

# Development of a multiplex AmpliDet RNA for the simultaneous detection of *Potato leafroll virus* and *Potato virus Y* in potato tubers

M.M. Klerks \*, G.O.M. Leone, M. Verbeek, J.F.J.M. van den Heuvel,  
C.D. Schoen

*Plant Research International BV, PO Box 16, 6700 AA Wageningen, The Netherlands*

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## Abstract

A novel isothermal multiplex AmpliDet RNA system is described for the simultaneous amplification and detection of *Potato leafroll virus* (PLRV) and *Potato virus Y* (PVY) in seed potatoes. The risk of contamination by carry-over during diagnostic screening is eliminated by performing the reaction in a single closed tube. The viruses present in a sample are identified using differently coloured molecular beacons directed to a selected virus-specific sequence within the amplicon formed during amplification. With this system, as little as 10 fg of purified PLRV or PVY can be detected. The presence of both viruses in a sample is detected by the multiplex assay within a high range of virus concentrations. The reliability of the multiplex assay was compared with the enzyme-linked immunosorbent assay for detection of PLRV- or PVY-antigens in potato tubers. The multiplex assay detected clearly the viruses present originally in the potato tubers in all samples, demonstrating its potential for routine diagnostic work and high-throughput screening. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** AmpliDet RNA; Fluorescent probes; Homogeneous assays; Molecular beacons; Multiplex detection; NASBA; Potato viruses

## 1. Introduction

In plant diagnostic work, the use of molecular techniques has increased in recent years, enabling a sensitive detection of plant pathogens, necessary for virus certification programs. However, most of

these systems are based on detection of only one pathogen, while different plant pathogens may occur at the same time. The two most important viral potato pathogens *Potato leafroll virus* (PLRV; genus *Luteovirus*) and *Potato virus Y* (PVY; genus *Potyvirus*) occur as single or mixed infections in potato cultivation and are responsible for major economic losses world-wide. Until now, the presence of these viruses in potato cultivars has been tested mainly by enzyme-linked

\* Corresponding author. Tel.: +31-317-476026; fax: +31-317-410113.

E-mail address: m.m.klerks@plant.wag-ur.nl (M.M. Klerks).

immunosorbent assay (ELISA) screening on leaf extracts of sprouted tubers, since direct detection on dormant tubers is not reliable (Hill and Jackson, 1984; Spiegel and Martin, 1993). During recent years, molecular-based techniques like RT-PCR have been developed (e.g. Schoen et al., 1996; Singh and Singh, 1996; Singh et al., 2000) enabling the direct detection of PLRV and/or PVY in dormant tubers, thus reducing the time for completion dramatically. Recently, the viability of the nucleic acid sequence-based amplification (NASBA) system for the direct detection of PLRV in potato tubers was also demonstrated, enabling a more rapid and more sensitive detection than the currently used techniques (Leone et al., 1997). NASBA is an isothermal nucleic acid amplification method that amplifies RNA (viral RNA, mRNA, rRNA) specifically at one temperature (41°C) using oligonucleotide primers and three enzymes: AMV RT, RNase H and T7 RNA polymerase (Compton, 1991; Kievits et al., 1991), generating mainly target-specific, antisense single-stranded RNA amplicons.

To improve the applicability of this system in high-throughput settings, a molecular beacon probe (Tyagi and Kramer, 1996) was incorporated in the PLRV-specific NASBA system, enabling simultaneous real-time amplification and detection of specific amplicons in a sealed tube (Leone et al., 1998).

Molecular beacons are single-stranded DNA oligonucleotides, having a stem-loop structure (Fig. 1). The loop consists of a probe sequence complementary to its target sequence and is embedded in the arm sequences, which are unrelated

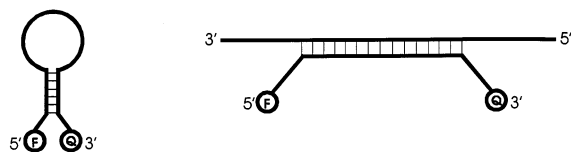


Fig. 1. Structure of molecular beacon with (right) and without (left) template. The molecular beacon in the closed state shows a typical stem-loop structure. DABCYL (Q) quenches the fluorescence of the fluorescent dye (F) when the molecular beacon is not hybridised to its target. When the loop-sequence is hybridised to the target-sequence, the stem structure is forced to open, and fluorescence is no longer quenched.

to the target sequence and complementary to each other, thus forming a double-stranded stem structure. A fluorophore is coupled to one arm of the stem, and the other arm is coupled with a non-fluorescent quencher. In the presence of a target sequence, the stem structure is forced to unfold by the energetically more favourable hybridisation of the probe sequence with its complementary target, enabling a molecular beacon to fluoresce. In the absence of a target sequence, the molecular beacon remains folded causing fluorescence to be quenched (Stryer, 1978). Fluorescence is measured in real-time during amplification enabling a simultaneous amplification and detection in a sealed tube. Moreover, molecular beacons can be synthesised carrying differently coloured fluorophores that allows the specific and simultaneous detection of multiple target sequences in one single tube (Tyagi et al., 1998). The above combination of molecular beacons and NASBA to enable a simultaneous amplification and detection in a closed tube was called AmpliDet RNA.

In this paper, the development of an isothermal multiplex AmpliDet RNA system is described for the simultaneous amplification and detection of PLRV and PVY in seed potatoes in a closed tube. Using this novel one-tube multiplex assay, we show that a separate gel-based detection of amplification products is no longer needed. This allows a more user-friendly diagnosis and eliminates the risk of carry-over contamination. The multiplex assay was tested on both purified PLRV and PVY and on potato tubers with a single or mixed infection of PLRV and PVY. A comparison of the performance of the multiplex AmpliDet RNA system with the current ELISA test on dormant potato tubers demonstrated the viability of the multiplex assay and its potential for routine diagnostic work.

## 2. Materials and methods

### 2.1. Plant material and virus isolates

PLRV and PVY virions were derived from the Plant Research International BV collection of plant viruses and used for optimisation of the

multiplex AmpliDet RNA system. From the same collection, potato tubers were derived infected with PLRV, PVY<sup>N</sup>, PVY<sup>O</sup>, PVY<sup>C</sup> or PVY<sup>NTN</sup>. Potato tubers infected with both PLRV and PVY were obtained by allowing 1 day old nymphs of *Myzus persicae* feeding on secondary PLRV-infected potato plants for an acquisition access period of 2 days. These aphids were then transferred to PVY-infected potato plants, with an inoculation pressure of 30 aphids/plant and left for an inoculation access period of 4 days. PLRV-infected potato plants were mechanically inoculated with PVY. After 4 months, the potato tubers were harvested and stored for 3 months at 4°C.

From the potato tubers infected with both PLRV and PVY, and from potato tubers infected with PLRV, PVY<sup>N</sup>, PVY<sup>O</sup>, PVY<sup>C</sup> or PVY<sup>NTN</sup>, four different tuber disks were taken per tuber using a potato peeler and a punch with a diameter of 17 mm. Disks were taken from the first and second peeling from both the rose-end and heel-end of the tuber, since PLRV is present mainly in the vascular tissues, and all vascular bundles are concentrated at both ends of a tuber, while PVY is supposed to be present in all potato cells (Weidemann, 1982). Using a Pollähne press, each tuber disk was homogenised separately in 1 ml of sample extraction buffer (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% PVP 44 000, 0.2% ovalbumin, 0.5% BSA and 0.05% Na-azide). From this sample, an aliquot of 100 µl was used for ELISA, and another aliquot of 100 µl was used for extracting total RNA using the RNeasy isolation kit following the manufacturer's instructions (Qiagen).

## 2.2. Primers and probes

For the amplification of viral RNA by NASBA, the virus-specific primers P1<sub>PLRV</sub> and P2<sub>PLRV</sub> for PLRV and P1<sub>PVY</sub> and P2<sub>PVY</sub> for PVY were designed within the coat protein (CP)-encoding region of each virus, enclosing a virus-specific sequence of 145 (PLRV) or 204 (PVY) nucleotides. The primers P1<sub>PLRV</sub> and P1<sub>PVY</sub> both consisted of a 3' target-complementary sequence and a 5' T7 polymerase recognition site sequence. Bi-

otinylated probes Bio<sub>PLRV</sub> and Bio<sub>PVY</sub> were developed to detect specific NASBA amplicons of PLRV and PVY, respectively, by chemiluminescent Northern blotting (see below). Sequences and locations of the primers and probes on the viral genomes are listed in Table 1.

## 2.3. Molecular beacons

For real-time detection of PLRV and PVY amplicons, virus-specific molecular beacons were designed. Each molecular beacon carried a 5' and 3' arm sequence of six nucleotides and a sequence of 20 (PLRV) or 19 (PVY) nucleotides complementary to the specific amplicon. The arm sequences of the molecular beacons formed a double-stranded structure at 41°C to avoid fluorescence from non-hybridised molecular beacons and were designed to hybridise with each other and not with the amplicon. The molecular beacon (MB<sub>PLRV</sub>) used to detect PLRV amplicons was coupled with fluorescein (FAM) and the non-fluorescent quencher DABCYL (4-[4'-dimethylaminophenylazo]-benzoic acid). For detection of PVY amplicons, the molecular beacon (MB<sub>PVY</sub>) was used, to which tetrachloro-6-carboxyfluorescein (TET) and the non-fluorescent quencher DABCYL were coupled. The emission maximum of the fluorescent labels FAM (530 nm) and TET (538 nm) can be discriminated by using an ABI Prism 7700 thermal cycler (Perkin Elmer). The sequences of both molecular beacons are listed in Table 1.

## 2.4. NASBA

For each reaction, the NASBA was carried out as described previously (Compton, 1991; Kievits et al., 1991). The NASBA reaction mix consisted of 4 µl of 5 × NASBA-buffer (200 mM Tris-HCl, pH 8.5, 60 mM MgCl<sub>2</sub>, 350 mM KCl, 2.5 mM DTT, 5 mM of each dNTP, 10 mM each of ATP, UTP and CTP, 7.5 mM GTP and 2.5 mM ITP), 4 µl of 5 × primer mix (75% DMSO and 1 µM of each primer) and 2 µl of RNase-free water per reaction. In case of a multiplex NASBA reaction, the primer mix consisted of two different target-specific primer pairs. A volume of 5 µl of

Table 1

Sequences of primers, biotinylated detection probes and molecular beacons for NASBA and AmpliDet RNA of PLRV and PVY<sup>a</sup>

Oligonucleotide primer	Sequence	Length (nt)	Positions	Type
PLRV primer 1 (P1 <sub>PLRV</sub> )	5'- <u>AAT TCT AAT ACG ACT CAC TAT AGG GAG GTA</u> TCA TCC GCG CTT GAT A-3'	46	3818–3839	Antisense
PLRV primer 2 (P2 <sub>PLRV</sub> )	5'-CGT CAG CGA GGC CTC TTC CA-3'	20	3944–3963	Sense
PLRV detection probe (Bio <sub>PLRV</sub> )	5'-B-GCA AAG TAT CAT CCC TCC AG-3'	20	4003–4022	Sense
PLRV molecular beacon (MB <sub>PLRV</sub> )	5'-FAM- <u>CCA AGC GCA AAG TAT CAT CCC TCC AGG</u> <b>CTT GG-DABCYL-3'</b>	32	4003–4022	Sense
PVY primer 1 (P1 <sub>PVY</sub> )	5'- <u>AAT TCT AAT ACG ACT CAC TAT AGG GGA ATT</u> AAA CCA TAT CGT GGC A-3'	46	9114–9134	Antisense
PVY primer 2 (P2 <sub>PVY</sub> )	5'-ACC TCG CCA AAT GTC AAC GGA-3'	21	8942–8962	Sense
PVY detection probe (Bio <sub>PVY</sub> )	5'-B-TGG CAC ATT TCT CAG ATG TTG CAG A-3'	25	9051–9075	Sense
PVY molecular beacon (MB <sub>PVY</sub> )	5'-TET- <u>CGA CGT TGG CAC ATT TCT CAG ATG TAC</u> <b>GTC G-DABCYL-3'</b>	31	9051–9069	Sense

<sup>a</sup> B, biotin; FAM, fluorescein; TET, tetrachloro-6-carboxyfluorescein; D, DABCYL. The underlined parts indicate the T7-promoter recognition-site sequence, and the bold sequences of the molecular beacons form the stem structures.

sample solution (purified virus, tuber extract) or water (negative control) was added to the NASBA reaction mix. The reactions were then pre-incubated at 65°C directly followed by 41°C for 5 min each. The NASBA reaction was started by adding 5 µl of enzyme mix (375 mM sorbitol, 2.1 µg of BSA, 0.08 U of RNase H, 32 U of T7 RNA polymerase and 6.4 U of AMV-reverse transcriptase) per reaction, incubated for 5 min at 41°C, then shortly centrifuged followed by incubation for 90 min at 41°C.

Real-time amplification and detection using AmpliDet RNA were carried out as described above, except that the 2 µl of RNase-free water were replaced by 1 µl of 8 µM ROX [5-(and -6)-carboxy-X-rhodamine] and 1 µl of the molecular beacon solution, consisting of 18 ng of MB<sub>PLRV</sub>/µl, 9 ng of MB<sub>PVY</sub>/µl, or both. The ROX and molecular beacons were all dissolved in RNase free water. The 90 min incubation at 41°C was performed in an ABI Prism 7700 thermal cycler, and the emission spectrum of either label (FAM and TET) was measured in real-time every 2 min.

### 2.5. Detection of NASBA amplicons by Northern blotting and enhanced chemiluminescence (ECL)

NASBA products were analysed by electrophoresis using a 1% pronarose gel containing 0.5 µg/ml of EtBr. Gels were run at 100 V for 15 min in buffer containing 40 mM Tris-acetate and 1 mM EDTA, pH 8.0 (1 × TAE). The gel was blotted onto a Z-probe nylon membrane in 0.3 M NaCl and 30 mM Na-citrate (2 × SSC) solution for 20 min. Nucleic acids were cross-linked onto the Z-probe by UV exposure (365 nm) for 2 min. Hybridisation of the amplicon-specific biotinylated probe solution (3 µM) to the NASBA products occurred at 50°C for 30–60 min in hybridisation-mix (5 × SSC, 7% SDS, 20 mM Na-phosphate, pH 6.7, 10 × Denhardt's solution). The blots were washed twice with 3 × SSC, 1% SDS at 50°C for 5 min and once with buffer containing 0.1% SDS with 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.36 M NaCl and 2 mM EDTA (2 × SSPE) at room temperature for 10 min. All subsequent steps were carried out at room temperature. The blot was incubated for 30 min with 2 µl of streptavidin/per-

oxidase conjugate in  $5 \times$  SSPE with 0.5% SDS, followed by washing three times with  $2 \times$  SSPE with 0.1% SDS for 1 min (twice) and 10 min, respectively. Subsequently the blots were washed twice with  $2 \times$  SSPE for 2 min, incubated in substrate solution (ECL detection reagents, Amersham Pharmacia Biotech) for 60 s and exposed to X-ray films.

### 3. Results

#### 3.1. Development of AmpliDet RNA assays for PLRV and PVY

For the development of a separate PLRV- or PVY-specific NASBA, CP-specific primer sets were designed for both viruses, and the sensitivity of both NASBA systems was determined by testing a 10-fold dilution series from 100 pg down to 10 fg of purified PLRV or PVY as template. Virus-specific amplicons were detected by Northern blotting and ECL using the biotinylated probes  $\text{Bio}_{\text{PLRV}}$  and  $\text{Bio}_{\text{PVY}}$ , respectively (Fig. 2). Using this gel-based system, a sensitivity of at least 10 fg of each virus was reached.

To assess whether a similar level of sensitivity could be obtained in a gel-free system, the PLRV- and PVY-specific molecular beacons  $\text{MB}_{\text{PLRV}}$  and  $\text{MB}_{\text{PVY}}$  were tested in NASBA by amplification of 10-fold dilution series from 100 pg down to 10 fg of purified PLRV or PVY as template. The increase of fluorescence due to hybridisation of the

molecular beacons  $\text{MB}_{\text{PLRV}}$  (FAM) or  $\text{MB}_{\text{PVY}}$  (TET) to specific amplicons was measured and plotted as function of time in a two-dimensional graph (Fig. 3). To ensure a similar level of fluorescent signal, the concentration of the molecular beacons  $\text{MB}_{\text{PLRV}}$  and  $\text{MB}_{\text{PVY}}$  was set on 18 and 9 ng, respectively per reaction. The results show that for both AmpliDet RNA systems a sensitivity of at least 10 fg of purified virus per reaction was obtained, thus being as sensitive as the separate NASBA amplification and gel-based amplicon detection systems.

#### 3.2. Development of a multiplex AmpliDet RNA for the detection of PLRV and PVY

The previously designed primers and molecular beacons were combined to enable an AmpliDet RNA system for the simultaneous detection of PLRV and PVY in one closed tube. The PLRV- and PVY-specific primers and molecular beacons were designed in a way to avoid any interference with each other and to maintain target specificity during a multiplex reaction.

To determine whether the viruses could be distinguished efficiently from each other by this multiplex assay, three separate reactions were carried out. Each reaction contained both molecular beacons  $\text{MB}_{\text{PLRV}}$  and  $\text{MB}_{\text{PVY}}$  and 1 ng of purified PLRV, 1 ng of purified PVY, or water. The fluorescence of the molecular beacons complementary to the expected amplicon clearly permitted the detection of the virus originally present in each reaction tube (Fig. 4). These results demonstrate that each molecular beacon binds specifically to its complementary target amplicon, and the emission spectra of both molecular beacons can be easily distinguished from each other in this multiplex AmpliDet RNA system.

#### 3.3. Sensitivity of the multiplex AmpliDet RNA assay

To determine whether both PLRV and PVY could be detected simultaneously in a single reaction, the ability of the multiplex assay to amplify and detect PLRV and PVY was evaluated when both were present in the same sample.

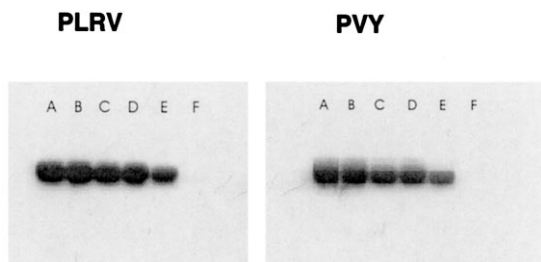


Fig. 2. Detection of PLRV (left) and PVY (right) amplicons using Northern blotting and ECL-detection. Two dilution series with each containing six samples were tested. The samples of both dilution series contained 100 pg (A), 10 pg (B), 1 pg (C), 100 fg (D), 10 fg (E) or no (F) purified virus.

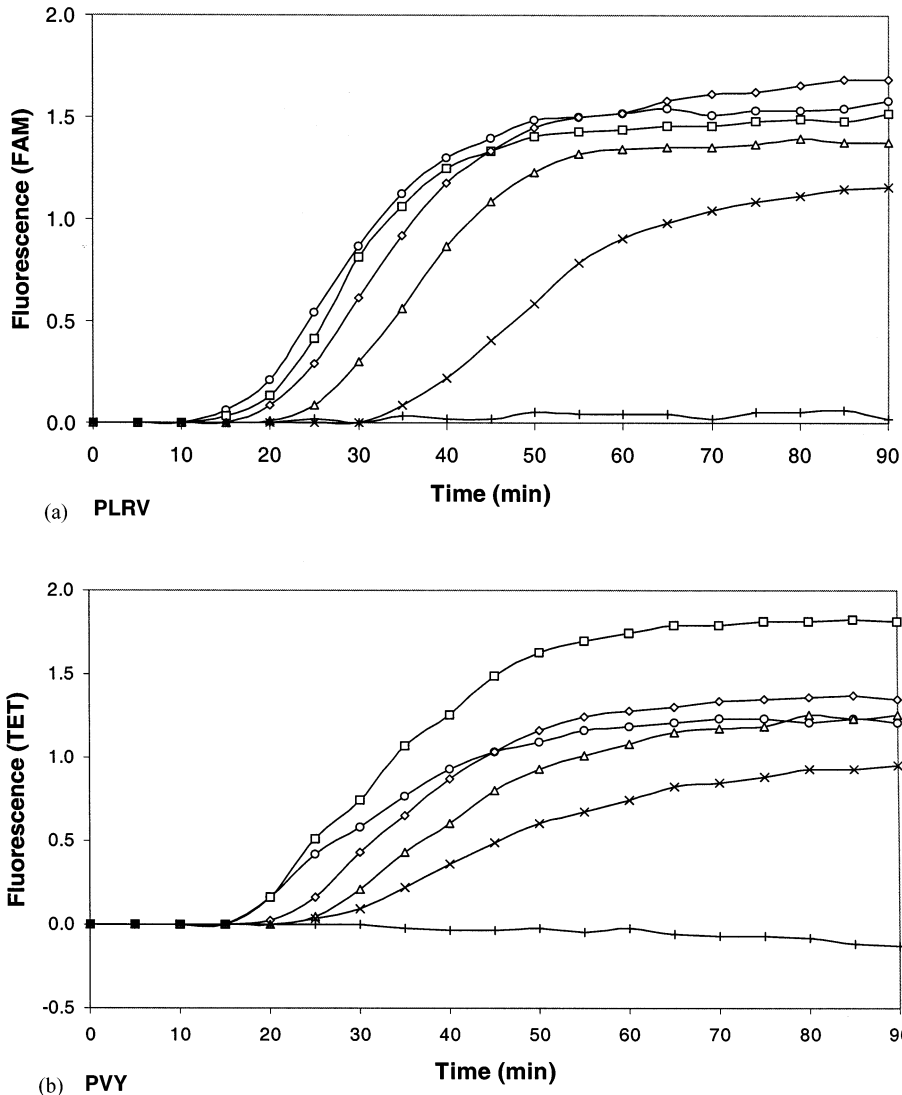


Fig. 3. Real-time detection of dilution series of either purified PLRV or PVY: Two dilution series each containing 100 pg (○), 10 pg (□), 1 pg (◇), 100 fg (△) or 10 fg (X) of either purified virus PLRV or PVY, and one negative control (+) sample containing water. (a) FAM fluorescence increase of the purified PLRV dilution series. (b) TET fluorescence increase of purified PVY dilution series.

Each concentration of a 10-fold dilution of purified PVY ranging from 1 ng to 100 fg was tested with a 10-fold dilution of purified PLRV ranging from 1 ng to 100 fg. The results of the multiplex assay performed on the dilution series of PVY containing 1 ng of purified PLRV are shown (Fig. 5). They indicate that the RNA of PVY was efficiently amplified and detected in the

presence of a larger amount of PLRV. The same results were obtained in all other cases (results not shown). A sensitivity of at least 100 fg of purified PVY and 1 pg of purified PLRV was obtained in the presence of at least 1 ng of the other virus. This indicated only a limited loss of sensitivity when both viruses were present in the test, while no reduction in sensitivity was observed if only

one virus was present (Fig. 3). This showed the ability of the multiplex AmpliDet RNA to amplify and detect PLRV and PVY simultaneously in a wide range of target concentrations.

#### 3.4. Simultaneous detection of PLRV and PVY in potato tubers using the multiplex AmpliDet RNA

To test the multiplex assay under simulated real-world conditions, infected potato tubers, stored for 3 months at 4°C, were used as a source for both viruses. In total, 24 potato tubers were tested (four healthy, five PLRV-, nine PVY- and six mix-infected tubers). From each tuber, four different samples were collected: two disks from the first and second peeling of either end. Each tuber disk was homogenised in sample extraction buffer using a Pollähne press. Total RNA was extracted separately from each homogenised tuber disk and tested in the multiplex AmpliDet RNA system. For confirmation, all tuber disks were also tested using ELISA. For ELISA and the multiplex assay, the samples were considered positive in case the value given by spectrophotometric detection (ELISA) or fluorescence intensity (multiplex assay) was above the calculated threshold of the average of values of healthy tubers plus four times the standard deviation of the sum of values of healthy tubers ( $P = 0.99$ ).

The results, listed in Table 2, showed that in all cases, the multiplex AmpliDet RNA assay de-

tected the viruses when present in the single- or mix-infected potato tubers. All healthy tubers gave negative results. Using ELISA, some PVY-infected samples gave false negative results (second peeling of rose-end of tuber 1 and 2, second peeling of heel-end of tuber 3 and 16, and all but the first peeling of rose-end of tuber 18) indicating a concentration of virus present in these samples that was too low to be detected by this method. The results of this small-scale test demonstrated that the multiplex AmpliDet RNA provides a more reliable detection of PLRV and/or PVY in dormant potato tubers relative to ELISA.

#### 4. Discussion

The novel isothermal multiplex AmpliDet RNA system described in this paper can be a powerful tool for rapid and simultaneous detection of low concentrations of PLRV and PVY in one tube. Introduction of target specific molecular beacons to the NASBA system enables simultaneous amplification and detection of RNA molecules in a closed tube format, eliminating the risk of carry-over contamination for amplicon analysis. The presence of PLRV and/or PVY in tubers with a low virus titre could be reliably detected by this multiplex assay irrespective of the sampling tissue (first or second peeling), thus demonstrating the potential of the multiplex AmpliDet RNA system.

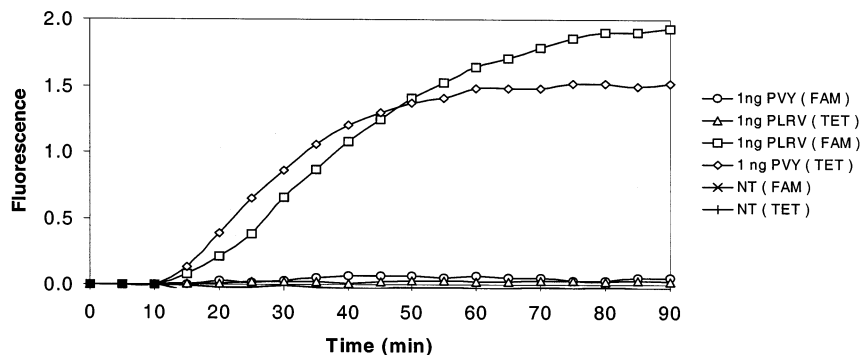
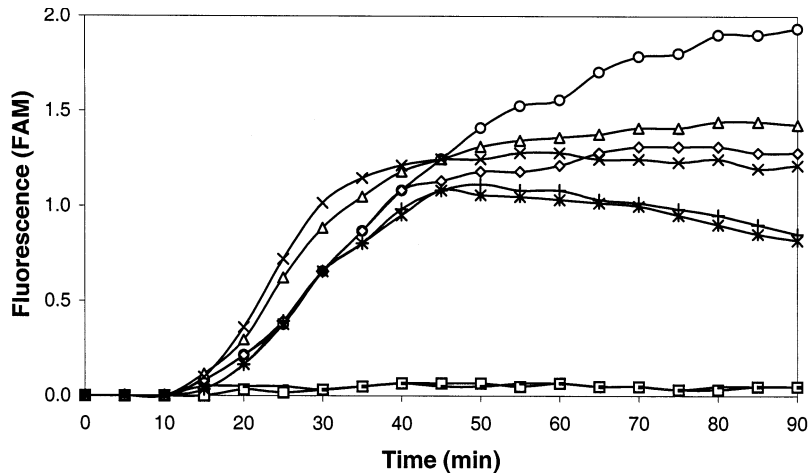
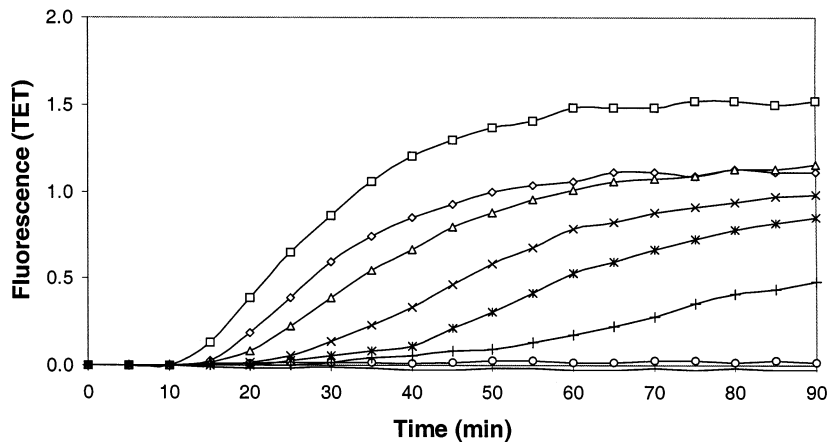


Fig. 4. Real-time amplification and detection of purified virus PLRV and PVY using the multiplex AmpliDet RNA system. Three samples are tested, containing either 1 ng of purified virus PLRV, 1 ng of purified virus PVY or water as a negative control. All samples contained both molecular beacons specific for PLRV amplicon and PVY amplicon. The FAM and TET fluorescence increase is independently measured in each sample and plotted separately on the graph.



(a) PLRV



(b) PVY

	○	□	◇	△	×	✱	+	-
PLRV	1 ng		1 ng	1 ng	1 ng	1 ng	1 ng	NT
PVY		1 ng	1 ng	100 pg	10 pg	1 pg	100 fg	NT

Fig. 5. Simultaneous detection of purified virus PLRV and PVY using the multiplex AmpliDet RNA system. Eight samples were tested containing 1 ng of purified PLRV and either 1 ng, 100 pg, 10 pg, 1 pg, 100 fg or no purified PVY, or only 1 ng purified PVY, and one sample contained water serving as a negative control (-). All samples were tested using the multiplex assay for detection of PLRV and PVY. FAM and TET fluorescence due to hybridisation of MB<sub>PLRV</sub> to PLRV and MB<sub>PVY</sub> to PVY amplicons was measured in real time in each tube (Fig. 5a and b, respectively). In the legend, the amount of purified virus is shown together with its corresponding mark.

For application for routine diagnostic work, a large-scale evaluation might be necessary to compare its efficiency with other molecular biology-

based techniques like RT-PCR and with the currently used ELISA.

The main advantage of the developed multiplex

AmpliDet RNA over published multiplex RT-PCR systems for simultaneous detection of PLRV and PVY (Singh et al., 1996; Singh et al. 2000) is that it allows a gel-free detection of the amplicons. Also, with regard to its isothermal nature for both real-time and end-point measurements, AmpliDet RNA offers an advantage in comparison with RT-PCR or PCR diagnostic systems containing a molecular beacon-like detection probe (Giesendorf et al., 1998; Whitcombe et al., 1999). In addition, NASBA has been shown to be more sensitive than RT-PCR techniques (e.g. Goossens et al., 2000; Lunel et al., 1999).

The multiplex AmpliDet RNA system also provides the possibility of discriminating false negative from true negative results within each reaction. Introduction of an internal positive control (IPC) in each reaction might be accomplished by incorporating a low concentration of the IPC having the same sequence as the target RNA, except for a randomly shuffled sequence of three to 20 nucleotides (Van Gemen et al., 1993). A differently coloured molecular beacon directed to the specific IPC sequence could then be added to detect the IPC amplicon. Such an assay will always show a positive signal from either the IPC

Table 2

Twenty-four tubers, of which four are healthy (tuber 21–24), 14 single (PLRV; tuber 7–11, PVY; tuber 12–20) and six mix infected (tuber 1–6), all tested in both ELISA and the multiplex assay for the presence of PLRV and/or PVY<sup>a</sup>

	PLRV detection								PVY detection							
	ELISA				Multiplex assay				ELISA				Multiplex assay			
	R1	R2	H1	H2	R1	R2	H1	H2	R1	R2	H1	H2	R1	R2	H1	H2
Tuber1/Mix	+	++	+	++	+	+	+	+	++	–	++	+	+	+	+	+
Tuber2/Mix	+	++	+	++	+	+	+	+	++	–	+	+	+	+	+	+
Tuber3/Mix	+	++	+	++	+	+	+	+	++	+	++	–	+	+	+	+
Tuber4/Mix	+	++	+	++	+	+	+	+	++	+	++	+	+	+	+	+
Tuber5/Mix	+	++	+	++	+	+	+	+	++	+	++	+	+	+	+	+
Tuber6/Mix	+	++	+	++	+	+	+	+	++	+	++	+	+	+	+	+
Tuber7/PLRV	+	++	+	++	+	+	+	+	–	–	–	–	–	–	–	–
Tuber8/PLRV	+	++	+	++	+	+	+	+	–	–	–	–	–	–	–	–
Tuber9/PLRV	+	++	+	++	+	+	+	+	–	–	–	–	–	–	–	–
Tuber10/PLRV	+	++	+	++	+	+	+	+	–	–	–	–	–	–	–	–
Tuber11/PLRV	+	++	+	++	+	+	+	+	–	–	–	–	–	–	–	–
Tuber12/PVY <sup>N</sup>	–	–	–	–	–	–	–	–	++	+	++	+	+	+	+	+
Tuber13/PVY <sup>N</sup>	–	–	–	–	–	–	–	–	++	+	++	+	+	+	+	+
Tuber14/PVY <sup>N</sup>	–	–	–	–	–	–	–	–	++	+	++	+	+	+	+	+
Tuber15/PVY <sup>N</sup>	–	–	–	–	–	–	–	–	++	+	++	+	+	+	+	+
Tuber16/PVY <sup>N</sup>	–	–	–	–	–	–	–	–	+	+	++	–	+	+	+	+
Tuber17/PVY <sup>N</sup>	–	–	–	–	–	–	–	–	++	+	++	+	+	+	+	+
Tuber18/PVY <sup>C</sup>	–	–	–	–	–	–	–	–	++	–	–	–	+	+	+	+
Tuber19/PVY <sup>O</sup>	–	–	–	–	–	–	–	–	+	++	+	++	+	+	+	+
Tuber20/PVY <sup>NTN</sup>	–	–	–	–	–	–	–	–	++	+	+	++	+	+	+	+
Tuber21/healthy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tuber22/healthy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tuber23/healthy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tuber24/healthy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

<sup>a</sup> From each potato tuber, four samples were tested, from both rose-(R) and heel-end (H), a first peeling (R1, H1) and a second peeling (R2, H2). For ELISA, (++) indicates a higher, and (+) a lower amount of virus present in the sample compared with the other peeling from the same rose- or heel-end of the same potato. A negative result is indicated by (–) for both ELISA and the multiplex assay; as for the multiplex assay, a positive result is marked with (+).

or the virus present in the sample. If none of the signals is visible the reaction did not take place, suggesting a false negative result.

The real-time measurements as described in this paper are commonly used for optimising a one-tube amplification and detection system but are not necessary for discriminating positive and negative reactions during routine diagnostic screenings. Since NASBA mainly generates single-stranded RNA (Compton, 1991; Kievits et al., 1991), fluorescence from hybridised molecular beacons can be measured after amplification without denaturing the amplicons. This can be seen in Fig. 3, where all positive samples show a high fluorescent signal at the final measure point at 90 min. compared to the negative sample. This enables end-point fluorescence reading, providing the possibility of using a relatively cheap thermostatic fluorimeter.

In conclusion, the multiplex assay described in this paper can be a good option for sensitive, user-friendly and reliable tools for plant pathogen diagnostic work. The detection of PLRV and PVY in potato tubers was used as a model system due to its economical importance and the fact that both viruses can occur concurrently in natural infections. The multiplex AmpliDet RNA has shown to be very suitable for the reliable detection of PLRV and PVY in seed potato tubers.

The multiplex AmpliDet RNA system is potentially very suitable also for large-scale testing due to its isothermal nature and the possibility of end-point fluorescence readings, although cost versus benefit aspects need to be better defined for seed potato certification in relation to RT-PCR and ELISA. Especially when ELISA is not sufficient, direct fields of application are screening for quarantine organisms and pathogen screening of very-high-quality propagative material like in-vitro cultured plants and/or mini- and micro-tubers.

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