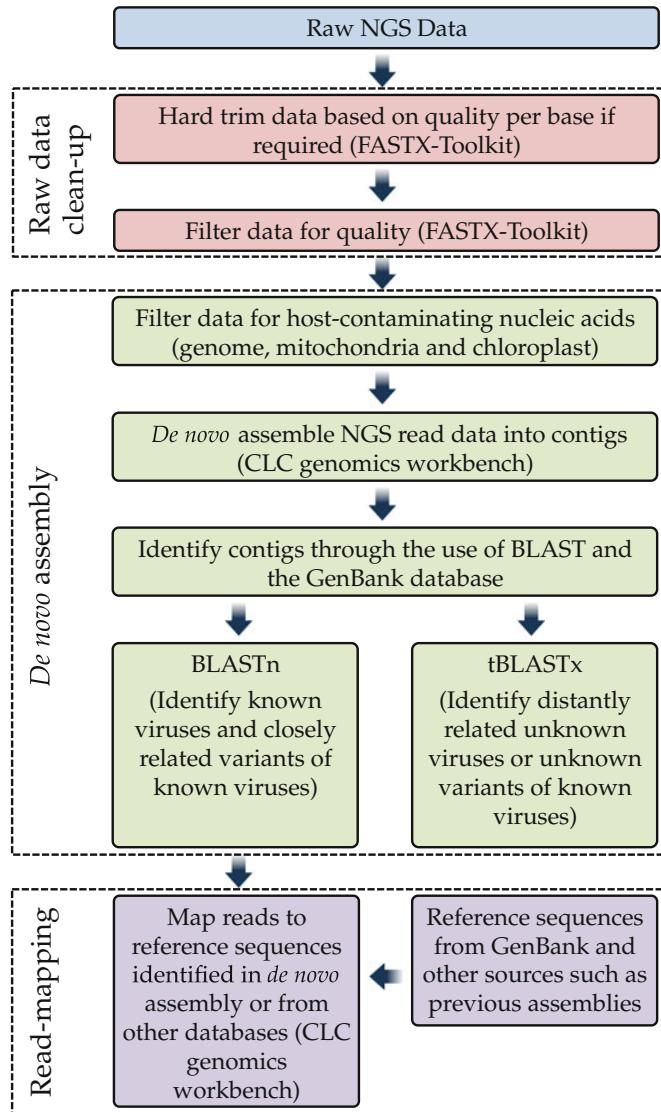
41. Add 50 µl TE buffer to resuspend the pellet.
42. Electrophoresis: 10 µl (20 %) of the dsRNA and 0.5 µl of the molecular marker (GeneRuler 1 kb) on a 1 % Agarose-TAE gel at 80 V for 60 min (*see Note 12*). Visualize the gel on a UV transilluminator. The quality and concentration of the dsRNA can be visually assessed by comparison to the DNA molecular weight marker (*see Note 13*).
43. Precipitate the remaining 40 µl of the dsRNA sample by adding 4 µl 3 M NaOAc and 110 µl EtOH and store at -20 °C until used for cDNA library construction (*see Note 14*).

### **3.3 NGS Library Construction and Sequencing**

The choice of sequencing platform can influence the sample prep kit selection. We have chosen the Illumina platform and the TruSeq® RNA sample preparation kit to sequence metagenomic dsRNA (*see Note 15*).

Once the library is constructed, several decisions need to be made:

- This library can be sequenced on a number of different Illumina platforms. What sequencing platform must be used? (This often depends on your service provider; *see Note 16*). The example reported here used the Illumina MiSeq platform.
- Will the sequencing be performed as single or pair-ended reads (PE)? (*see Note 17*.) The example reported here was performed as pair-ended reads.
- How much data is required? (*see Note 18*.) For the example reported here, 2.5 million PE reads were generated.
  1. Pellet dsRNA at 12,000×g for 60 min.
  2. Wash pellet with 70 % EtOH (be careful not to lose the pellet, it will be very small).
  3. Air-dry pellet for 20 min at RT.
  4. Add 19.5 µl of Elute, Prime Fragment mix (EPF) and pipette gently up and down 6 times. Proceed with RNA fragmentation as described in the TruSeq® RNA sample preparation v2 guide.
  5. Proceed with first- and second-strand synthesis followed by end-repair, adenylation of the 3' ends and primer ligation ending off with PCR amplification. Details of these steps can be found in the TruSeq® RNA Sample Preparation v2 Guide ([http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry\\_documentation/samplepreps\\_truseq/truseqrna/truseq-rna-sample-prep-v2-guide-15026495-f.pdf](http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqrna/truseq-rna-sample-prep-v2-guide-15026495-f.pdf)).
  6. Prepared library can then be shipped to an external Illumina service provider or sequenced in-house on an Illumina Platform, such as MiSeq.



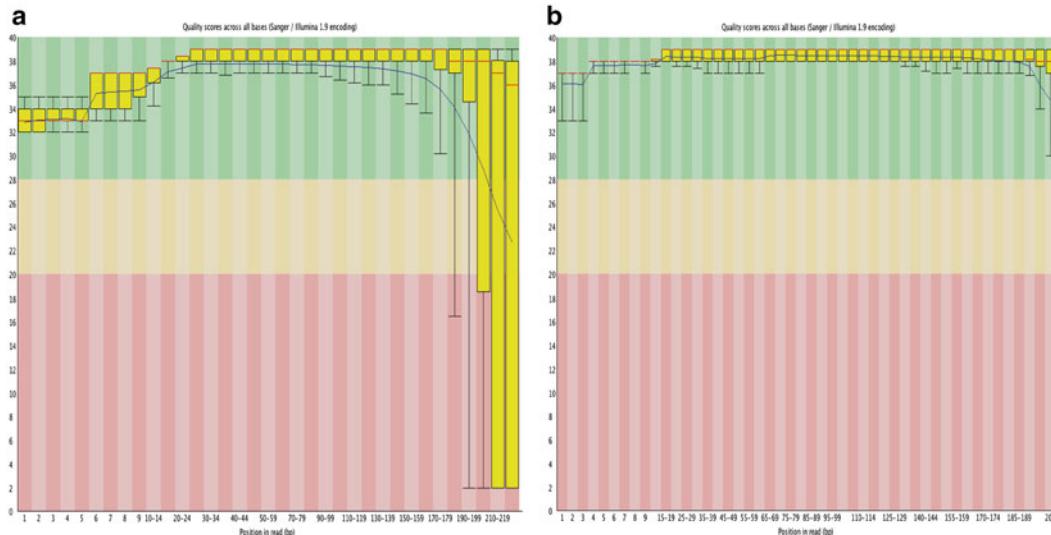
**Fig. 2** General workflow of the bioinformatic analysis of NGS data

### 3.4 Bioinformatic Data Analysis

#### 3.4.1 Raw Data Clean-up

The bioinformatic data analysis can be divided into three sections: raw data clean-up (Subheading 3.4.1), de novo assembly (Subheading 3.4.2) and read-mapping (Subheading 3.4.3) (Fig. 2).

1. The overall quality per base can be evaluated visually using FastQC. Several modules are available to evaluate the sequence quality and help identify possible problems such as adaptor contamination. Viewing the “per base sequence quality” gives a visual representation of the overall quality of the data. We use the mean value (Fig. 3, represented as a blue line) as an indicator of where to perform a hard trim of the data. All nucleotides 3' of the intersection of the blue line and a quality score of 28



**Fig. 3** The “per base sequence quality score” Box whiskerplot output graphs by FastQC of the same data set before (a) and after (b) data clean-up. The *central red line* is the median value, the *yellow box* represents the inter-quartile range (25–75 %), the *upper* and *lower* whiskers represent the 10 % and 90 % points and the *blue line* represents the mean quality. The Y-axis is the quality score and the X-axis the nucleotide base position. In panel A, an Illumina MiSeq dataset with wide whiskers and inter-quartile range towards the 3' end of the read data. Panel B show the same dataset on which a hard trim of the first 9 nt and last 11 nt was performed followed by quality filtering

are removed (Fig. 3). The “per base sequence content” module will most likely also indicate an imbalance in nucleotide compositions in the first few bases ( $\pm 9$  nt) (see Note 19). FastQC will also identify sequences that are overexpressed (usually remnants of adaptors).

2. Hard trimming, when required, can easily be performed using the “trimmer” tool in the FASTX-Toolkit. Trimming is performed on the 5' and 3' ends of reads simultaneously by supplying the first and last base that should be retained in the command line (see Note 20).
3. Quality filtering: the data can also be filtered for quality using the quality filter tool in the FASTX-Toolkit. General setting: minimum quality score of 20 (99 % accuracy) for 96 % of the nucleotides in a read. Reads that do not comply with these standards are automatically filtered from the dataset.

### 3.4.2 De Novo Assembly

The de novo assembly described here is as performed in CLC genomics workbench 7.0.

1. Data is imported into CLC genomics workbench.
2. Additional filtering of host-contaminating nucleic acids is recommended to aid downstream analysis. The data set is read-mapped

to the *Vitis vinifera* genome (NC\_012007 to NC\_012025), as well as the *Vitis vinifera* mitochondrial (NC\_012119) and chloroplast genomes (NC\_007957) simultaneously using the default parameters with nonspecific matches mapped randomly. Reads that do not match these genomes are written to a separate file and used for further analysis.

3. De novo assembly can be performed on the reduced dataset using the automatic detection function for word and bubble size. The recommended minimum contig length is 2× the read length +1.
4. Contigs can be identified through BLAST analysis directly from CLC genomics workbench. Alternatively, contigs can be exported and identified using Blast2GO. Blast2GO allows for BLASTn and tBLASTx searches. Specific viruses and closely related unknown variants of known viruses can be identified using BLASTn, however more distantly related unknown viruses or unknown variants of known viruses can only be identified using tBLASTx.

#### 3.4.3 Read-Mapping

The read-mapping described here is as performed in CLC genomics workbench.

1. Data can be mapped to reference sequences using the read-mapping module of CLC genomics workbench. A typical application would be to identify the virus variants of known viruses present in a sample. Reads are mapped to the reference genomes of all virus variants simultaneously and allowing for only unique binding events (nonspecific matches ignored). It is important to note that reference genomes will not be fully covered in this approach. Virus variants present in a sample should be clearly distinguishable from the other reference sequences that were used based on the percentage/number of reads that mapped. This approach is not quantifiable and results can be skewed if unknown virus variants or viruses at low titer are present.

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## 4 Notes

1. Depending on the age of the absolute EtOH, the percentage purity will range between 96 and 99 %. The percentage EtOH in “16 %” solutions will range between 16 and 16.5 %. This small variation does not affect the selective binding of dsRNA to the cellulose. Add 100 ml 10× STE to 800 ml RO-H<sub>2</sub>O and 160 ml EtOH for a final volume of 960 ml.
2. Preparation of STE-saturated phenol: Extreme care must be taken during preparation and take the necessary safety precautions.

Although STE-saturated phenol is recommended, H<sub>2</sub>O-saturated phenol can be used and can be purchased in liquid form. Start with 500 g of phenol crystals and fill the bottle (original packaging) with 1× STE. Shake until the crystals are dissolved. Store the phenol at 4 °C overnight. After the overnight incubation, shake the phenol again and pour into a separation funnel that is protected from light and allow two phases to separate. Pour the phenol into dark safety bottles and layer with 1 cm 1× STE. Store at 4 °C until use.

3. Selection of cellulose powder: CF11 (Whatman) has been the standard cellulose for dsRNA extractions for decades, however there are alternatives that have the same performance and can be used. We have had good results with cellulose from Sigma and cellulose powder MN2100 from Macherey-Nagel.
4. To perform these analysis we are currently using a MacBook Pro with a 2.7 GHz Intel Core i7 Processor and 16 GB (1,600 MHz) RAM. Additionally, we also use a High Performance Cluster Computer. Although it is recommended it is not essential to be able to perform the analysis.
5. Free software:  
FastQC:  
<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>  
FASTX-Toolkit: [https://hannonlab.cshl.edu/fastx\\_toolkit/](https://hannonlab.cshl.edu/fastx_toolkit/)  
Blast2GO: <https://wwwblast2go.com/b2gome/aboutblast2go?eprivacy=1>  
The support documents (help files) are very useful and clear to understand.
6. Bioinformatic software is constantly being developed and commercial software packages are generally adequate and often have a user-friendly graphical user interface. CLC genomics workbench (Qiagen) is recommended. An alternative commercial software package is Geneious (Biomatters). There are also free software available that can be used to perform the basic de novo assembly (Velvet, <https://www.ebi.ac.uk/~zerbino/velvet/>) and read-mapping (Bowtie, <https://bowtie-bio.sourceforge.net/index.shtml>).
7. If nondestructive sampling is required, petioles can be used but it is not recommended. The concentration of dsRNA is generally much lower in petioles compared to the phloem and likely also subject to seasonal fluctuations.
8. Take the necessary safety precautions when preparing the extraction buffer.
9. Depending on the age of the absolute EtOH the percentage purity will range between 96 and 99 %. The volume of EtOH

to add to the S/N can be determined by dividing the volume of the S/N by 5. The percentage EtOH will range between 16 and 16.5 %. This small variation does not affect the selective binding of dsRNA to the cellulose.

*Example:* For 53 ml S/N 10.6 ml of Ethanol will be added.

$$\% \text{ EtOH} = 10.6 \text{ ml} / 63.6 \text{ ml final volume} \times 96 \% = 16 \% (\text{16.5 \% if EtOH is 99 \%})$$

10. Add RO-H<sub>2</sub>O to the pellet if DNase/RNase treatments are going to be performed, or TE buffer if proceeding directly to evaluation and final precipitation.
11. Relevance of DNase treatment is arguable. The percentage of viral reads in preparations does not seem to increase significantly with DNase treatment and remains at ±30 %. The loss in sample might outweigh the potential increase in percentage viral reads. The host reads can easily be removed from the data set bioinformatically.
12. To ensure good separation of the dsRNA, increase volumes of the dsRNA and molecular marker to 16 µl with TE buffer and add 4 µl of 6x loading dye. This is calculated on 6 mm wide wells with a 1.5 mm thick comb.
13. The quality and concentration can be visually assessed by comparison to the DNA molecular marker. 0.5 µl of the molecular marker (GeneRuler 1 kb ladder, Thermo Scientific) translates to 3 ng for the 10 kb marker and 7 ng for the 6 kb marker. A concentration of >2× the 6 kb marker is a good sample, sufficient for library preparation. This translates to a weight for the remaining sample (80 %) equivalent to ~50 ng DNA.
14. If NGS is outsourced, dsRNA can be shipped by rapid courier (overnight or less than 24 h) delivery. During shipment, for extra precaution, samples can be shipped with an icepack.
15. The TruSeq® RNA sample preparation kit v2 uses additional RNA purification steps with oligo-dT magnetic beads to bind polyadenylated mRNA. During the final elution step of purification, RNA is also fragmented and primed for cDNA synthesis. Since most grapevine viruses are not polyadenylated, and to avoid additional bias, the initial steps of the library preparation in the TruSeq® RNA sample preparation kit are skipped. The sample enters the regular library preparation protocol at the RNA fragmentation step as a pellet. 19.5 µl EPF mix is added to the dsRNA pellet and sample is further treated as any other RNA sample as described by the kit manufacturer.
16. Using the Illumina TruSeq library preparation allows for the sequencing on any Illumina MiSeq or HiSeq platform. The

selection of platform will influence the average read length in the data, cost of sequencing, and time required to generate the data. The impact of Illumina platform on the quality and diversity of the data is limited and either can be used. Comparisons between samples should however be made with data generated by the same platform.

17. Either single reads or paired-end (PE) read data could be used. The aim of metagenomic sequencing is to determine the diversity and not necessarily to assemble full genomes. PE read data will assemble into longer and more informative contigs. The possibility to generate long reads by overlapping PE reads is an interesting prospect and would be the most useful (Currently, 2x 300 nt PE sequencing is available on the MiSeq v3 reagent kits).
18. It is difficult to determine how much data is required in a metagenomic sample. It is not only dependent on viral genome sizes, but also on their relative abundance in the sample. In our experience 10 million unique data points (clusters) (i.e. 10 million single reads or 20 million PE reads) seems to be sufficient to generate data that reflect the full complexity of the sample. This means that it is possible to perform virus discovery experiments that can identify different viruses as well as virus stains in the dataset. However, if the aim of the experiment is to determine the viral diversity up to species level for diagnostics, less data should be sufficient (such as the example described here).
19. Experience has shown that RNA-Seq libraries have a bias if random hexamers were used. Although these primers should theoretically have good diversity through the sequence, these libraries always have a selection bias in the first few bases [12] of each run. This bias is due to the selection of primers and does not represent any individually biased sequences. Although this is true sequence we have experienced better de novo assemblies if the first 9 bases are trimmed.
20. Hard trimming of data might not be required for good quality datasets that have an average quality score of over 28 for the full length of the reads.

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## Acknowledgements

We hereby acknowledge financial support from Winetech and the THRIP programme of the National Research Foundation. We thank Beatrix Coetzee for critical reading of the manuscript.

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# Chapter 24

## Droplet Digital PCR for Absolute Quantification of Pathogens

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### Abstract

The recent advent of different digital PCR (dPCR) platforms is enabling the expansion of this technology for research and diagnostic applications worldwide. The main principle of dPCR, as in other PCR-based methods including quantitative PCR (qPCR), is the specific amplification of a nucleic acid target. The distinctive feature of dPCR is the separation of the reaction mixture into thousands to millions of partitions which is followed by a real time or end point detection of the amplification. The distribution of target sequences into partitions is described by the Poisson distribution, thus allowing accurate and absolute quantification of the target from the ratio of positive against all partitions at the end of the reaction. This omits the need to use reference materials with known target concentrations and increases the accuracy of quantification at low target concentrations compared to qPCR. dPCR has also shown higher resilience to inhibitors in a number of different types of samples. In this chapter we describe the droplet digital PCR (ddPCR) workflow for the detection and quantification of pathogens using the droplet digital Bio-Rad platform QX100. We present as an example the quantification of the quarantine plant pathogenic bacterium, *Erwinia amylovora*.

**Key words** Digital PCR, Droplet digital PCR, Plant pathogens, Absolute quantification, Viruses, Bacteria

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### 1 Introduction

During the past decade real-time quantitative PCR (qPCR) has been progressively accepted as one of the golden standards for molecular detection and quantification of pathogens [1, 2]. The advantages of qPCR that have contributed to its wide use include reduced possibility of contamination by eliminating the need for post-reaction processing, improved multiplexing and throughput, real-time monitoring, and significantly its potential for quantification [3]. However, for quantification in qPCR a standard curve with known concentrations of the target is necessary to transform the output values of qPCR, quantification cycles (Cq), into actual concentration values (target copies/ $\mu$ l). The unavailability of standardized reference materials in plant pathology can lead to a

lack of harmonization and significant inter-laboratory quantification biases. Moreover, the co-extraction of substances that influence the amplification efficiency (inhibitors and facilitators) influences the Cq value obtained in qPCR which can lead to lower accuracy of quantification [4]. qPCR also shows limitations when rare alleles or mutants are to be quantified or detected in a high background of wild type targets [5].

Digital PCR (dPCR), which originated from the studies of target quantification using limiting dilutions [6], has the potential to improve the above mentioned limitations of qPCR. In the dPCR format, the reaction mixture containing primer/probes and nucleic acid sample is divided into hundreds (BioMark™ HD System Fluidigm) to millions (RainDrop® System RainDance) of partitions (also called compartments or droplets). Depending on the dPCR platform, these partitions are constituted by chambers or droplets. A fraction of the partitions is occupied by a single to few copies of the target DNA/RNA, while a fraction of compartments must remain target free. After amplification, positive and negative compartments are counted and the absolute concentration of target copies in the initial sample is derived by applying Poisson's distribution without the need for any standard [7]. Compartmentalization facilitates the detection and quantification of rare mutants in a background of wild type sequences [8], because, in contrast to qPCR, low frequency targets do not need to compete for reaction mixture resources (primers, probes, ...) with those present in high frequency leading to a higher signal to noise ratio. In dPCR, the number of positives and negatives is counted after end point PCR amplification, and therefore, the final result is independent of variations in the PCR amplification efficiency, making dPCR potentially more accurate, repeatable, and less prone to inter-laboratory variations than qPCR [9]. Several recent studies also suggest that dPCR can be more resilient to inhibitors than its non-digital counterpart [10–12].

Different dPCR platforms are available, which differ mainly in the strategy they use to produce partitions (chambers or droplets) and in their number [13]. Among the first instruments available, Fluidigm Corporation (San Francisco, CA) and Life Technologies (Carlsbad, CA) offer microfluidic chip based compartmentalization resulting in hundreds to thousands of compartments, while Bio-Rad (Hercules, CA) and RainDance (Lexington, MA) focus on emulsion based compartmentalization that can generate from tens of thousands (QX100 and 200 systems, Bio-Rad) to up to millions of droplets (RainDrop® System, RainDance). New platforms are also starting to emerge as well as upgrades of existing ones. The number of partitions affects the accuracy of quantification. This is of importance primarily in for example rare event detection in a high background. For the majority of applications

involving quantification of plant pathogens, the number of partitions in the range of 12,000–20,000 is sufficient.

Plant pathology field can benefit from dPCR features both for research and diagnostic applications. dPCR can enable accurate quantification of plant pathogens without standards, and is thus a promising method of choice for calibration of reference materials to be used in laboratories worldwide. dPCR can be applied to detect and study the dynamics of low frequent mutants within a given pathogen population, i.e., in quasispecies studies. Moreover dPCR can allow overcoming problems associated with the presence of inhibitors associated with certain plant materials and other difficult matrices (e.g., soil). Studies describing the application of dPCR to the quantification of pathogens are increasing [10, 14–16], among them also first examples dealing with plant pathogens, such as phytoplasma [17], *Erwinia amylovora* and *Ralstonia solanacearum* [18]. In our laboratory, pathogen quantification was successfully undertaken using droplet digital PCR (ddPCR). We found that optimized qPCR assays could be easily transferred to ddPCR format. In these assays, ddPCR offered higher quantification accuracy at lower concentrations [10, 17, 18] and lower sensitivity to inhibitors [10]. It has also been demonstrated that RNA quantification is possible using a one-step reverse transcription-ddPCR (RT-ddPCR) [10].

In summary, dPCR in general and in particular ddPCR have great potential for practical use in plant pathogen detection: (1) for quality control of in-house reference materials to be used in qPCR and other PCR-based diagnosis methods, (2) for quality control of materials used in test performance studies, (3) in optimizing and assessing qPCR and DNA/RNA extraction methods [18], and finally (4) for routine quantification of nucleic acid target in a wide variety of samples. While running costs of ddPCR currently remain slightly above qPCR, this is compensated by an improved performance (i.e., lower susceptibility to inhibition), ease of interpretation, and absolute quantification independent of reference materials. Among other advantages, multiplexing potential has been demonstrated for accurate quantification of genetically modified organisms, bacteria, and viruses [11, 19, 20], and can be of benefit also in plant health field. ddPCR is also a useful method for library quantification in next generation sequencing applications. In addition to the minimum information for publication of quantitative real-time PCR experiments (MIQE) [21], guidelines describing the minimum information for the publication of dPCR experiments have been also made available [9].

In this chapter we focus on the ddPCR workflow for pathogen (DNA or RNA) quantification using the Bio-Rad QX100 (or QX200) system, introducing also as an example the case of *E. amylovora*. In addition to the workflow we show examples of the different signals that can be obtained and give some hints on the data analysis.

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## 2 Materials

### 2.1 Samples

#### and Controls

(See Note 1)

1. Extracted DNA or RNA from the plant or matrix under analysis (*see Note 2*).
2. Buffer extraction control. Buffer is added to the nucleic acid extraction procedure to monitor for potential contamination occurring during extraction.
3. Negative matrix control. Same material or matrix is added to the nucleic acid extraction, to assess the background signal inherent to the matrix.
4. No template control. Nuclease-free water used for master mix preparation is applied to the ddPCR reaction, to monitor for eventual contamination introduced during master mix preparation.
5. Positive control. A known concentration of the target sequence is added to the ddPCR reaction, to assess ddPCR performance.

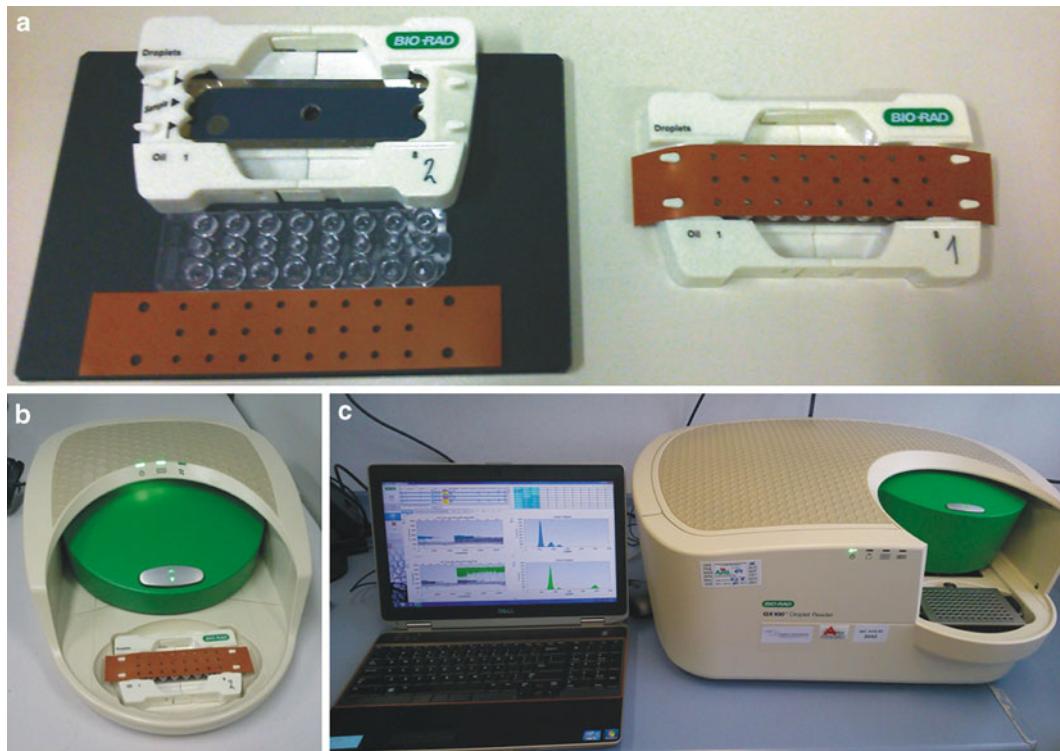
### 2.2 ddPCR Reagents

(See Note 3)

1. 2× ddPCR Super Mix for probes (Bio-Rad) for DNA amplification or 2× One-Step RT ddPCR Supermix kit (Bio-Rad) for RNA to cDNA transcription and amplification in one step (*see Note 4*).
2. Droplet generation oil for probes (Bio-Rad).
3. Molecular grade nuclease-free water.
4. Primers and probe.

### 2.3 Equipment

1. QX100 (or QX200) Droplet Digital PCR system (Bio-Rad) including droplet generator, droplet reader, and QuantaSoft data acquisition and analysis software (Fig. 1b, c).
2. Thermal cycler, i.e., T100 (Bio-Rad).
3. Droplet generator cartridge holder (Bio-Rad) (Fig. 1a).
4. DG8 droplet generator cartridges and gaskets (Bio-Rad, Cat. No. 186-4008 and 186-3009) (Fig. 1a).
5. Easy Pierce foil plate seals (Thermo, Cat. No. AB-0757).
6. 96-well PCR plate (Eppendorf Cat. No. 951020362).
7. PCR plate sealer (Eppendorf, Model No. 5390 000.024).
8. Set of pipettes. Manufacturer recommends Rainin (L-20, L-50, L8-50, L8-200) for the steps of droplet generation and droplet handling (*see Note 5*).
9. Tips for pipettes with aerosol barrier filters. Manufacturer recommends Rainin for the steps of droplet generation and droplet handling (*see Note 5*).
10. Microfuges and vortex for master mix preparation and appropriate nuclease-free plasticware.



**Fig. 1** (a) *Left side*: droplet generator cartridge holder, cartridge and gasket. *Right side*: assembled components prepared for droplet generation. (b) Droplet generator with inserted cartridge holder. (c) Droplet reader prepared for insertion of a 96-well plate and Quantasoft software

### 3 Methods

The workflow for detection and quantification of any RNA/DNA target using the QX100 system consists of four main steps: (1) preparation of the reaction mixture, (2) droplet generation, (3) PCR amplification, and (4) droplet reading and analysis of results.

#### 3.1 Preparation of the Reaction Mixture

1. Mix, in nuclease-free tubes of appropriate volume, the concentrations of primers and probe for each assay with the corresponding 2× ddPCR master mix (Tables 1 and 2). Preparation of duplex or multiplex assays is also possible (*see Note 6*). Note that manganese acetate needs to be added in the case of the one-step master mix. Final reaction volume should be planned to 20 µl. Follow good laboratory practice in order to avoid contamination during mix preparation.
2. Before adding the sample, distribute the prepared mix into nuclease-free tubes, strips, or 96-well plates.
3. Add each sample (DNA/RNA samples and controls) into each of the tubes containing master mixes. Mix thoroughly by pipetting up and down, or by vortexing followed by a brief

**Table 1**  
**Reaction setup for DNA amplification in ddPCR**

Component	Volume ( $\mu\text{l}$ )	Final concentration
2× ddPCR super mix for probes	10	1×
20× Target primers/probe (FAM)	1	1× (i.e., 900 nM/150 nM) <sup>a</sup>
20× 2nd Target primers/probe (VIC) <sup>b</sup>	1	1× (i.e., 900 nM/150 nM)
Nuclease-free water	Variable	–
DNA sample	Variable	50 fg to 100 ng <sup>c</sup>
<i>Final volume</i>	20	–

<sup>a</sup>Concentrations of primers and probe should be preferably optimized previously (*see Note 7*)

<sup>b</sup>Duplex assay can be included to monitor for a second target or reference gene (*see Note 6*)

<sup>c</sup>For quantification purposes the target concentration should be within the linear range of the method (*see Note 8*)

**Table 2**  
**Reaction setup for RNA amplification in one step RT-ddPCR**

Component	Volume ( $\mu\text{l}$ )	Final concentration
2× one-step RT-ddPCR supermix	10	1×
Manganese acetate	0.8	1× (i.e., 900 nM/250 nM)
20× Target primers/probe (FAM)	1	1× (i.e., 900 nM/150 nM) <sup>a</sup>
20× 2nd Target primers/probe (VIC) <sup>b</sup>	1	1× (i.e., 900 nM/150 nM) <sup>a</sup>
Nuclease-free water	Variable	–
RNA sample	Variable	50 fg to 5 ng <sup>c</sup>
<i>Final volume</i>	20	–

<sup>a</sup>Concentrations of primers and probe should be preferably optimized previously (*see Note 7*)

<sup>b</sup>Duplex assay can be included to monitor for a second target or reference gene (*see Note 6*)

<sup>c</sup>For quantification purposes the target concentration should be within the linear range of the method (*see Note 8*)

centrifugation. Each tube should contain 20  $\mu\text{l}$  of reaction mixture (*see Note 9*).

### 3.2 Droplet Generation

1. Place a DG8 droplet generation cartridge into the cartridge holder (Fig. 1a).
2. Transfer 20  $\mu\text{l}$  of each prepared reaction mixture to each of the 8 wells indicated as “sample” in the droplet generation cartridge (*see Note 5*). There should be no empty wells left (*see Note 10*). Precautions should be taken not to form bubbles in the bottom

of the well, as they could interfere with the droplet formation. Add 70 µl of droplet generation oil, in all the wells indicated as “oil.” Do not leave the oil bottle open for extended periods of time to avoid evaporation and stability of components. Hook the gasket over the cartridge holder using the holes in both sides.

3. Place the holder with the cartridge in the QX100 droplet generator unit (Fig. 1b). Initiate droplet generation. Oil and sample are pushed through microfluidic channels and mixed in the cartridge in the process forming droplets. Droplets are accumulated in the droplet well. The process takes 2–3 min for each cartridge.
4. Once droplets are generated the gasket is removed and 40 µl of the droplet suspension (“droplets” lane in the cartridge) are transferred from the cartridge to a 96-well PCR plate. The pipetting both for collecting the droplet suspension and for dispensing it in the PCR plate wells should be slow to protect the integrity of the droplets (*see Note 5*).
5. After all the samples (*see Note 11*) have gone through droplet generation and have been transferred to the 96-well PCR plate, the plate is heat sealed with a pierceable foil (*see Note 12*). Do not spin down the plate. This would break down the droplets.

### 3.3 PCR Amplification

1. Transfer the sealed plate to the thermocycler and run the PCR in the conditions shown in Table 3. Modifying the manufacturer provided cycling conditions can serve to improve the performance of certain assays (better cluster separation, higher specificity, *see Note 7*).

**Table 3**  
**PCR cycling conditions**

Step	DNA samples			RNA samples		
	T (°C)	Time	Cycle #	T (°C)	Time	Cycle #
RT	–	–	–	60	30 min	Hold
Enzyme activation	95	10 min	Hold	95	5 min	Hold
Denaturation	94	30 s	40	94	30 s	40
Annealing and extension	60	1 min		60	1 min	
Heat deactivation	98	10 min	Hold	98	10 min	Hold
Hold	4	∞	Hold	4	∞	Hold

Ramp rates should be adjusted to 2–3 °C/s

### **3.4 Droplet Reading and Analysis of Results**

1. Transfer the PCR plate to the QX100 droplet reader (Fig. 1c) and close the lid (*see Note 13*).
2. In the QuantaSoft software (Bio-Rad), click “Setup” and define the information for each well/sample, including name, type of experiment, type of sample and detectors or channels (FAM and/or VIC).
3. Click Run to start the reading. The droplet reader acts as a flow cytometer and reads each droplet to determine their signal and amplitude in the selected detectors.
4. Click “Analyze.” The critical point is to set a threshold that allows the software to differentiate between negative and positive droplets. The software offers the possibility of defining this automatically or manually however, other approaches to analyses are available and may in specific cases be more suitable (*see Note 14*).
5. The software offers different ways of viewing the results, (1D amplitude of one channel, 2D amplitudes of both channels, copy number in each well/channel ...) that are more or less informative depending on the type of experiment (absolute quantification, allele discrimination, multiplexing, ...). It ultimately gives a table with parameters resulting from the analysis, such as the concentration of target copies/microliter of reaction, the number of total accepted droplets, the positive ones and the negative ones (*see Note 15*).

### **3.5 Case Study, Quantification of the Quarantine Pathogen *E. amylovora***

As a practical example, we describe a recently published ddPCR based protocol for the quantification of the DNA quarantine bacterial pathogen *E. amylovora* [18] (Table 4 and Fig. 2). The method was transferred from a previously published qPCR assay [22]. There are also examples in the literature of one step RT-ddPCR assays that target RNA pathogens, such as Rotavirus [10] and Pepper Mild Mottle virus ([12] and Fig. 4).

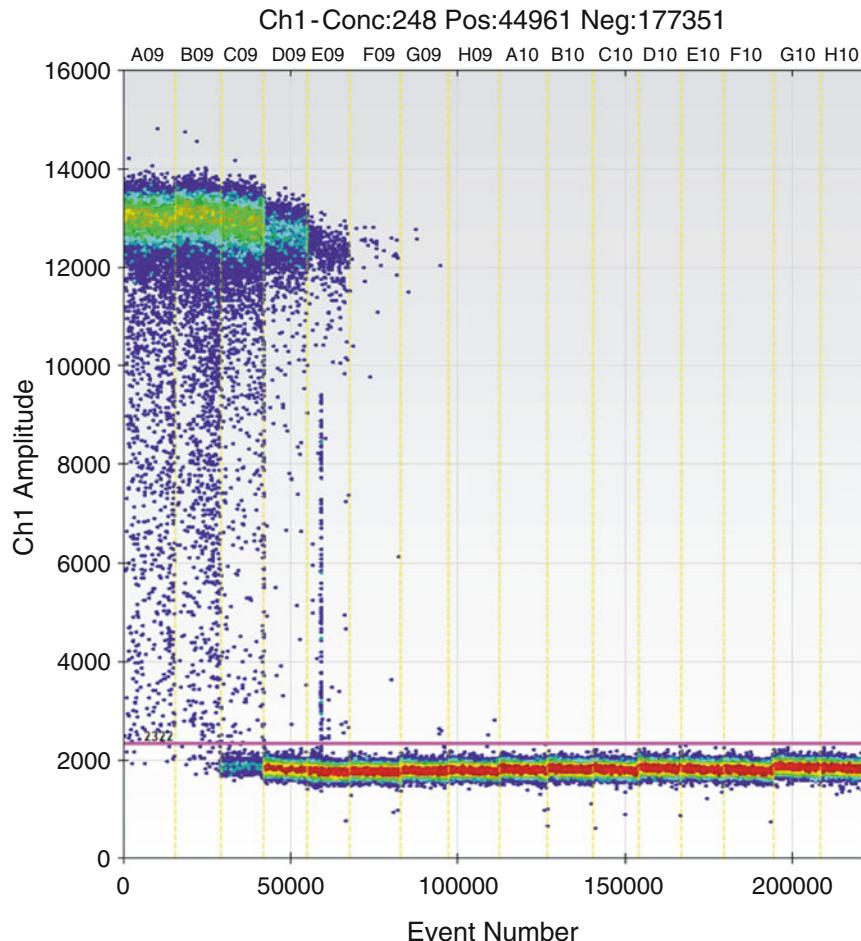
1. DNA from the pure bacterial cultures and plant extracts was extracted using magnetic bead based QuickPick™ SML Plant DNA kits (Bio-Nobile, Turku, Finland) in a King Fisher™ automated system (Thermo Labsystem) as described previously for *E. amylovora* [22], and with a minor modification (440 µl lysate used in the purification).
2. Reaction components are mixed as shown in Table 1 with 4 µl of DNA in a final 20 µl volume. Concentrations of primers and probe are the same as the previously optimized by Pirc et al. for the qPCR assay, 900 nM and 250 nM, respectively [22]. For droplet generation and PCR, the same steps and cycling conditions as the ones described above for a DNA target were followed.

**Table 4**  
Selected parameters calculated for the *E. amylovora* samples

Sample	Positive droplets	Accepted droplets	$\lambda$	Quantity-Reaction (copies)	P	Effective reaction size	Positive mean	Negative mean
A09	15,699	15,708	7.5	117,255.50	0.999	71.5	12,716.00	2,060.70
B09	13,728	13,757	6.2	84,770.70	0.998	62.6	12,700.80	1,997.30
C09	12,122	12,881	2.8	36,472.60	0.941	58.6	12,700.80	1,873.90
D09	2,832	13,042	0.2	3,192.80	0.217	59.3	12,544.30	1,814.40
E09	532	12,464	0	543.7	0.043	56.7	10,382.70	1,760.70
F09	36	15,398	0	36	0.002	70.1	11,769.40	1,767.10
G09	8	14,110	0	8	0.001	64.2	7,404.90	1,782.70
H09	3	15,491	0	3	0	70.5	2,547.30	1,788.50

Parameters were calculated using an R script [18] for the positive samples shown in Fig. 2 and include parameters listed in the minimum information for the publication of dPCR experiments as previously described [9]. The highest concentration shown corresponds to  $3 \times 10^7$  target DNA copies per mL. See [18] for calculation of additional parameters describing the quality of separation of positive and negative droplets.

Parameters: Positive drop = number of positive droplets, Accepted drop = number of accepted droplets,  $\lambda$  = mean copies per partition, calculated using the number of positive partitions, as described in Huggett et al. [9]:  $-\ln(1 - (\text{number of positive partitions}) / (\text{number of accepted droplets}))$ , Quantity-Reaction(copies) = target concentration expressed in copies per ddPCR reaction, P = fraction of positive droplets (number of positive droplets / (number of accepted droplets)), Effective reaction size = total volume of partitions measured. It is calculated by multiplying the number of accepted droplets with the volume of partition. The volume of droplets is assumed to be 0.91 nL, consistent with the instrument manufacturer's software; Positive mean = mean of the signal in positive droplets and Negative mean = mean of the signal in negative droplets.



**Fig. 2** Results of amplification of *E. amylovora* DNA in ddPCR. Serial tenfold dilution of *E. amylovora* DNA (A09 to H09) and no template controls (A10 to H10) are shown. For each sample droplets are depicted according to the event (number of droplet as read during reading) and its fluorescence (Ch1 Amplitude). The concentration of droplets is seen as a heat map with the highest concentration depicted in red and the lowest in blue. At higher concentrations of the target DNA the number of negative droplets is low and the test is approaching its upper limit of the dynamic range. Note the distribution of positive droplets over a range of fluorescence values that is caused by small differences in the amplification efficiencies or delayed amplification (Monte Carlo effect) and the absence of positive droplets in no template controls. The threshold discriminating between negative and positive droplets is set at fluorescence of 2,322 and was chosen as previously described [18]. Because of the Poisson modelling that is used for calculating concentration, different threshold have little effect on the concentrations however they can have pronounced effect on the plus/minus detection. Concentrations determined and parameters calculated for the positive samples shown here are listed in Table 4

3. After thermal cycling the plate is transferred to the droplet reader. The software package (Quanta Soft) from Bio-Rad was used for data acquisition and analysis.
4. A manual threshold value of 2.322 was defined [18], after evaluation of different types of negative samples (no template

controls and negative plant material) and positive samples (plant material containing low concentrations of target bacteria and serial dilutions of target DNA). The mentioned threshold value allowed proper detection and quantification and of all the tested samples. The automatic threshold assignment by the software yielded very similar results to the manual threshold, as well as the use of the “Defining the rain” tool (<http://definetherain.org.uk/>). Therefore, for this particular assay any of these three strategies can be used for data analysis however, in particular with suboptimal assays, the analysis approach can significantly influence the results [18].

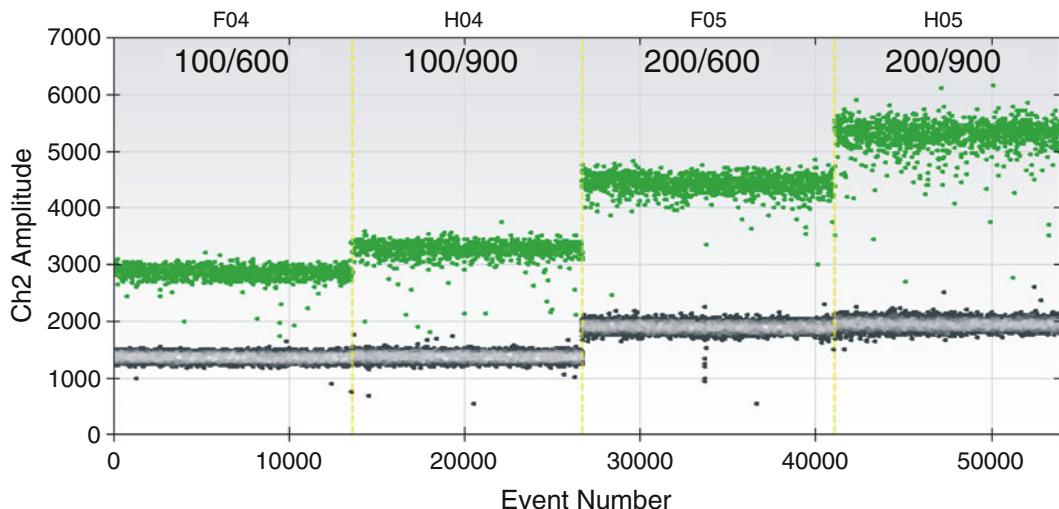
5. In Fig. 2 examples of the results obtained with different samples are shown: NTC, negative matrix, low target concentration, and high target concentration. In Table 4 the ddPCR parameters corresponding to each of the samples from Fig. 2 are shown: total accepted droplets, positive droplets, negative droplets, concentration, and average number of copies per partition ( $\lambda$ ). The QuantaSoft software gives a list of most of these parameters in tabular form. Dreo et al. [18] developed an open source R script that allows automation of the ddPCR data analysis under different settings and calculates additional important data (i.e.,  $\lambda$ ), thus significantly simplifying the optimization of this step and allowing for high-throughput analysis of samples (*see Note 15*).

---

## 4 Notes

1. Depending on the nature of the ddPCR experiment, all of the mentioned controls may not be necessary, or alternatively, some controls that have not been listed here may be needed, but this would be subject of another chapter. Diagnostic applications are likely to require more controls than research ones. For example useful controls consist of spiking a negative sample with target pathogen at concentrations close to the limit of detection and process it in parallel to other samples through DNA extraction and PCR. Assay optimization runs will also require additional controls, in order to evaluate the different signal amplitudes that are generated and associate those with positive and negative droplets. It is not uncommon to observe a small number of droplets [1–3] with higher amplitude than the negative droplets in negative samples. Sometimes the amplitude of such droplets can equal the one of the real positives. In this case based on negative sample observations a minimum number of droplets to make a sample positive should be defined. A previous report defined two droplets as minimum for considering a sample positive [18].

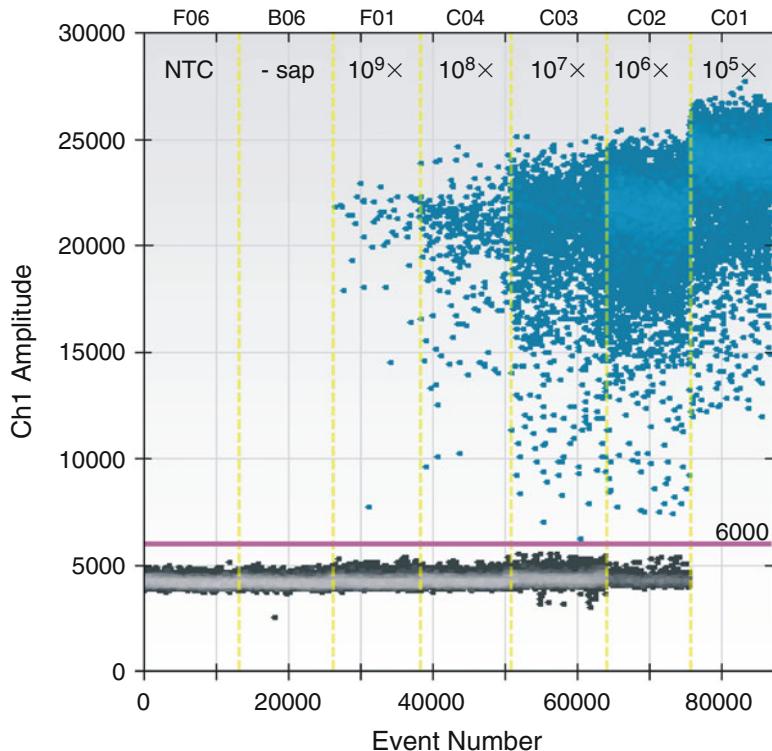
2. For RNA or DNA isolation same protocols or kits as those used for qPCR can be used for ddPCR. The higher resilience of ddPCR to inhibitors reported in several recent studies [10–12, 23] allow the use of a broader range of nucleic acid purification methods, including more simple extraction protocols. The possibility of direct ddPCR analysis and quantification of bacteria without DNA extraction has been also confirmed [18].
3. At present, the Bio-Rad platform requires the use of their proprietary master mixes enabling stable droplets formation, while platforms based on partitioning into chambers allow for testing different commercially available PCR mixes.
4. In the case of analysis of an RNA pathogen in a two-step format, a reverse transcriptase (RT) or RT kit with confirmed optimal performance should be selected. For the RT optimizations known concentrations of the RNA target, i.e., an in vitro transcript quantified spectrophotometrically, should be applied to different RT systems, and the yield of the RT estimated. The RT yield should preferably be as close as 100 % as possible, otherwise the quantification with ddPCR requires a correcting factor to be genuinely absolute.
5. The highest accuracy when pipetting is required. In addition, when handling the droplets, the pipetting should be done very carefully to preserve their integrity. For example when transferring the freshly generated droplets from the cartridge to the PCR plate, the pipetting should be executed at slow and constant speed, taking 10 s both for the collection and dispense. The use of regularly calibrated pipettes that ensure an accurate liquid handling is highly recommended. Bio-Rad, for example, recommends using Rainin pipettes (single and multichannel).
6. The QX100 and QX200 systems allow duplexing with fluorescence data acquisition in both the FAM and HEX-VIC spectral regions, respectively. There is increasing number of studies where multiplexing with ddPCR has been reported [11, 19, 20, 24]. In addition, multiplexing of up to four assays using the same reporter dye has been demonstrated by optimizing primer and probe concentrations that lead to positive signals of different fluorescence intensity. QX100 is not compatible with the use of intercalating dyes such as SYBR Green or Eva Green, but this issue has been addressed in the newer model QX200.
7. Assays (primer and labeled probe) for dPCR are designed in the same way as for qPCR and same tools can be used (i.e., Primer Express from life Technologies, or others). Assays that have been optimized in qPCR format are easily transferred to ddPCR format [1, 10, 11, 17, 18], while assays that have shown limitations in qPCR (cross-amplification, low efficiency



**Fig. 3** Influence of primer and probe concentration. ddPCR analysis of the same viral target using the same assay with four different primer/probe concentrations (100 nM/600 nM, 100 nM/900 nM, 200 nM/600 nM, 200 nM/900 nM). Increase of primer concentrations results in an increase in the amplitude of positive droplets. Increase in the probe concentrations results in an increase in the amplitude of both positive and negative droplets. With the highest primer/probe concentrations the highest separation between positive and negative droplets is achieved but an increase in the dispersion (rain effect) of the positive droplets is observed. Optimal primer/probe concentration for this particular assay was 200 nM/600 nM

of amplification) are more likely to fail in a ddPCR assay as well [18]. Note that the closer the cycling protocol of qPCR is to the cycling protocol in ddPCR, the easier the transfer of assays. Primers and probe concentrations have an influence on the amplitude and clustering of the droplets (*see Fig. 3*), and therefore, testing different concentrations of primers and probe may be a wise thing to do in case the assays do not show the desired performance in ddPCR format. Annealing temperature is also a critical point that offers space for optimization and changing other cycling conditions such as elongation time or cycle number. Additional validation study includes testing of the matrices where the pathogen of interest will be detected/quantified to assess the background signal amplitude from the matrix and for any cross-reaction [18]. Testing of a higher number of NTC of a given assay is also necessary to evaluate the possibility of false positive results. All this information will be of help when deciding on the threshold cutoff (*see Note 14*).

8. Poisson distribution accounts for the presence of more than one target copy inside a droplet but a minimum number of negative droplets are necessary in order to calculate the target concentration. The theoretical upper limit of the linear range is defined by the number of available partitions (droplets in this case). For this reason, to achieve accurate quantification, if a

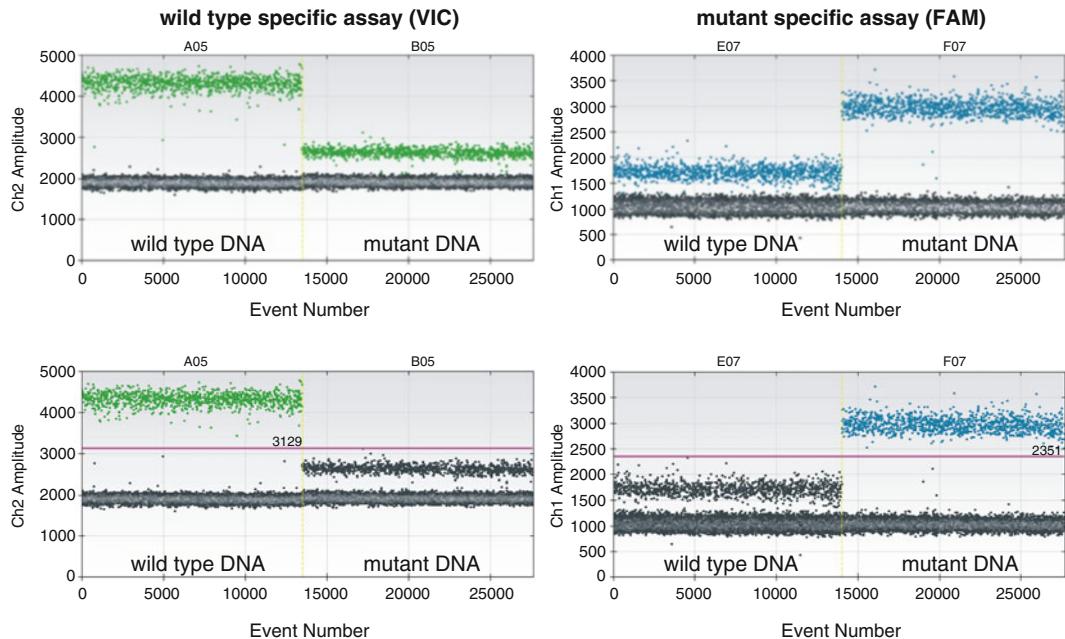


**Fig. 4** Dose dependence of ddPCR. Tenfold dilution series of *Pepper mild mottle virus* (PMMoV) infected plant sample analyzed in one step RT-ddPCR format. The clusters of negative and positive droplets are easily recognized. A manually set threshold separates both types of droplets. Droplets associated to the “rain” effect observed in some of the dilutions were considered positive in this experiment after evaluation of droplet amplitudes in negative samples. *NTC* non template control, *sap* healthy plant sap

saturation of the droplets is observed, the target should be diluted to a concentration within the linear range of the method (see Fig. 2 A09 and B09 samples and Fig. 4, 10<sup>5</sup>-fold diluted sample). The theoretical dynamic range of ddPCR is broad and up to 100,000 copies per reaction can be quantified which is higher than the commonly examined concentrations of plant pathogens in samples.

9. When preparing the master mix it is wise to account for excess volume for each component in order to ensure the presence of at least 20 µl in the reaction mixture before transferring it to the droplet generator cartridge.
10. If there are not enough samples as to fill all of the wells with the reaction mixture a special buffer (ddPCR™ Buffer Control Kit—Bio-Rad) should be used to fill the remaining wells. In spite of this we recommend that experiments are designed in a way that all the wells are filled with reaction mix, so that DG8 droplet generation cartridges are used at their full capacity.

11. The process of droplet generation is repeated as many times as required by the sample number. When longer preparation times are expected due to high number of samples it is recommended to keep both, samples and droplet suspensions, at 4 °C using refrigerated blocks.
12. Plates should only be sealed when the plate sealer has reached its optimal temperature. In our experience, pressure to the plate with the heated sealer should be applied 2× for 5 s. The foil on the plate should be inspected for the presence of circles from the wells beneath the foil. If the pressure is applied for too long the seal will be broken and all the oil and the reaction mixture will evaporate during PCR reaction.
13. In the case that the droplet reader is unavailable after PCR cycling, the plate can be stored at 4 °C for several hours or even overnight.
14. The variation of signals depends on many factors, e.g., assay characteristics [18], matrix or presence of inhibitors [10], single nucleotide polymorphisms (SNP) in the probe annealing region (Fig. 5) and others. All these factors can affect the separation between negative and positive droplets in different ways, resulting for example in the presence of higher signal droplets in negative samples or in the induction of the so-called droplet rain effect (Figs. 2 and 4). Therefore, in certain cases automatic analysis can be sometimes misleading, and setting the threshold manually can be necessary. An example of the influence of a single nucleotide polymorphism (SNP) in the probe annealing region is shown in Fig. 5. In this example wild type and mutant assays differ by one SNP in the probe. When wild type DNA is applied to mutant specific assay and vice versa, positive droplets with signal amplitude higher than the negative ones are observed due to cross-reactivity. Automatic analysis considers the cross-reactive droplets as positives (Fig. 5 upper panels), by defining manually the threshold can assign those amplitudes as negative (Fig. 5 lower panels). Observed cross-reactivity could also be improved by modifying the assay (annealing temperature, cycle number, ...) or redesigning it (LNA probes, SNP in the 5', ...). Manual definition of the threshold can also help overcoming other droplet particularities, such as rain effect. Both in diagnostic and quantitative applications, it is important to evaluate the signal amplitude obtained with negative samples (NTC, isolation controls, matrix controls, closely related pathogens). The threshold can be then manually defined to consider such signals as negative. For correctly designed assays that perform optimally automatic analysis is sufficient. A comprehensive study showing different ways of analyzing the ddPCR data based on a case study with two assays targeting two bacterial plant pathogens has been



**Fig. 5** Effect of single nucleotide polymorphisms. Analysis of a wild type and mutant viral sequences using two assays specific for the wild type (FAM) and mutant (VIC) sequences, respectively. Note that the mutant sequence still cross-reacts with the wild type assay and similarly the wild type sequence cross-reacts with the mutant assay (*upper panels*). An optimal separation of the clusters of droplets with specific signal and cross-reactive signal was achieved, allowing a correct quantification by manually setting the threshold (*lower panels*). The manual threshold was set at the lowest possible value that resulted in 0 positive droplets when applying mutant sequence to wild type assay and vice versa

recently published [18]. Automatic analysis was compared with manual threshold definition and the Web-based tool “definethereain” by challenging all three to the correct assigning of known positive and negative samples. Results show that the choice of the analysis depends on each particular assay’s performance and characteristics. More detailed information on the stringency and characteristics of each analysis tool can be found in ref. [18].

15. The data obtained from the results table (concentration, number of droplets: total, negative, positive, accepted, ...) can be used to calculate and optimize different parameters (Table 4) that are requested in the dPCR MIQE guidelines [9]. Some of these parameters are the mean number of copies per partition ( $\lambda$ ), the individual partition volume, or the effective reaction size [9]. An R-based script that automates these calculations has been recently described [18]. Low number of total accepted droplets can negatively affect the precision of the result and, thus, several published studies define a limit of 10,000 accepted droplets, below which the result in that well is rejected [10, 11, 18].

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## ERRATUM TO

# **Diagnostics of Tree Diseases Caused by *Phytophthora austrocedri* Species**

**Vincent Mulholland, Matthew Elliot,  
and Sarah Green**

Christophe Lacomme (ed.), *Plant Pathology: Techniques and Protocols*, Methods in Molecular Biology, vol. 1302, DOI 10.1007/978-1-4939-2620-6\_5, © Springer Science+Business Media New York 2015

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**DOI 10.1007/978-1-4939-2620-6\_25**

The publisher regrets that in the print and online versions of the Table of Contents and the title page of chapter 5 incorrectly appears in the book as “Diagnostics of Tree Diseases Caused by *Phytophthora austrocedri* Species”. The correct title is: “Diagnostics of Tree Diseases Caused by *Phytophthora* Species”.

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The online version of the original chapter can be found at  
[http://dx.doi.org/10.1007/978-1-4939-2620-6\\_5](http://dx.doi.org/10.1007/978-1-4939-2620-6_5)

# INDEX

## A

Agarose gel electrophoresis..... 9, 77, 88, 130–133, 189, 208, 211, 216, 218, 221, 309

### Antibodies

monoclonal..... 177  
polyclonal..... 166, 177, 189, 192, 210

Ash dieback..... 75, 78  
*Chalara fraxinea*..... 78

## B

Bacteria..... 1–15, 26, 85, 87, 91, 110, 113–117, 124, 128, 230, 255, 274, 333, 338, 341, 342

Bioassay..... 19  
Blackleg

*Dickeya*  
*D. dadantii*..... 2  
*D. dianthicola*..... 2, 3, 13  
*D. solani*..... 2, 3, 12–13

*Pectobacterium*  
*P. atrosepticum*..... 2, 3, 6, 10–14  
*P. carotovorum* subsp. *brasiliensis*..... 2, 6, 10  
*P. carotovorum* subsp. *carotovorum*..... 2, 6, 10, 14  
*P. wasabiae*..... 2, 3, 6, 10

BLAST search..... 133

## C

### *Candidatus* spp.

*Ca. Liberibacter solanacearum*..... 85–96  
*Ca. Phytoplasma americanum*..... 116  
*Ca. Phytoplasma australiense*..... 116

Capillary electrophoresis..... 188, 190, 192, 197–199, 202

*Chalara fraxinea*. See Ash dieback

## D

DAS-ELISA. See Enzyme-linked immunosorbent assay (ELISA)

*Dickeya* species  
*D. dadantii*..... 2  
*D. dianthicola*..... 2, 3, 13  
*D. solani*..... 2, 3, 12–13

Digoxigenin RNA probe (DIG-Probe)..... 262, 266

## DNA

bar-coding ..... 123–134  
cloning ..... 204, 250  
16Sr genes bar-code ..... 128  
*tuf* genes bar-code ..... 125, 128, 130

### extraction

BioSprint 96 DNA Plant Kit (QIAGEN) ..... 140  
CTAB method ..... 64, 91–93, 117, 118  
KingFisher ..... 64–65  
kit ..... 130, 138, 214, 234  
leaf tissues ..... 106, 264  
pathogen culture ..... 114  
phloem tissues ..... 64, 66  
sequence analysis ..... 133  
sequencing ..... 61–62  
soil material ..... 61  
tubers tissues ..... 26

Droplet digital PCR ..... 331–346  
absolute quantification of pathogens ..... 331–346

## E

### Enzyme-linked immunosorbent assay

(ELISA)..... 31, 100, 163–167, 170–172, 174, 178, 193, 203, 212, 221, 229, 240, 242, 244, 245, 283, 284, 286, 291, 292, 297, 298, 318

## F

Float material ..... 138, 141–143, 146, 148

Fluorescent techniques  
chemiluminescence substrate ..... 263, 267  
fluorescent-labelled ssDNA ..... 188  
Luminex Magplex ..... 286  
SNAPshot ..... 188, 190

Fungus ..... 17, 21, 29, 31, 35, 38

## G

### *Gangrene*. See *Phoma*

Gel electrophoresis ..... 5, 9, 14, 77, 82, 88, 89, 91, 95, 99, 109, 130–133, 189, 205, 208, 211, 216, 218, 221, 309  
SybrSafe™ ..... 2211  
*Globodera pallida* ..... 137, 139

<i>Globodera rostochiensis</i> .....	137, 139
Grapevine disease	
Grapevine fanleaf degeneration.....	149, 316, 317
Grapevine leafroll disease (GLD).....	315–317
Rugose wood complex .....	316, 317
Shiraz disease.....	317
<b>H</b>	
High-throughput MICROLAB® STARlet Liquid Handling Workstation .....	140, 141, 147
Hybridization	
anti-DIG-alkaline phosphatase.....	267
Luminex xTAG .....	286–287
microarray.....	276
nylon membrane.....	263
<b>I</b>	
Immunoassays .....	151
In vitro culture.....	125
Isothermal amplification	
LAMP .....	75–82, 85–96, 99–110
RPA	
PVY diagnostic .....	210
WDV diagnostic.....	211
ITS amplification .....	71
<b>L</b>	
LAMP. <i>See</i> Loop-mediated isothermal amplification (LAMP)	
Locked nucleic acid (LNA) probe.....	113–120
Loop-mediated isothermal amplification (LAMP) .....	75–82, 85–96, 99–110
Luminex Magplex	
biotinylated secondary antibodies.....	284–286
flow cytometer .....	286
LED-based image analyser.....	286
paramagnetic microspheres.....	284, 297, 298
target specific primer extension .....	284, 287, 290, 294, 295
xMAP serological assay .....	284–286
xTAG molecular assay .....	286–287
<b>M</b>	
Media	
carrot broth.....	63, 65
CVPM.....	3, 5, 8, 11, 14
D-PEM.....	4, 8, 14
Luria-Bertani Agar (LBA) .....	4, 9
nutrient agar (NA).....	4
selective plating media.....	4–5
SMA medium.....	62, 63
SMA + MRP.....	62, 71
V8 broth .....	63, 65
<b>N</b>	
Nematodes	
MEKU nematode carousel extraction	
method.....	138, 140, 141
Oostenbrink method .....	152, 155
potato cysts nematodes (PCN)	
<i>Globodera pallida</i> .....	137, 139
<i>Globodera rostochiensis</i> .....	137, 139
<i>Xiphinema</i> sp.	
<i>X. diversicaudatum</i> .....	150–154
<i>X. index</i> .....	150–154
<i>X. italiae</i> .....	150–154
<i>X. vittenezi</i> .....	150–154
Next generation sequencing (NGS)	
dsRNA extraction.....	319–323, 327
Illumina MiSeq platform.....	323
library construction.....	319, 320, 323
NGS data analysis .....	319
Nylon membrane	
chemiluminescence substrate.....	263, 267
DIG-labelling.....	263
dot blot .....	273
<b>O</b>	
On-site (in field) testing.....	108
<b>P</b>	
PCR	
asymmetric .....	280
conventional .....	3, 5–10, 19, 62, 76, 87–89, 99, 106, 138
degenerate oligonucleotide primed (DOP) RT-PCR.....	303
immuno capture (IC) RT-PCR.....	177, 179, 182, 183, 203
inhibition .....	333
multiplex asymmetric.....	286–287
multiplexing.....	280, 308, 342
nested .....	115, 128, 129, 131–132, 134, 243
random (anchored) amplification .....	280
reverse transcription (RT).....	151
target specific primer extension .....	284, 287, 290, 294, 295
<i>Pectobacterium</i>	
blackleg.....	1–3

- P. atrosepticum* ..... 2, 3, 6, 10–14  
*P. carotovorum* subsp. *brasiliensis* ..... 2, 6, 10  
*P. carotovorum* subsp. *carotovorum* ..... 2, 6, 10, 14  
*P. wasabiae* ..... 2, 3, 6, 10  
 16S rRNA ..... 6
- Phoma**
- gangrene ..... 17, 18, 20, 26
  - P. eupyrena* ..... 17, 18, 22–24, 26
  - P. exigua* var. *exigua* ..... 17–19, 22, 23, 26
  - P. foveata* ..... 17, 18, 21–24, 26
  - soil borne fungus ..... 17
- Phytophthora**
- larch dieback ..... 59
  - P. austrocedri* ..... 59–61, 65–67, 72
  - P. kernoviae* ..... 59–61, 66, 68
  - P. lateralis* ..... 59–61, 66, 69
  - P. ramorum* ..... 59–61, 66, 68
  - tree diseases ..... 59–73
- Phytoplasma**
- Ca. P. asteris* ..... 125
  - Ca. P. aurantifolia* ..... 124, 125
  - Ca. P. pruni* ..... 125
  - Ca. P. prunorum* ..... 124
  - Ca. P. solani* ..... 126
  - Ca. P. ulmi* ..... 126
  - Ca. P. ziziphi* ..... 126
  - flavescence doreé (FD) ..... 126
  - lime witches broom ..... 123, 124
  - potato stolbur ..... 116
  - 16 ribosomal gene SrDNA group I, II, III, V, VI and XII ..... 116
  - virescence ..... 123, 124
- Plexor® ..... 19–26
- Polymorphism ..... 127, 182, 200, 201
- single nucleotide polymorphisms (SNPs) ..... 188, 191, 192, 195–200, 203, 219, 345, 346
- Pospiviroids. *See* Viroids
- Potato cyst nematode (PCN)
- Globodera pallida* (Stone) ..... 137, 139
  - Globodera rostochiensis* (Wollenweber Behrens) ..... 137, 139
- Potato purple top wilt ..... 114, 116
- Probes
- fluorescent TaqMan ..... 67, 68
  - locked nucleic acid ..... 113–120
  - TaqMan MGB ..... 139
- Psyllid ..... 85, 86, 90, 91, 93, 114, 126
- Q**
- QBOL ..... 127, 128, 131
- Quantification ..... 10, 12, 48, 49, 99, 158, 331–346
- Quarantine regulated pathogens ..... 61
- R**
- Ramularia collo-cygni* ..... 29–35
- Ramularia leaf spot (RLS) ..... 29, 30, 35
- Raspberry crumbly fruit disease ..... 228
- Real time PCR
- fungi ..... 113, 116
  - LNA real time PCR ..... 113–120
  - nematode ..... 140–141, 143–145, 156
  - phytoplasma ..... 113–120
  - Plexor® ..... 20, 24
  - qPCR ..... 10, 31, 34, 39, 153, 164, 331
  - TaqMan ..... 10, 12, 61, 143, 153
  - viruses ..... 167–170
- Recombinase polymerase amplification (RPA) ..... 207–224
- Rhynchosporium secalis* ..... 35
- Rhynchosporium* leaf scald ..... 30
- RNA
- double stranded (ds) RNA ..... 303–305, 307–308, 319, 321
  - extraction
    - buffer for extraction from soft fruit ..... 231, 233, 236
    - kit ..... 210, 214
    - protocol for extraction from tuber skin tissues ..... 163–164
    - genomic ..... 195
    - reverse transcription ..... 164
- RPA. *See* Recombinase polymerase amplification (RPA)
- S**
- Serological assays
- DAS-ELISA ..... 284, 286
  - immuno capture (IC)-(RT) PCR ..... 177
  - Luminex xMAP ..... 284–286
- Single-strand conformation polymorphism (SSCP) analysis ..... 192, 205
- SNAPshot assay ..... 197, 203
- Soft rot ..... 1–3
- Soil
- DNA extraction from soil ..... 61
  - nematode extraction ..... 155, 156
- T**
- Tilletia* species
- Karnal bunt ..... 37
  - T. caries* ..... 41, 43–46, 48, 54, 55
  - T. ehrhartae* ..... 38, 41, 43–48, 54, 55
  - T. horrida* ..... 38, 41, 43–45, 48, 54, 55
  - T. indica* ..... 37, 38, 41–49, 54, 55
  - T. walkeri* ..... 38, 41, 43–46, 48, 54, 55
- Translation elongation factor (*tuf*) ..... 23, 125, 127–133

Tubers

- direct virus diagnostic by real-time PCR ..... 163, 167–173
- growing-on DAS-ELISA ..... 164–167, 170–172
- virus diagnosis ..... 161–175

**V**

Validation ..... 100, 107–109, 128, 175, 199, 280, 343

Vectors

- fungus ..... 17, 21, 29, 31, 35, 38
- insect ..... 87, 113, 114, 123, 125, 127, 163
- nematodes ..... 152, 316

Virion-associated nucleic acids (VANA) ..... 249–257

Viroids

- pospiviroids ..... 259–262, 268, 287
- potato spindle tuber viroid ..... 259–270

Virus

- Alfalfa mosaic virus (AMV) ..... 238
- Apple mosaic virus (ApMV) ..... 238
- Arabis mosaic virus (ArMV) ..... 149, 151, 238
- Beet pseudoyellows virus (BPYV) ..... 238
- Blackberry chlorotic ringspot virus (BCRV) ..... 239, 302
- Blackberry virus E (BVE) ..... 239
- Blackberry virus S (BIVS) ..... 239
- Blackberry virus X (BIVX) ..... 239
- Blackberry virus Y (BIVY) ..... 239
- Blackberry yellow vein associated virus (BYVaV) ..... 229, 239
- Blackcurrant reversion virus (BRV) ..... 237, 239
- Black raspberry cryptic virus (BrCV) ..... 238
- Black raspberry necrosis virus (BRNV) ..... 238
- Blueberry latent spherical virus (BLSV) ..... 240
- Blueberry latent virus (BBLV) ..... 240
- Blueberry mosaic virus (BIMV) ..... 240
- Blueberry necrotic ring blotch virus (BNRBV) ..... 240
- Blueberry red ringspot virus (BRRSV) ..... 240
- Blueberry scorch virus (BlScV) ..... 240
- Blueberry shock virus (BlShV) ..... 241
- Cherry leafroll virus (CLRV) ..... 241
- Cherry raspberry leaf virus (CRLV) ..... 241
- contact transmitted ..... 60, 85, 114, 123, 126, 149, 151, 162, 208, 228, 230, 260, 261, 301
- Cucumber mosaic virus (CMV) ..... 241
- fanleaf degeneration ..... 149, 316, 317
- Fragaria chiloensis cryptic virus (FCILV) ..... 241
- Fragaria chiloensis latent virus (FCiLV) ..... 241
- Gooseberry vein banding associated virus (GVBaV) ..... 241
- Grapevine fanleaf virus (GFLV) ..... 149–151, 316
- Grapevine Syrah virus-1 ..... 245
- Impatiens necrotic spot virus (INSV) ..... 241
- Plum pox virus (PPV) ..... 188, 201, 274
- population analysis ..... 302
- Potato leaf roll virus (PLRV) ..... 161, 165

- Potato mop top virus (PMTV) ..... 161–163, 165–167, 174
- Potato virus A (PVA) ..... 161–163, 165, 174
- Potato virus M (PVM) ..... 161, 162
- Potato virus S (PVS) ..... 161, 162, 174
- Potato virus V (PVV) ..... 161–163, 165, 166, 174
- Potato virus X (PVX) ..... 161–163, 165, 166, 174
- Potato virus Y (PVY)

  - PVY<sup>C</sup> ..... 166, 174
  - PVY<sup>E</sup> ..... 179, 182
  - PVY<sup>EU-NTN</sup> ..... 174
  - PVY<sup>NA-NTN</sup> / PVY<sup>NA-N</sup> ..... 174, 179, 182
  - PVY<sup>N-O</sup>, PVY<sup>N-Wilga</sup> ..... 174, 179, 182
  - PVY<sup>NTN</sup> ..... 179, 181, 182, 184
  - PVY<sup>O</sup> ..... 166, 174, 179, 182, 220, 221
  - recombinant variants ..... 174, 178, 179, 181, 182

- Raspberry bushy dwarf virus (RBDV) ..... 228, 229, 242
- Raspberry latent virus (RpLV) ..... 228, 242
- Raspberry leaf blotch virus (RLBV) ..... 228, 242
- Raspberry leaf mottle virus (RLMV) ..... 228, 229, 242
- Raspberry ringspot virus (RpRV) ..... 228, 230, 242
- Raspberry vein chlorosis virus (RVCV) ..... 242
- Rubus canadensis virus-1 (RuCV-1) ..... 243
- Rubus yellow net virus (RYNV) ..... 243
- Sowbane mosaic virus (Rubus chlorotic mottle virus) SoMV (RuCMV) ..... 243
- Strawberry chlorotic fleck virus (SCFV) ..... 243
- Strawberry crinkle virus (SCV) ..... 243
- Strawberry latent ringspot virus (SLRSV) ..... 243
- Strawberry mild yellow edge virus (SMYEV) ..... 243
- Strawberry mottle virus (SmoV) ..... 243
- Strawberry necrotic shock virus (SNSV) ..... 243
- Strawberry pallidosis-associated virus (SpaV) ..... 244
- Strawberry vein banding virus (SVBV) ..... 233, 236, 244
- Tobacco necrosis virus-D (TNV) ..... 244
- Tobacco rattle virus (TRV) ..... 161–163, 165, 167, 174, 244
- Tobacco ringspot virus (TRSV) ..... 244
- Tobacco streak virus (TSV) ..... 244
- Tomato black ring virus (TBRV) ..... 245
- Tomato spotted wilt virus (TSWV) ..... 174, 277, 278
- transmission by vectors

  - fungus-transmitted ..... 152
  - insect-transmitted ..... 114, 123, 162, 228, 230, 260, 301
  - insect-transmitted: aphid-transmitted ..... 208, 230
  - insect-transmitted: non-persistent transmission ..... 162
  - insect-transmitted: persistent transmission ..... 114, 162, 163
  - insect-transmitted: leafhopper-transmitted ..... 208
  - nematode-transmitted ..... 149

- Wheat dwarf virus (WDV) ..... 207, 208, 220, 221

**Z**

- Zebra chip (ZC) disease ..... 85–96