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# Development of a multiplex AmpliDet RNA assay for simultaneous detection and typing of *potato virus Y* isolates

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## Abstract

A multiplex AmpliDet RNA assay was developed for the specific detection of *potato virus Y* (PVY), and for the differentiation of the PVY<sup>N</sup>, PVY<sup>O/C</sup> strains and the tuber necrotic isolates (PVY<sup>NTN</sup>). The assay is based on the generic amplification of a region within the coat protein coding region of all known PVY isolates by nucleic acid sequence-based amplification (NASBA<sup>TM</sup>) and strain-specific detection by molecular beacons. PVY<sup>NTN</sup>-specific diagnosis is achieved by detecting PVY<sup>N</sup> and PVY<sup>O</sup>-specific sequences flanking a recombination site that is associated with the tuber necrotic pathotype. The assay exhibited good specificity toward the various PVY strains in both single and mixed infections. The technique was validated by the use of 47 PVY isolates originating from six countries. The results of the AmpliDet RNA assay were identical or consistent with those of biological characterisation in the decisive majority of cases. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Potato virus Y; AmpliDet RNA; Molecular beacons; NASBA; Strain differentiation

## 1. Introduction

*Potato virus Y* (PVY) is a widespread deleterious viral pathogen, which causes severe losses in potato, pepper, tomato and tobacco crops. On the basis of symptoms in indicator plants, the PVY isolates infecting potato were classified into three strains, PVY<sup>N</sup>, PVY<sup>O</sup> and PVY<sup>C</sup> (Munro, 1955; Le Romancer et al., 1994; De Bokx and Huttinga, 1981). A fourth group of isolates, resembling the

members of the PVY<sup>N</sup> strain, were reported to cause potato tuber necrotic ringspot disease (Beczner et al., 1984) and were designated PVY<sup>NTN</sup> (necrotic, tuber necrosis). These isolates were also stated to break the resistance of potato cultivars that were immune to PVY or possessed high levels of field resistance (van den Heuvel et al., 1994).

The three strains of PVY can be separated by serological methods. PVY<sup>NTN</sup> isolates, however, cannot be distinguished from PVY<sup>N</sup> by serology or by symptomatology on tobacco indicator plants (van den Heuvel et al., 1994; Weidemann

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and Maiss, 1996). To control successfully the outbreaks of PVY<sup>NTN</sup> infection, differential diagnosis of these isolates is pivotal. Biological testing, i.e. monitoring of the development of tuber necrotic symptoms in susceptible potato cultivars, is time-consuming and not entirely reliable since the severity of the symptoms depends strongly on environmental conditions, such as temperature and humidity (Le Romancer et al., 1994). A PCR-based assay has also been developed to detect PVY<sup>NTN</sup>-specific nucleic acid motifs within the P1 gene region (Weilguny and Singh, 1998). However, as more sequence data became available, the target nucleic acid motifs were also found in isolates that were not tuber necrotic.

AmpliDet RNA is a novel diagnostic procedure based on the combination of nucleic acid sequence-based amplification (NASBA<sup>TM</sup>) and detection by molecular beacons (MBs; Leone et al., 1998). NASBA is an isothermal amplification method, which uses single-stranded RNA as template, single-stranded complementary RNA being amplified in the course of the reaction (Compton, 1991; Kievits et al., 1991). It is particularly suited for the detection of RNA viruses, because there is no need for a separate reverse transcription step. Furthermore, the amplification power of NASBA has been reported to be comparable to, or sometimes even higher than that of PCR (Lunel et al., 1999).

Molecular beacons belong to a new generation of hybridisation probes; they fluoresce upon binding to the target, permitting real-time monitoring of the amplification reaction (Tyagi and Kramer, 1996). They possess a stem-and-loop structure and carry a fluorescent dye at the 5' end and a quencher group at the 3' end. In the absence of a hybridisation target, these probes are in a closed state. Upon excitation, direct energy transfer takes place between the fluorophore and the quencher; consequently, no light is emitted. However, when they hybridise to their target, the fluorophore and the quencher are separated and the dye emits light upon excitation. Fluorescence of the samples is recorded during the amplification, and the accumulation of the product can be followed in real-time. The use of different fluorophores attached to different molecular beacons allows multiplex detection in one reaction (Tyagi et al., 1998).

The present paper describes the development of a multiplex AmpliDet RNA assay for real-time detection and differentiation of isolates of PVY<sup>N</sup>, PVY<sup>O/C</sup> and PVY<sup>NTN</sup> in a single reaction. Generic NASBA amplification was combined with detection by molecular beacons specific for the PVY<sup>N</sup> and PVY<sup>O/C</sup> strains. The coat protein (CP) coding region of PVY<sup>NTN</sup> is chimerical, presumably in consequence of a recombination event between a PVY<sup>N</sup> and a PVY<sup>O</sup> genome. The differentiation of PVY<sup>NTN</sup> isolates was based on the detection of this rearrangement by molecular beacons specific for the parental sequences flanking the recombination site. Previously, it was found that this rearrangement correlates perfectly with the tuber necrotic pathotype (Revers et al., 1996).

The specificity and the sensitivity of the method were evaluated with viral isolates characterised biologically. The method developed was validated by testing a collection of 47 PVY isolates from six countries.

## 2. Materials and methods

### 2.1. Virus isolates

The PVY isolates used in this study are listed in Table 1. Twelve isolates originated from the Plant Research International B.V. collection (Nos. 1–12). Sixteen PVY isolates (Nos. 13–28) were kindly provided by Dr S.L. Nielsen; these represented Danish isolates mostly, but also contained PVY isolates from Lebanon, Romania and Hungary. PVY<sup>O</sup> (U09509) was a gift from Dr R.P. Singh. The isolates were transferred to and maintained on *Nicotiana tabacum* plants. A collection of 18 PVY<sup>NTN</sup> isolates, maintained on *in vitro* potato plant cultures, were kindly provided by Dr I. Wolf (Nos. 30–47). Biological characterisation of the isolates was carried out previously (Singh and Singh, 1996; Wolf and Horváth, 2001; Nielsen, personal communication).

### 2.2. Nucleic acid sequence analysis

The coat protein coding nucleotide sequences of 40 PVY isolates were retrieved from GenBank

Table 1  
Comparison of the results of AmpliDet RNA typing with those of biological characterisation

| Number | Isolate code | Country of origin | Biological strain | AmpliDet |
|--------|--------------|-------------------|-------------------|----------|
| 1      | 751          | Netherlands       | NTN               | NTN      |
| 2      | PRI_NTN1     | Netherlands       | NTN               | NTN      |
| 3      | Nicola 250   | Netherlands       | NTN               | NTN      |
| 4      | Kondor 417   | Netherlands       | NTN               | NTN      |
| 5      | Liseta 67    | Netherlands       | NTN               | NTN      |
| 6      | Premiere100  | Netherlands       | NTN               | NTN      |
| 7      | 601          | Netherlands       | N                 | N        |
| 8      | 602          | Netherlands       | N                 | N        |
| 9      | 603          | Netherlands       | N                 | N        |
| 10     | 604          | Netherlands       | N                 | N        |
| 11     | 606          | Netherlands       | N                 | N        |
| 12     | 607          | Netherlands       | N                 | N        |
| 13     | 53–46        | Denmark           | NTN               | NTN      |
| 14     | 53–47        | Denmark           | NTN               | N        |
| 15     | NTN-Attica   | Hungary           | NTN               | NTN      |
| 16     | NTN-Lb       | Lebanon           | NTN               | NTN      |
| 17     | NTN-R        | Romania           | NTN               | NTN      |
| 18     | 53–17        | Denmark           | N                 | N        |
| 19     | 53–24        | Denmark           | N                 | N        |
| 20     | 53–25        | Denmark           | N                 | N        |
| 21     | 53–15        | Denmark           | O                 | O/C      |
| 22     | 53–16        | Denmark           | O                 | O/C      |
| 23     | 53–28        | Denmark           | O                 | O/C      |
| 24     | 53–19        | Denmark           | O                 | O/C      |
| 25     | 53–31        | Denmark           | O                 | O/C      |
| 26     | 53–45        | Denmark           | O                 | O/C      |
| 27     | 53–38        | Denmark           | C                 | O/C      |
| 28     | D-40         | Denmark           | C                 | O/C      |
| 29     | U09509       | Canada            | O                 | O/C      |
| 30     | KE 1         | Hungary           | NTN               | NTN      |
| 31     | KE 3         | Hungary           | NTN               | NTN      |
| 32     | KE 9         | Hungary           | NTN               | NTN      |
| 33     | KE 17        | Hungary           | NTN               | NTN      |
| 34     | KE 20        | Hungary           | NTN               | NTN      |
| 35     | KA 2         | Hungary           | NTN               | NTN      |
| 36     | KA 3         | Hungary           | NTN               | NTN      |
| 37     | KA 5         | Hungary           | NTN               | NTN      |
| 38     | KA 6         | Hungary           | NTN               | NTN      |
| 39     | KA 8         | Hungary           | NTN               | NTN      |
| 40     | KA 9         | Hungary           | NTN               | NTN      |
| 41     | D 1          | Hungary           | NTN               | NTN      |
| 42     | D 2          | Hungary           | NTN               | NTN      |
| 43     | D 3          | Hungary           | NTN               | NTN      |
| 44     | D 5          | Hungary           | NTN               | NTN      |
| 45     | D 6          | Hungary           | NTN               | NTN      |
| 46     | D 9          | Hungary           | NTN               | NTN      |
| 47     | D 10         | Hungary           | NTN               | NTN      |

Table 2

Sequences and positions of the oligonucleotide primers, probes and molecular beacons used in this study

| Name      | Sequence   | Position  |
|-----------|--|-----------|
| P1A-3'    | <i>AATTCTAATACGACTCACTATAGGGGAAAAGTCGAGGTTGGGCTGA</i>      | 9250–9270 |
| P2D-5'    | TCTCAGATGTTGCAGAAGCGTA                                     | 9059–9080 |
| PVY-T7-5' | <i>AATTCTAATACGACTCACTATAGGGGGCTTATGGTTTGGTGCAT</i>        | 8911–8930 |
| RNA-3'    | GTACAGGAAAAAGCCAAAATACTTA                                  | 9419–9442 |
| P-PVY     | Biotin—CACGAACACCAGTGAGGGCTAG                              | 9191–9212 |
| MB-N1     | Texas Red— <u>GCAACCTGGAAGTTTGGCTCGSTATGGGTTGC</u> —DABCYL | 9147–9166 |
| MB-N2     | HEX— <u>CGGTCCTAGAGAGGCACACATTCAAATGGGACCG</u> —DABCYL     | 9209–9231 |
| MB-O1     | TAMRA— <u>GCAGCAGGTPTAGCGCGPTATGCCTTIGCTGC</u> —DABCYL     | 9151–9172 |
| MB-O2     | FAM— <u>CGACGTTGAGGTCACATCACGAACACACGTCG</u> —DABCYL       | 9180–9199 |

T7 polymerase recognition sites are indicated by italicised letters. Sense primers have the suffix 5', and the antisense ones the suffix 3'. The sequences forming the stem structure of the molecular beacons are underlined. Positions are given relative to PVY<sup>NTN</sup>-Hu genomic sequence (M95491).

and were aligned by ClustalW (Thompson et al., 1994). The sequences of PVY viruses isolated from non-potato hosts were excluded from this analysis, unless they were biologically characterised and classified into PVY<sup>NTN</sup> or into one or other of the three strains. The sequences were clustered by using the PHYLIP package 3.5 (Felsenstein, 1993). On the basis of the longest overlapping region of the alignment (full-length coat protein coding sequences except for the 5' eight nucleotides), pairwise distances were calculated by means of DNADIST, using the bootstrap option with 100 replicates. Clustering was carried out from the distance matrices with NEIGHBOR. A consensus tree was generated by CONSENSE and was visualised by TREEVIEW (Page, 1996). As a result of the analysis, an unrooted tree was generated, which depicts the similarity-based grouping between the sequences, but does not infer phylogenetic relationships.

### 2.3. Design of oligonucleotide primers for NASBA

Target sequences for two reverse (P1), and three forward (P2) PVY-specific oligonucleotide primers flanking the diagnostic region were selected. Their performance in NASBA was tested in all possible combinations, using serial dilution of RNAs, representing the four viral types, as template. The amplicon RNAs were detected with a generic, biotin-labelled probe by northern hybridisation. Primer combination P1A-3' + P2D-5'

(Table 2) was found to be the most effective and was selected for use in the later experiments.

### 2.4. Molecular beacon design

Formation of the required stem-and-loop structure of the molecular beacons and the stability of the stem was predicted by the MFold program for single-stranded DNA (SantaLucia, 1998). The strength of the interaction between the RNA amplicon and the DNA MB probe was predicted by using the OligoWalk program (Mathews et al., 1999).

The target regions of the strain-specific molecular beacons (Fig. 2) were selected to contain a minimum of three discriminatory nucleotides in order to prevent duplex formation with non-cognate amplicon RNAs. The lengths of the hybridising sequences of the molecular beacons were chosen so that the molecular beacons could be expected to tolerate one mismatch. To compensate for known sequence polymorphisms present in the MB binding sites, the nucleotide compositions of two molecular beacons were modified. The target area of MB-O1 was the most divergent, therefore two universal pyrimidine analogues (Lin and Brown, 1989) were incorporated to compensate for C/T polymorphisms at two positions. The P analogues form base pairs with the corresponding A and G nucleotides in the amplicon RNA, which is complementary to the

PVY genomic RNA. The second MB (MB-N1) was degenerated in one position.

The fluorescent dyes (FAM, HEX, TAMRA and Texas Red) coupled to the molecular beacons were selected in a way that their excitation and emission spectra should overlap as little as possible. All the molecular beacons carried DABCYL as a quencher moiety. The sequences and positions of the molecular beacons are shown in Table 2. The molecular beacons and oligonucleotides were purchased from Isogen Bioscience B.V., Netherlands.

### 2.5. RNA extraction from plant material and cDNA synthesis

Total RNA was extracted from 100 mg of tobacco or potato leaf, or potato tuber tissue, using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

RNA was denatured in the presence of 0.5  $\mu\text{M}$  oligo dT oligonucleotide primer, 1.67  $\times$  Taqman buffer A (Applied Biosystems), 6.67 mM  $\text{MgCl}_2$  and 4 U RNA Guard (Amersham Pharmacia Biotech) in 12  $\mu\text{l}$  final volume for 5 min at 65  $^\circ\text{C}$ . After cooling at 25  $^\circ\text{C}$  for 15 min, 2 U MuLV reverse transcriptase (Applied Biosystems), 4 U RNA Guard and dNTP were added to 300 nM final concentration, each, in 8  $\mu\text{l}$  volume. cDNA synthesis was carried out for 100 min at 37  $^\circ\text{C}$ , followed by an inactivation step at 95  $^\circ\text{C}$  for 5 min.

### 2.6. Synthesis of *in vitro* RNA

Positive-sense PVY RNA was used as template in NASBA and was synthesised *in vitro* according to the following protocol.

In a PCR reaction, a 557 nucleotide-long region of the 3' part of the coat protein gene was amplified with PVY-T7-5' and RNA-3' oligonucleotide primers, using PVY cDNA as template (Section 2.5) in a 50  $\mu\text{l}$  reaction mixture containing 1  $\times$  GeneAmp PCR buffer, 2 mM  $\text{MgCl}_2$ , 300 nM dNTP each, 0.1  $\mu\text{M}$  primers, 2  $\mu\text{l}$  cDNA and 1.25 U AmpliTaqGold polymerase. After initial denaturation and activation at 95  $^\circ\text{C}$  for 10 min, the following cycle was

repeated 30 times: 0.5 min at 93  $^\circ\text{C}$ , 0.5 min at 55  $^\circ\text{C}$  and 1 min at 72  $^\circ\text{C}$ . At the end of the reaction, a final extension step at 72  $^\circ\text{C}$  for 10 min was included. All the reagents were purchased from Applied Biosystems.

The PCR product was purified with the High Pure PCR Product Purification Kit from Roche and eluted in 50  $\mu\text{l}$  sterile, DEPC-treated water.

RNA was transcribed in a reaction mixture containing 1  $\times$  T7 RNA polymerase transcription buffer, 0.5 mM rNTP each, 100 mM DTT, 10 U RNA Guard, 150 U T7 RNA polymerase and 5  $\mu\text{l}$  of the purified PCR product for 2 h at 37  $^\circ\text{C}$ . The reagents were purchased from Amersham Pharmacia Biotech. The synthesised RNA was purified by using the RNeasy Mini Kit from Qiagen. Its purity and concentration were determined by measuring the UV absorption at 260 and 280 nm on a Beckman spectrophotometer.

### 2.7. NASBA amplification

The NASBA reaction mixture consisted of 4  $\mu\text{l}$  5  $\times$  NASBA-buffer (200 mM Tris-HCl, pH 8.5, 60 mM  $\text{MgCl}_2$ , 350 mM KCl, 2.5 mM DTT, 5 mM of each dNTP, 10 mM each of rATP, rUTP and rCTP, 7.5 mM rGTP and 2.5 mM rITP), 4  $\mu\text{l}$  5  $\times$  primer mix (75% DMSO and 1  $\mu\text{M}$  of each primer) and 2  $\mu\text{l}$  of RNase-free  $\text{H}_2\text{O}$ . When NASBA was carried out in the presence of molecular beacons (0.05  $\mu\text{M}$  MB-N1, 0.02  $\mu\text{M}$  MB-N2, 0.05  $\mu\text{M}$  MB-O1 and 0.02  $\mu\text{M}$  MB-O2), the water was replaced by the molecular beacons mix. Five microlitre RNA extract or water (negative control) was added and the samples were denatured at 65  $^\circ\text{C}$  for 5 min. After incubation at 41  $^\circ\text{C}$  for 5 min, 5  $\mu\text{l}$  enzyme mix (375 mM sorbitol, 2.1  $\mu\text{g}$  BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase) was added to each tube. After 5 min equilibration at 41  $^\circ\text{C}$ , the samples were transferred to a Fluoroskan FL fluorometre, to monitor the amplification in real-time, and were incubated at 41  $^\circ\text{C}$  for 1.5 h. The reagents were provided by Organon Teknika, Netherlands.

## 2.8. Detection of NASBA amplicon RNA by Northern hybridisation and enhanced chemiluminescence

NASBA products were analysed by electrophoresis on a 1% pronarose gel containing 0.5 µg/ml EtBr. Gels were run at 10 V/cm for 20 min in 1 × TAE buffer, and were 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 to the membrane by UV exposure at 365 nm for 2 min. The biotinylated P-PVY probe (Table 2) was added to the NASBA products to give a final concentration of 1.5 nM and the mixture was incubated at 50 °C for 60 min in the presence of 5 × SSC, 7% SDS, 20 mM Na-phosphate, pH 6.7 and 10 × Denhardt's solution. The filter was washed twice with 3 × SSC, 1% SDS at 50 °C for 5 min, and once with 0.1% SDS and 2 × SSPE (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.36 M NaCl and 2 mM EDTA) at room temperature for 10 min. The blot was then incubated for 30 min with 2 µl streptavidin/peroxidase conjugate in 5 × SSPE and 0.5% SDS, followed by two washes with 2 × SSPE, 0.1% SDS for 1 min and one for 10 min. Subsequently, the blot was washed twice with 2 × SSPE for 2 min, followed by an incubation in substrate solution (enhanced chemiluminescence (ECL) detection reagent, Amersham Pharmacia Biotech) for 60 s, and exposed to X-ray films.

## 2.9. Fluorescence measurement and correction for overlapping spectra

Fluorescence was measured with a Fluoroskan FL instrument. FAM, HEX, TAMRA and Texas Red fluorophores were excited at 485, 530, 544 and 584 nm and the emitted light was measured at 538, 555, 590 and 612 nm, respectively. The signals were corrected for the overlap of excitation and emission spectra.

## 3. Results

### 3.1. Design of the assay

The coat protein coding region of PVY was reported to be characteristic for the three strains

(van den Heuvel et al., 1994), and also contained a rearrangement associated with the PVY<sup>NTN</sup> pathotype. To compare all available sequences to date, the coat protein coding regions of PVY isolates were aligned. Pairwise distances were determined and the sequences were clustered on the basis of similarity. An unrooted tree showing the relationships between the isolates is presented in Fig. 1. In agreement with previous studies (Revers et al., 1996; Boonham et al., 1999), four major clusters were found. Three clusters were homogeneous, containing PVY<sup>NTN</sup>, PVY<sup>N</sup> and PVY<sup>C</sup> sequences, respectively. The fourth contained both PVY<sup>O</sup> and PVY<sup>N</sup> isolates and most of the biologically not characterised viruses. The clustering of three PVY<sup>N</sup> sequences with the PVY<sup>O</sup> strain was not surprising, since certain PVY<sup>N</sup> isolates (PVY-Wilga and PVY-Fr) were previously reported with PVY<sup>O</sup>-characteristic coat proteins (Chachulska et al., 1997). A single sequence (S74813) was not assigned to any of the major clusters. This isolate had a PVY<sup>N</sup> phenotype and contained a rearrangement in the coat protein coding region different from that found in PVY<sup>NTN</sup> isolates (Revers et al., 1996). Since the PVY<sup>NTN</sup> isolates formed a well-separated, homogeneous group, and the remaining three clusters generally correlated with the biological grouping, the analysed genome region appeared suitable for the development of an AmpliDet RNA assay for the strain-specific detection of PVY and the differentiation of PVY<sup>NTN</sup>.

The region of the alignment containing the sequence rearrangement is shown in Fig. 2. PVY<sup>N</sup> and PVY<sup>O</sup> cluster-specific sequence motifs were selected immediately adjacent to the presumed recombination site as target areas for the strain-specific molecular beacons. The molecular beacons were named MB-N1, MB-N2, MB-O1 and MB-O2, according to their specificity and position. Flanking this diagnostic region, conserved sequences were selected as binding sites for PVY-specific NASBA primers. Three groups of isolates can be detected: one containing all the PVY<sup>NTN</sup> isolates (by MB-N1 and MB-O2), one with PVY<sup>N</sup> isolates (by MB-N1 and MB-N2) and a third one with PVY<sup>O</sup> and PVY<sup>C</sup> isolates (by MB-O1 and MB-O2, as shown in Fig. 3(A)). The use of four molecular beacons was

required to recognise mixed infections of PVY strains. Since MB-N2 and MB-O1 are unique for the PVY<sup>N</sup> and PVY<sup>O/C</sup> strains, respectively, a mixed PVY<sup>N</sup> and PVY<sup>O</sup> infection can be correctly identified and is not mistaken for PVY<sup>NTN</sup> (Fig. 3(B)).

### 3.2. Performance of the assay

The specificity of the molecular beacons for the different PVY clusters was determined by using RNAs of well-defined representatives of each viral strain and that of the PVY<sup>NTN</sup> group, as template.

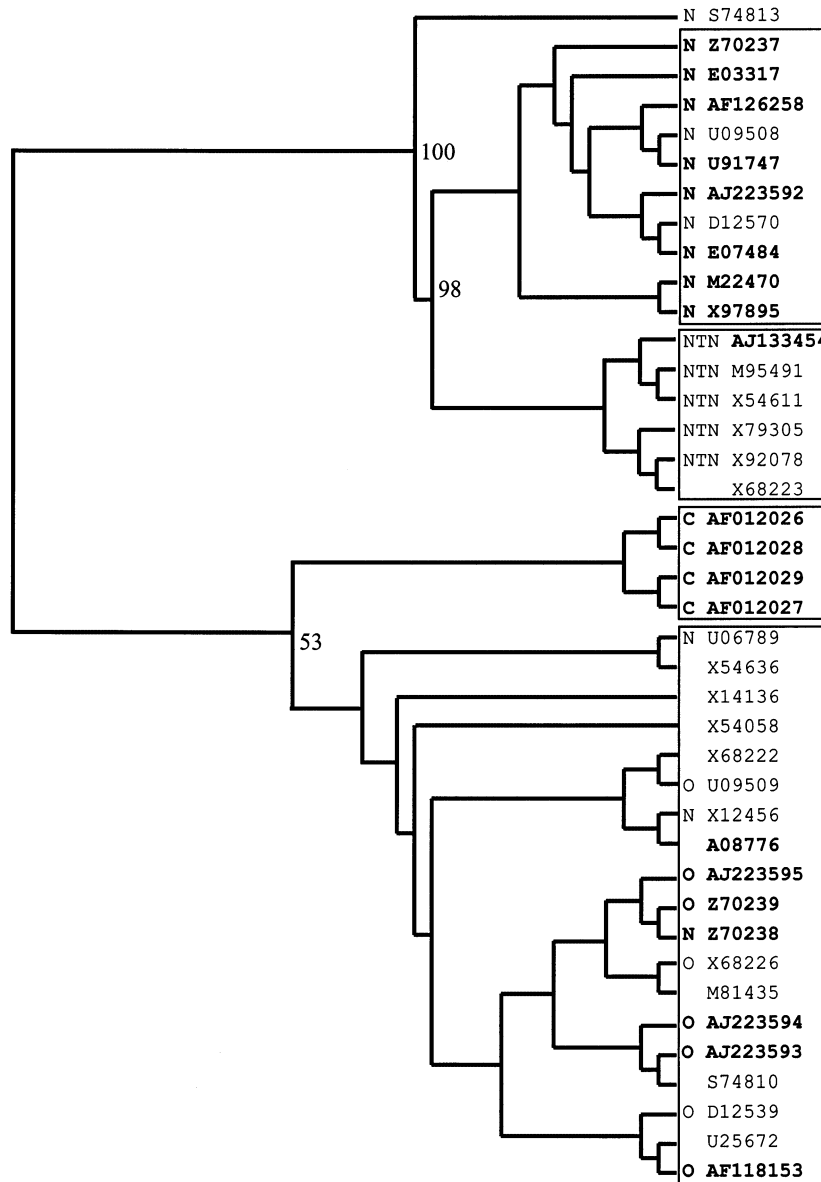


Fig. 1. Clustering of PVY coat protein gene sequences. The numbers at the forks between the major clusters indicate the bootstrap values of the branching. The biological classification of the isolates, when available, is shown in front of the accession numbers of the sequences. The four major clusters are boxed. The sequences not clustered by Revers et al. (1996) are shown in bold.



exception was the binding of MB-O1 to PVY<sup>C</sup> (53–38), in which case an A/G change in the amplicon RNA was not tolerated.

The detection of mixed infections was also tested. The signals of assays carried out on artificial mixtures of RNA samples are shown in Fig. 6. Mixtures of PVY<sup>NTN</sup> with either PVY<sup>O</sup> or PVY<sup>N</sup> could be diagnosed unambiguously and correctly. A PVY<sup>N</sup>/PVY<sup>O</sup> mixed infection results in positive signals with all molecular beacons, which does not rule out the presence of a PVY<sup>NTN</sup> virus. However, in this case it must be present as a third infecting virus. Such cases are expected to be rare and demand additional examination.

The sensitivity of NASBA was determined without molecular beacons and in the presence of one or four MBs, to establish how the binding of the probes affects the sensitivity (Fig. 7). The detection

threshold was determined by using a serial dilution of *in vitro* transcribed, positive-sense PVY RNA as template, representing PVY<sup>NTN</sup>, PVY<sup>N</sup> and PVY<sup>O</sup> sequences. In the absence of molecular beacons and in the presence of one molecular beacon, the sensitivity of the assay was 10<sup>1</sup> copies of template RNA per reaction, which indicates that NASBA was not hindered significantly by the hybridisation of a molecular beacon in the concentration range used. When four molecular beacons were used, the sensitivity was lowered by one order of magnitude to 10<sup>2</sup> copies of RNA per reaction.

### 3.3. Validation of the assay

The performance and reliability of the assay were assessed by analysing 47 PVY isolates originating from six countries (Table 1). First, a set of isolates characterised biologically were tested (Table 1: 1–29). The results of AmpliDet RNA were identical or consistent (PVY<sup>O/C</sup> diagnosis in the case of a PVY<sup>O</sup> or a PVY<sup>C</sup> infection) with those of biological strain determination in all cases, but one. Nine PVY<sup>NTN</sup>, nine PVY<sup>N</sup>, eight PVY<sup>O</sup> and two PVY<sup>C</sup> viruses, representing isolates from six countries, were diagnosed correctly. A single exception (isolate 53–47) gave PVY<sup>N</sup> characteristic signals, but was described previously as PVY<sup>NTN</sup> (Nielsen, personal communication).

Eighteen isolates collected from independent PVY outbreaks in Hungary were also analysed. On the basis of symptoms in susceptible potato cultivars Vital and Murillo, they were classified as PVY<sup>NTN</sup> (Wolf and Horváth, 2001). All these isolates were detected successfully and were identified correctly by AmpliDet RNA assay as PVY<sup>NTN</sup>.

Taken together, the AmpliDet RNA assay gave correct strain identifications in 46 cases: 27 PVY<sup>NTN</sup>, nine PVY<sup>N</sup>, eight PVY<sup>O</sup> and two PVY<sup>C</sup> isolates.

## 4. Discussion

An AmpliDet RNA assay was developed for the specific detection of PVY, and for the differentiation of its strains and the tuber necrotic isolates.

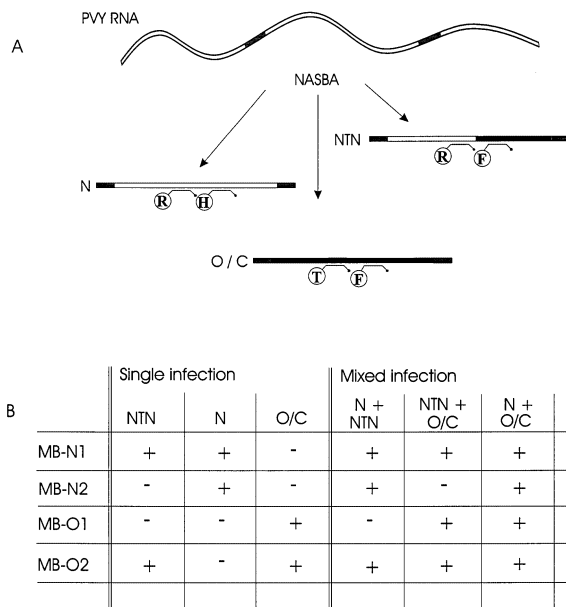


Fig. 3. (A) Scheme of the AmpliDet RNA assay for PVY<sup>NTN</sup>. In a PVY-specific NASBA, a region of the coat protein gene is amplified for all PVY isolates. With four MBs, three PVY clusters are identified: PVY<sup>NTN</sup> isolates are detected by MB-N1 and MB-O2, which carry a Texas Red (R) and a FAM (F) label, respectively. The PVY<sup>N</sup> strain is signalled by the two PVY<sup>N</sup>-specific beacons, MB-N1 and the HEX-labelled MB-N2 (H), and the PVY<sup>O/C</sup> strains by MB-O1 (TAMRA, T) and MB-O2.

(B) Diagnosis based on MB signals in the event of single or mixed infections of PVY strains.

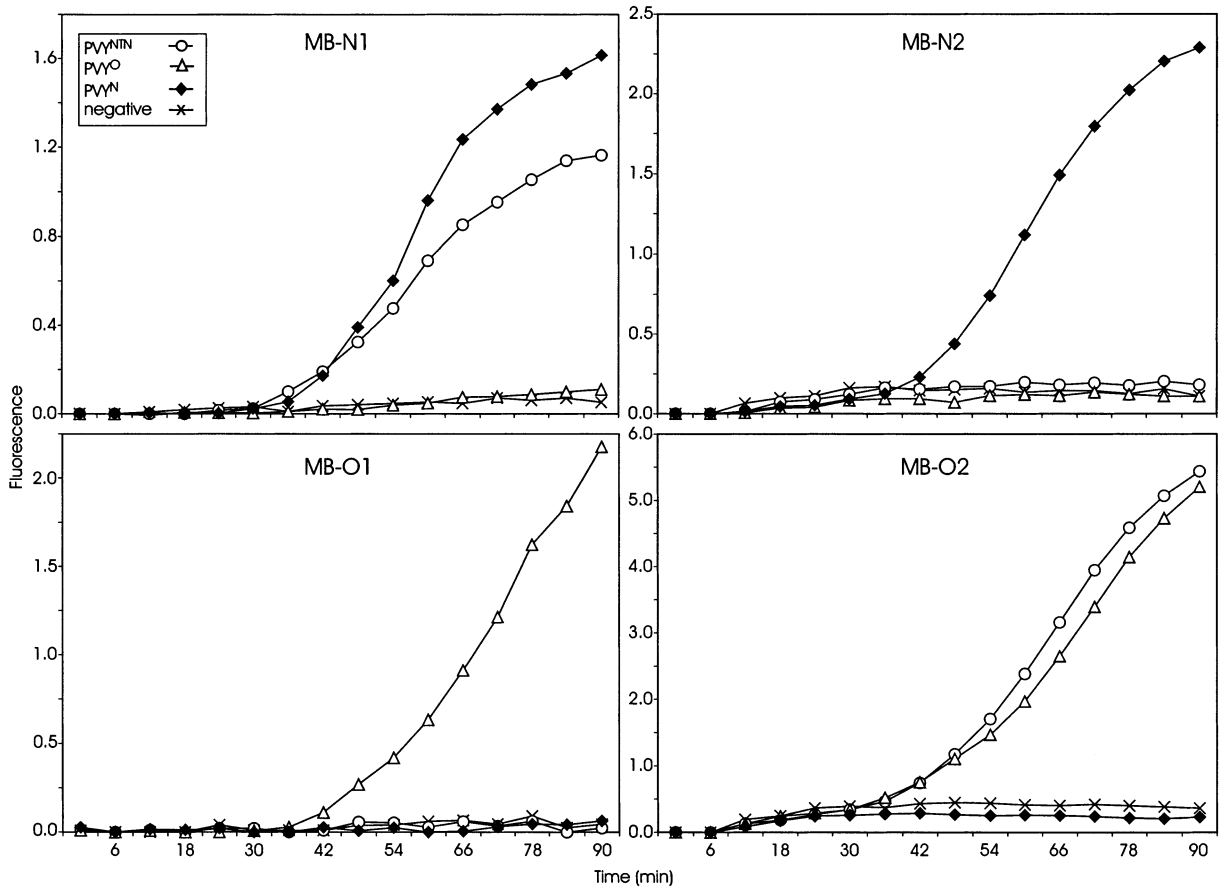


Fig. 4. Strain-specific diagnosis by real-time AmpliDet RNA assay. The signal of each MB is shown in a separate panel. Templates are labelled as shown in the index box.

The assay consists in a PVY-specific NASBA amplification followed by strain-specific identification by four molecular beacons.

The performance of the AmpliDet RNA assay was evaluated by using RNAs of viruses characterised biologically as templates. The method was found to be perfectly specific for the targeted PVY clusters; the molecular beacons gave signals only with their respective target molecules. Artificially mixed infections were also diagnosed successfully.

The clusters were defined on the basis of coat protein coding sequences, and mostly corresponded to the biological classification. The only exceptions were a few PVY<sup>N</sup> isolates that grouped in the PVY<sup>O</sup> cluster. These isolates, however, would also be

diagnosed as PVY<sup>O</sup> by other methods detecting characteristic features of the coat protein region, such as ELISA or an RT-PCR assay developed recently (Walsh et al., 2001).

In the course of molecular beacon design, different strategies were followed to make the molecular beacons tolerant to the known sequence polymorphisms present in the molecular beacon hybridisation site. The mismatch tolerance of the molecular beacons was tested by using isolates containing a mutation in the target regions. All mismatches, but one were tolerated and the molecular beacons gave specific, but reduced signals. The one exception was an A/G transition affecting the hybridisation site of MB-O1, the molecular beacon that contained two P analogues.

The universal pyrimidines were earlier reported to bind less efficiently to guanines than to adenines (Hill et al., 1998) and, incidentally, the amplicon of the mutant isolate contained guanines as hybridising counterparts to P in both positions. We assume that this additional destabilising of the probe-target duplex contributed to the lack of molecular beacon binding. As a result, some PVY<sup>C</sup> isolates do not give the MB-O1 signal; they are detected solely via MB-O2. In fact, the signals of PVY<sup>C</sup> isolates are quite characteristic: a reduced MB-O2 signal with or without MB-O1. Since the probes were shown to produce positive, but reduced signals with isolates containing a single mismatch, the use of molecular beacons not only enables the detection

of the viral types, but also would indicate polymorphisms in the molecular beacon target regions.

The combination of generic oligonucleotide primers and group-specific molecular beacons offers a convenient means of multiplex detection. The number of molecular beacons used in a single assay is mostly limited by the ability to distinguish between the fluorescent signals of the different dyes. In this study, four molecular beacons were used and their signals could be distinguished perfectly. Two molecular beacons bound to each amplicon RNA, and it was found that, although the sensitivity of NASBA was lowered, the assay retained sufficient sensitivity for diagnostic purposes.

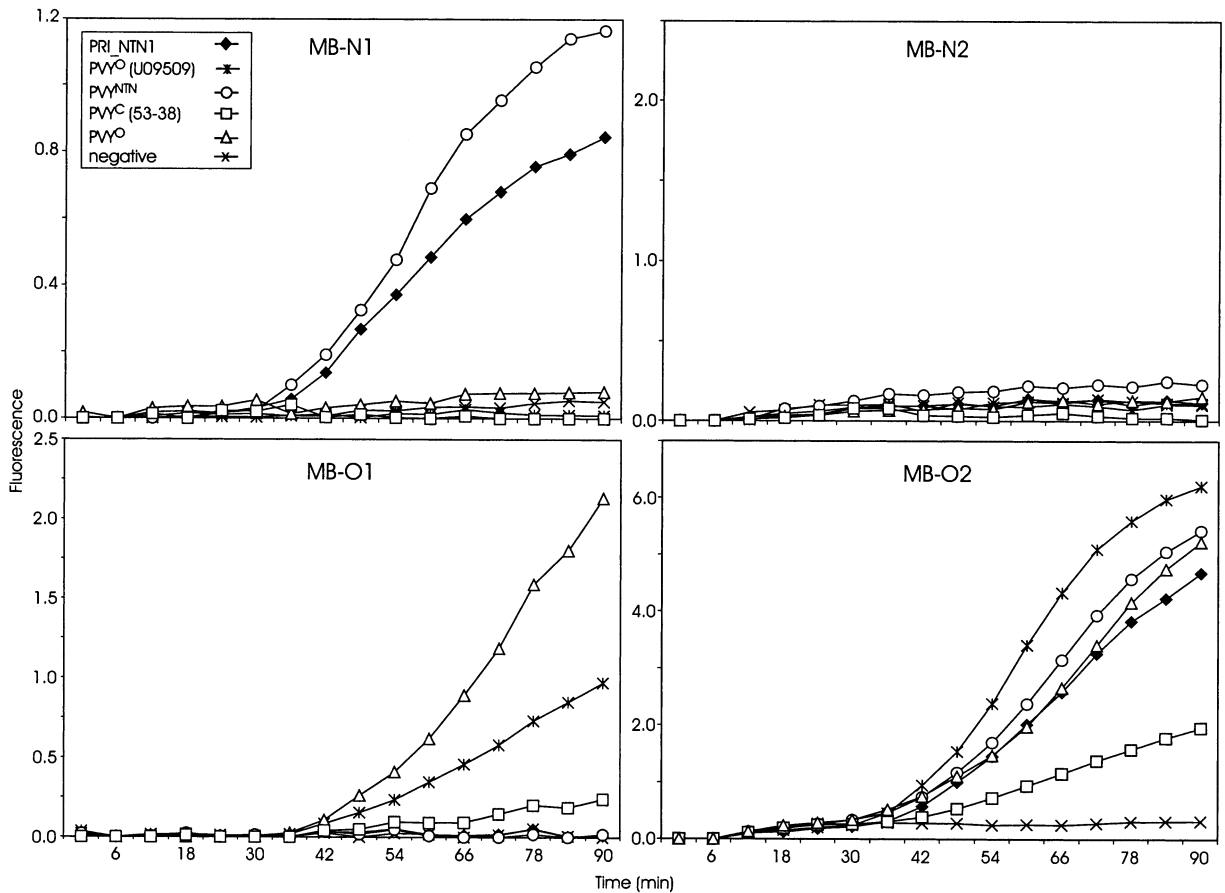


Fig. 5. AmpliDet RNA assay with PVY isolates containing a mutation in one of the MB target areas. PRI\_NTN1 has a mutation in the MB-N1 site, PVY<sup>O</sup> (U09509) in the MB-O1 site, and PVY<sup>C</sup> (53–38) in both the MB-O1 and MB-O2 sites. The signals with non-mutant PVY<sup>NTN</sup> and PVY<sup>O</sup> isolates are included for comparison.

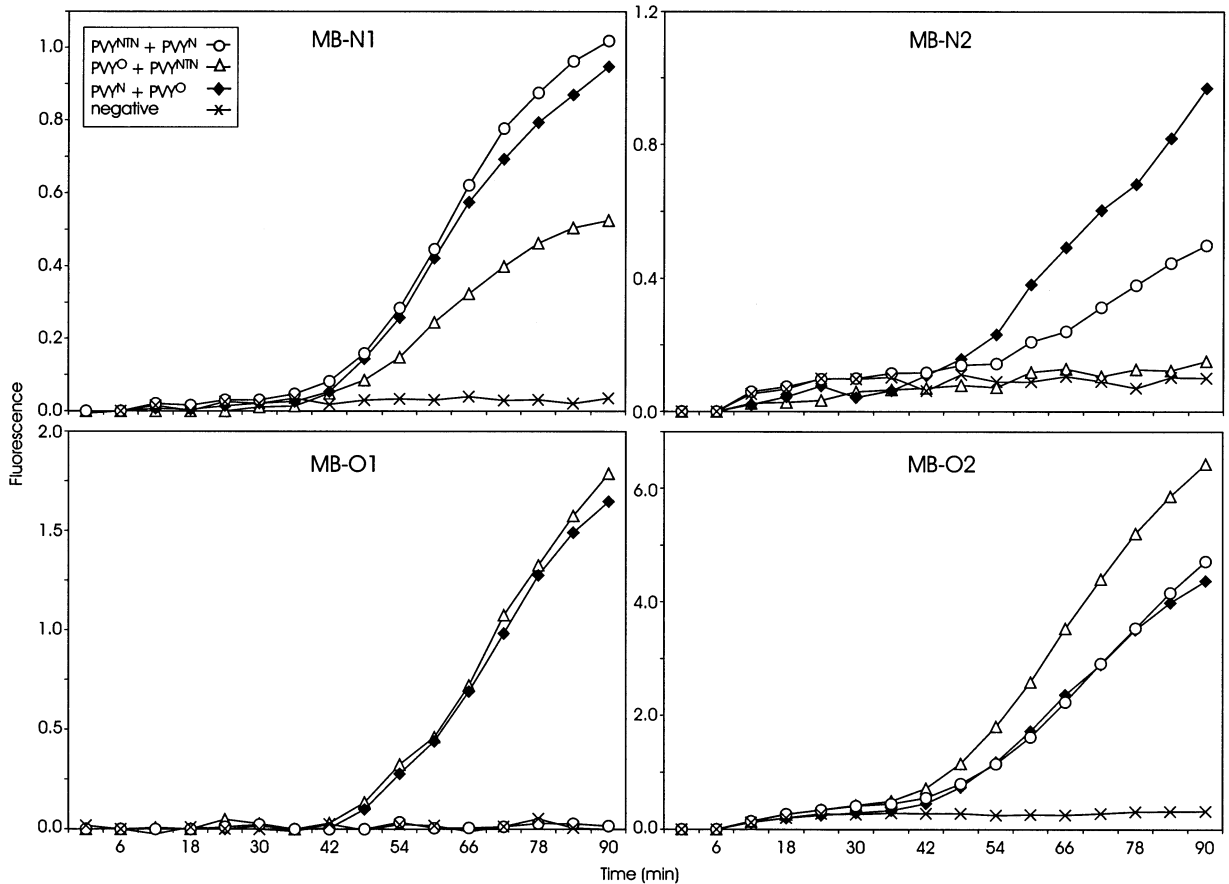


Fig. 6. AmpliDet RNA assay with artificially mixed infections.

The assay was validated by testing 47 PVY isolates originating from six countries. With the exception of one PVY isolate, all were detected successfully, among them 27 PVY<sup>NTN</sup>, nine PVY<sup>N</sup>, eight PVY<sup>O</sup> and two PVY<sup>C</sup> viruses. The exceptional isolate (53–47) belonged in the PVY<sup>NTN</sup> group and gave signals characteristic of PVY<sup>N</sup>.

Since the completion of the experimental phase of this study, a number of PVY coat protein coding sequences, representing isolates from various countries, have been submitted to GenBank. The analysis of these sequences indicated that the assay developed would identify correctly the newly described PVY<sup>N</sup>, PVY<sup>O</sup> and PVY<sup>C</sup> isolates. Some PVY<sup>NTN</sup> isolates, however, did not contain the characteristic rearrangement targeted, thus they would not be typed correctly. These isolates had

distinct geographical origins, namely North America, Northern Europe and Japan. It is noticeable that the exceptional PVY<sup>NTN</sup> isolate that failed to give an NTN-specific signal in our assay originated also from Northern Europe (Denmark). The polymorphism of tuber necrotic isolates is not without an example: the distinct biological features and nucleic acid patterns in the P1 coding region of North American PVY<sup>NTN</sup> viruses were also reported (McDonald and Singh, 1996; Weilguny and Singh, 1998). Therefore, we suggest that the chimerical nature of the PVY<sup>NTN</sup> coat protein merely correlates, but does not have a causal relationship with the phenotype. Nevertheless, the diagnostic procedure described in this paper would identify most of the viruses with PVY<sup>NTN</sup> pathotype, mainly those originating from Europe.

The developed AmpliDet RNA assay is amenable to high-throughput testing enabling the detection and cluster-specific differentiation of PVY isolates in one reaction vessel within < 2 h. It is suitable for extensive screening studies for routine phytosanitary analyses and for epidemio-

logical purposes, although it should be borne in mind that exceptions may occur.

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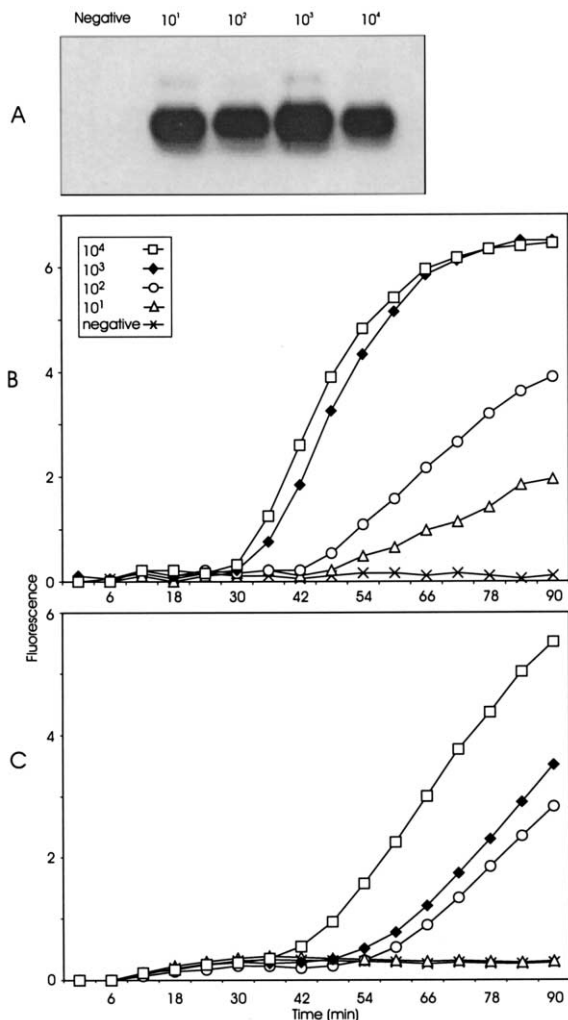


Fig. 7. Sensitivity of PVY-specific NASBA reaction and AmpliDet RNA assay with 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup> and 10<sup>4</sup> copies of in vitro transcribed RNA (Section 2.6). (A) Northern hybridisation of NASBA reaction products. (B) Sensitivity of AmpliDet RNA assay in the presence of one MB (MB-O2). (C) Sensitivity of the assay with four MBs, where two molecular beacons hybridise to the amplified RNA molecules. The signal of MB-O2 is shown.

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