European Journal of Plant Pathology **108:** 293–298, 2002.

Asymmetric PCR ELISA: increased sensitivity and reduced costs for the detection of plant viruses

Gustavo Nolasco[1], Zita Sequeira[1], Claudia Soares[1], Ana Mansinho[1], Ana M. Bailey[2] and Charles L. Niblett[3]
[1]*FERN, Universidade do Algarve, Campus de Gambelas, 8000 Faro, Portugal (Phone: +351 289800960; Fax: +351 289818419; E-mail: gnolasco@ualg.pt);* [2]*Department of Ingenieria Genetica de Plantas, CINVESTAV-IPN, Irapuato, Mexico;* [3]*Department of Horticultural Sciences, University of Florida, USA*

Accepted 18 December 2001

Key words: asymmetric PCR, CTV, nucleic acids detection, RSPaV, RT-PCR ELISA

Abstract

PCR ELISA is the immunodetection of the products of a polymerase chain reaction (PCR). It is effective for detecting and differentiating plant viral nucleic acids, but as currently performed, it is laborious and expensive. The procedure has been modified and simplified by using asymmetric PCR. This eliminated the need to denature and neutralize samples prior to hybridization. It also increased the relative concentration of the target DNA species, making PCR ELISA more sensitive than TaqMan™, a fluorescence-based detection method. Reducing the reaction volumes to half and the concentration of the dNTPs and the digoxigenin label by tenfold significantly reduced the costs of PCR ELISA without reducing its sensitivity. The usefulness of these modifications was demonstrated for the detection of *Citrus tristeza virus* and *Rupestris stem pitting-associated virus*. We expect that with only minor modifications asymmetric PCR ELISA could be used effectively for the detection of most nucleic acid molecules of interest.

Introduction

The ability of the polymerase chain reaction (PCR) to amplify minute quantities of specific genomic sequences makes it the technique of choice for the detection of pathogens. However, detection of the amplified products has been one of the major bottlenecks which has impaired its widespread use on a routine basis, especially where large numbers of samples are necessary. Two strategies have been developed for the rapid detection of DNA. These are immunoenzymatic detection of labeled DNA or PCR ELISA (Alard et al., 1993) and fluorescence resonance energy transfer (FRET) using two approaches: molecular beacons (Tyagi et al., 1996) or a 5′-exonuclease assay (Holland et al., 1991). Both strategies have been used for the detection of plant viruses (Schoen et al., 1996; Nolasco et al., 1997, 1999; Rowhani et al., 1998). PCR ELISA incorporates a non-radioactive label such as digoxigenin (DIG) into the PCR products during the amplification, followed by hybridization of the amplified products in a microplate to an immobilized capture probe complementary to a sequence of the amplicon. The immobilized label is detected by an ELISA-like (Clark et al., 1977) procedure. A typical protocol (Rowhani et al., 1998) for the detection of diverse plant viruses involves labeling of PCR products with DIG, chemical or heat denaturation of PCR products, hybridization of the PCR products to the capture probe in the microplate, addition of an enzymatic conjugate specific for DIG, and finally, addition of the colorimetric substrate and measuring the reaction in an ELISA plate reader. In this work, the combination of PCR ELISA with asymmetric PCR is described. This results in increased availability of the target strand for the capture probe and avoids the denaturation/neutralization steps. This novel assay is more rapid, more sensitive and less expensive than conventional PCR ELISA. This procedure is demonstrated with immunocaptured virions of *Citrus tristeza virus*

(CTV, genus *Closterovirus*, family *Closteroviridae*) and double-stranded RNA (ds-RNA) from plants infected with *Rupestris stem pitting-associated virus* (RSPaV, genus *Foveavirus*, family *Closteroviridae*).

Materials and methods

Templates for viral gene amplification were the cloned capsid protein gene (672 nt) of CTV (isolates B249 and B53) or CTV virions obtained by immunocapture (Nolasco et al., 1999) or ds-RNA extracted from grapevines infected with RSPaV (Nolasco et al., 2000).

RT-PCR amplification

Normal or symmetric (ratio of forward : reverse primer = 1 : 1) reverse transcription PCR (RT-PCR) experiments were done as a single step protocol in 50 µl of a reaction mixture containing 10 mM Tris (pH 8.8), 50 mM KCl, 0.08% Nonidet P40, 4 mM $MgCl_2$, 200 µM each dNTP, 200 nM each primer, 3 U RNAguard (Pharmacia), 7.5 U M-MLV reverse transcriptase (Perkin–Elmer) and 1 U of *Taq* polymerase (MBI-Fermentas). The reverse transcription (RT) reactions were incubated for 45 min at 38 °C. The PCR amplification was then preceded with a 2 min incubation at 94 °C, followed by 30 cycles of 92 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, followed by a final 72 °C incubation for 5 min.

Incorporation of the DIG label through asymmetric RT-PCR

Asymmetric RT-PCR for incorporation of DIG was performed similarly to 'normal' RT-PCR except that (1) the dNTP mix was replaced with a DIG-labeling mix containing 200 µM each dATP, dGTP, dCTP, 190 µM dTTP and 10 µM Digoxigenin-11-dUTP (DIG-dUTP) (Roche Molecular Biochemicals), (2) one PCR primer was at a fixed concentration of 200 nM while the other primer was used in various proportions from 1 : 25 to 1 : 1, and (3) the thermal cycling was extended to 50 cycles. The primers used were CTV1 and CTV14 for CTV (Nolasco et al., 1999) and RSPaV 13 and RSPaV 14 for RSPaV (Meng et al., 1999) (Table 1). In experiments using the cloned capsid protein gene of CTV B249, the RT step was omitted and the primers used were CTV1 and CTV10 (Table 1). In this case CTV1 (forward primer) was used in excess.

TaqMan™ assay

The TaqMan™ assays with CTV were done using a developed protocol (Nolasco et al., 1999) with minor changes. Briefly, the PCR mixture was the 'normal' mixture referred to above, containing in addition 200 nM of the TaqMan™ (FAM labeled) probe CTV E (Table 1). The fluorescence was recorded in a 7200 ABI PRISM Sequence Detector (Perkin Elmer) before and after the PCR reaction and the net fluorescence increase was obtained by the subtraction of the pre-PCR value. The positive/negative threshold was defined as the mean of the negative controls (three replicates) plus 6.965 times the standard deviation as described in the ABI PRISM 7200 Sequence Detector user's manual.

PCR ELISA detection of amplified products

All PCR ELISA reactions were performed in Microlon 600 microplates with round bottom wells (Greiner).

Table 1. Primers and probes used. The sequences are listed from the 5′ to 3′ end

Primer or probe	Sequence	Position[b]
Specific for CTV		
CTV1	ATGGACGACGAAACAAAGAA	1
CTV14	GGTCAAGAAATCYGCACACA	524
CTV10	ATCAACGTGTGTTGAATTTCC	653
Probe CTV E (TaqMan)	ACGGGTATAACGTACACTCGG	301
Probe[a] CTV BE	GGGTATAACGTACACTCGGGAGGG	303
Probe[a] CTV V[c]	ACACCCGTGGTATCATCGT	290
Specific for RSPaV		
RSPaV13	GATGAGGTCCAGTTGTTTCC	4373
RSPaV14	ATCCAAAGGACCTTTTGACC	4692
Probe[a] RSPaV B1	TGATGCTTTCATTTCACTTGC	4432

[a]Probes biotinylated at the 5′ end. [b]Positions are relative to the Genebank accession numbers AF057136 (RSPaV) and AF184118 (CTV). [c]Antisense probe.

All final reaction volumes were 100 µl. Detection of DIG-labeled 'normal' PCR products was done for comparative purposes using the protocol described by Rowhani et al. (1998), which includes the denaturing and neutralizing steps. Detection of asymmetric PCR DIG-labeled products was done omitting the denaturing and neutralizing steps, as described below. The wells were coated with streptavidin (10 µg/ml and 100 µl/well in 50 mM sodium carbonate buffer (pH 9.6) at 37 °C for 1–1.5 h or overnight at 4 °C). The plates were washed three times and between succeeding steps with PBS–Tween buffer (Clark et al., 1977). The biotinylated capture probe (20 pmol per well in hybridization buffer – 5× SSPE, 0.5 M NaCl, 0.1% n-lauroylsarcosine) was added and incubated at 37 °C for 30 min to immobilize it in the wells of the microplate. Variable amounts (5–35 µl) of DIG-labeled PCR products were added to the wells and the volume adjusted to 100 µl with the hybridization buffer. Hybridization was performed at 37 °C for 90 min. An anti-DIG-F(ab')$_2$ alkaline phosphatase conjugate (Roche Molecular Biochemicals), 0.075 units in PBS–Tween plus 2% PVP-40 and 0.2% BSA, was added per well and incubated at 37 °C for 30 min. The enzyme substrate (p-nitrophenyl phosphate) was used at 0.75 mg/ml of substrate buffer (9.7% diethanolamine, pH 9.8). The colorimetric reaction was developed at room temperature for about one hour and read in a conventional ELISA plate reader at 405 nm. The negative/positive threshold was defined in a similar way as for the TaqMan™ assay.

Results and discussion

Initial experiments following the protocol described by Rowhani et al. (1998) were time-consuming and yielded low absorbance values. We reasoned that it might be beneficial to increase the proportion of the DIG-labeled target strand of DNA by asymmetric PCR.

Asymmetric PCR ELISA for detection of cloned CP *gene*

This was tested by amplifying the cloned CP gene of CTV B249 in the presence of DIG at different ratios of the forward and reverse primers. As shown in Figure 1, lane 7, a 1 : 1 ratio of the primers yields an intense double-stranded (ds) DNA band of the expected size, 672 bp. As the amount of reverse primer decreases to a ratio (forward : reverse) of 25 : 1 (Figure 1, lane 3) the intensity of this band decreased, accompanied by the appearance of a slower migrating band characteristic of single-stranded (ss) DNA. The maximum intensity of the ssDNA band was reached at a primer ratio of 10 : 1 (forward : reverse) (Figure 1, lanes 4 and 5). The PCR products from this experiment, with and without prior denaturing and neutralization, were applied to the wells of a microplate coated with capture probe CTV V, which was designed as an antisense probe (Table 1). The absorbance data in Figure 1 shows a significant increase for the samples amplified by asymmetric PCR, with the maximum values obtained at a forward : reverse primer ratio of 10 : 1 (lanes 4 and 5). Furthermore, their similar absorbance values indicate that there was not a major effect of denaturing the PCR products prior to hybridization when using the protocol described by Rowhani et al. (1998). To confirm that the increase in absorbance was due to the increase in the positive (target) strand, a similar experiment was performed in which the reverse primer was in excess. The ss- and ds-DNA PCR products were detected by polyacrylamide gel electrophoresis (PAGE) and were similar to those in Figure 1, but the absorbance values all remained at the level of the negative controls (data not shown). Therefore, the nearly fourfold enhancement of the absorbance (hence sensitivity) shown in lanes 4 and 5 of Figure 1 resulted from increased availability of the target strand. All subsequent asymmetric PCR

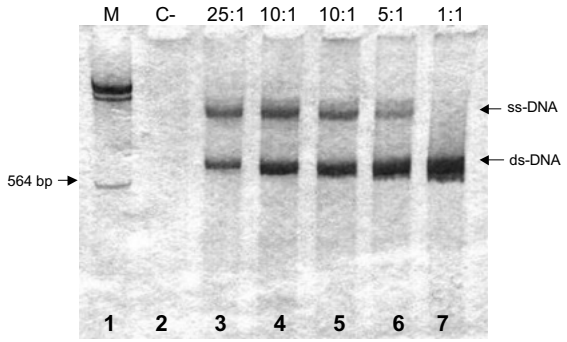

Figure 1. Electrophoretic (PAGE and silver staining) and colorimetric detection of PCR products amplified from clone CTV-B249. The concentration of the forward primer (CTV1) was maintained constant at 200 nM while the concentration of the reverse primer (CTV10) was varied according to the ratios (forward : reverse) shown above lanes 3–7. Lane 1 (M) is *Hind*III restricted Lambda DNA used as the marker, and lane 2 (C^-) is the template-free negative control, reacted with a primer ratio 10 : 1. Below the gel are the respective absorbance ($A_{405\,nm}$) readings for the hybridization of the non-denatured and denatured PCR products to the CTV-V capture probe.

experiments for CTV were performed at a primer ratio of 10 : 1, and the blocking, denaturation and neutralization steps described in the initial protocol (Rowhani et al., 1998) were omitted.

Asymmetric RT-PCR ELISA for detection of RNA viruses

Detection of RNA viruses by PCR ELISA should include an initial reverse transcription step. To ensure that this would not be impaired by the inclusion of DIG and the imbalanced primer ratio, we tested the asymmetric PCR ELISA for the detection of RSPaV from ds-RNA templates (Nolasco et al., 2000) and for the detection of CTV from immunocaptured virions (Nolasco et al., 1999), thus covering two diverse but widely used template preparation methods. We designed biotinylated probe RSPaV B1, a genome sense probe (Table 1), following sequencing confirmation that the putatively conserved region of RSPaV (Meng et al., 1999) was indeed conserved in the Portuguese virus isolates used in these studies. In a preliminary experiment similar to that described in Figure 1, the best asymmetric RT-PCR primer ratio (forward : reverse) was found to be 1 : 10 (results not shown). Asymmetric and symmetric PCR reactions were then performed for each of 17 grapevine samples (15 field samples and two control samples known to be free of RSPaV). These samples were then analyzed by PCR ELISA and by PAGE. The results in Table 2 demonstrate excellent correlation between the two methods for the detection of RSPaV, as well as their comparable sensitivity.

In previous research a fluorescent 5′-exonuclease assay (TaqMan™ system) was developed for the detection of CTV (Nolasco et al., 1999). In that assay it was possible to reliably detect samples composed of one part of an extract from an infected plant mixed with 999 parts of an extract from uninfected plants. To compare their relative sensitivities, the TaqMan™ assay, PAGE and asymmetric PCR ELISA were performed using the same preparation of immunocaptured virions of Portuguese CTV isolate 25 as a template. Symmetric and asymmetric PCR were as described above, but in this case with capture probe CTV BE. The TaqMan™ assay used probe CTV E which is designed for the same genomic region as probe CTV BE. At the highest dilution assayed (one part of an extract from an infected plant mixed with 999 parts of an extract from uninfected plants), both assays were able to detect the virus. However the ratio of signals of the infected sample over the healthy control (I/H) was much higher for the asymmetric PCR ELISA ($I/H = 8.4$) than for the TaqMan™ assay ($I/H = 1.4$). A more precise comparison was prepared using known amounts of cloned DNA (Figure 2). As can be seen, 10^{-3} pg of the target still produces a strong signal with the asymmetric PCR ELISA, whereas the TaqMan™ values are already very close to the positive/negative threshold; at 10^{-4} pg the TaqMan™ value is already below the threshold, while a positive signal was still obtained with the asymmetric PCR ELISA. Thus, the asymmetric PCR ELISA assay appears to be more sensitive than the TaqMan™ assay.

Table 2. Detection of RSPaV in grapevine samples by asymmetric PCR ELISA and by PAGE

Sample	A_{405}	PAGE
1	0.102	−
2	0.107	−
3	0.095	−
4	0.112	−
5	0.113	−
6	0.098	−
7	0.124	−
8	0.268	+
9	0.284	+
10	0.478	+++
11	1.022	+++
12	1.261	+++
13	2.283	++++
14	2.308	++++
15	2.943	++++
16	0.098	−
17	0.092	−
C^+	3.743	++++
C^-	0.071	−

The absorbance readings at 405 nm (A_{405}) are given for each sample, including those known to be not infected with RSPaV (16 and 17). The intensity of the bands in the gels is indicated as − = not visible; + = visible; ++ = obvious; + + + = strong, and + + ++ = intense. The positive controls (C^+) contained 10 pg of cloned DNA in each of three replicates. The negative controls consisted of three reactions without added template. The mean value is presented.

Reducing the concentration of the DIG label

A major constraint to the use of asymmetric PCR ELISA for large-scale assays is the cost per reaction, in which the DIG label is one of the most expensive including *Taq* polymerase and

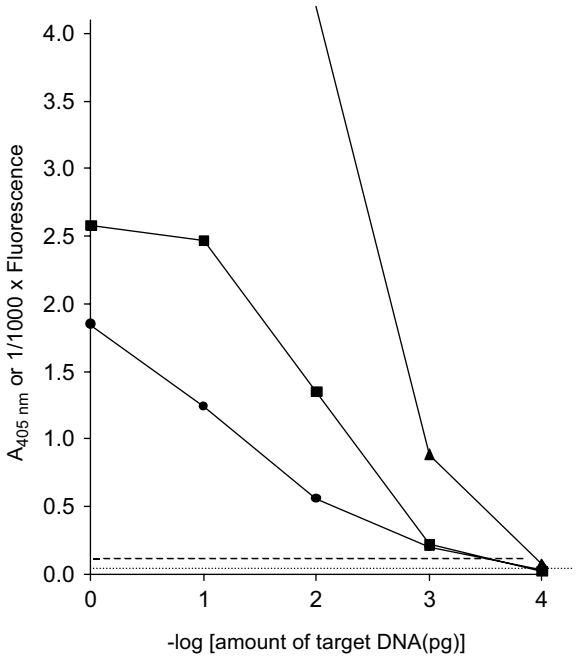

Figure 2. Comparison of the fluorescent exonuclease assay (TaqMan™ system, closed circles) and asymmetric PCR ELISA (after 15 min of substrate incubation – closed squares and 1 h – closed triangles; note that at the highest concentrations the absorbance readings at 1 h were already higher than 4 OD) for the detection of decreasing amounts of a cloned coat protein gene of CTV isolate B53. Each point represents the mean of three replicates. The A_{405} values of the mean of three negative controls (non-template) have been subtracted. The positive/negative threshold is represented at its approximated position as the horizontal dashed line for TaqMan™ (threshold = 0.143) and the dotted line for asymmetric PCR ELISA (threshold = 0.014). The values obtained with the lowest amount of target were 0.023 and 0.075 for TaqMan™ and asymmetric PCR ELISA, respectively.

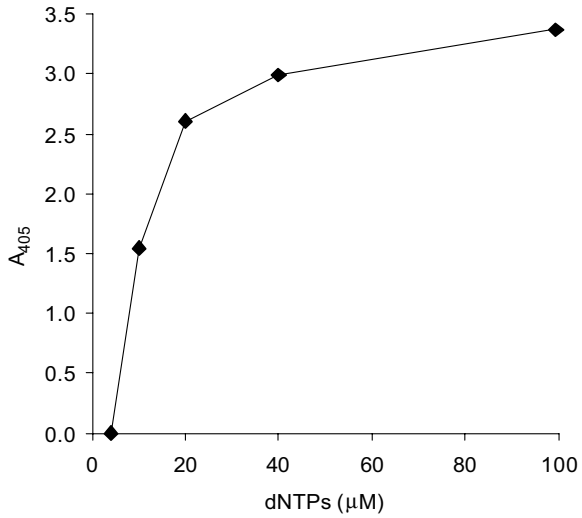

Figure 3. The effect of the concentration of dNTPs and DIG-dUTP on the absorbance obtained by asymmetric PCR ELISA. The capsid protein gene of CTV was amplified from immunocaptured virions by RT-PCR and hybridized with capture probe CTV BE. Absorbance values were recorded after 30 min of incubation with the substrate, and the values for the negative controls have been subtracted. Each point is the mean of two replicate assays. The concentration of Dig-11-dUTP was always one-twentieth of the concentration of the other dNTPs.

M-MLV RTase. Therefore, we designed experiments to determine if reduced amounts of DIG could be used in asymmetric PCR. The results using immunocaptured virions of CTV are shown in Figure 3. It was surprising that the curve depicted a saturation behavior at 20–40 μM of each dNTP and 1 μM of DIG-dUTP, far below the usual concentration of dNTPs (200 μM) and DIG label recommended by the manufacturer or customarily used. Concentrations of 20 μM of each dNTP (including 1 μM of DIG-dUTP and 19 μM dTTP) could be used routinely in asymmetric RT-PCR to label probes with only a slight reduction in absorbance readings and without compromising the ability to detect the viral RNA. Dilutions of each dNTP to 10 μM caused much greater decreases in absorbance and there was no absorbance at 5 μM of each dNTP. Similarly, the PCR products were still detected by PAGE and silver staining with each dNTP at 10 μM, but not at 5 μM (data not shown).

We have investigated several parameters to improve the easiness and efficiency of PCR ELISA for the detection of two woody plant viruses. The blocking, denaturation and neutralization steps of the original procedure can be omitted and the reaction volumes reduced to 100 μl without detriment. Significant increases in sensitivity were obtained by increasing the proportion of target strand by asymmetric PCR. Reducing the amount of dNTPs and DIG label used per reaction by tenfold and incorporating the DIG label into only the target strand significantly reduced the costs of the assay. This enables the analysis of approximately 10 times as many samples for the same reagent costs as the procedure described by Rowhany et al. (1998) and with increased sensitivity. In summary, under the conditions described, the sensitivity of asymmetric PCR ELISA was found to be superior to the TaqMan™ procedure and equivalent in sensitivity to PAGE and silver staining. In addition, asymmetric PCR ELISA utilizes laboratory reagents and equipment which are more commonly

available, thus avoiding the large initial investments in equipment and specific probes required for TaqMan™ and other FRET-based methods. Therefore, we expect that asymmetric PCR ELISA can be used extensively for the detection of not only viral genomes but of most nucleic acid molecules of plant pathogens.

Acknowledgements

Parts of this research were supported in Portugal by the grant PRAXIS XXI – 3/3.2/HORT/2159-95 provided by FCT and were supported in Florida by grants from the Florida Citrus Research Advisory Committee, a T-STAR-CBAG special Grant in Tropical Agriculture #94-34135-0652 and USDA Specific Cooperative Agreement 58-6617-0-102.

References

Alard P, Lantz O, Sebagh M, Calvo CF, Weill D, Chavanel G, Senik A and Charpentier B (1993) A versatile ELISA-PCR assay for mRNA quantitation from a few cells. Biotechniques 15: 730–736

Clark MF and Adams AN (1977) Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 33: 165–167

Holland P, Abramson RD, Watson R and Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 3′–5′-exonuclease activity of Taq polymerase. Proceedings of National Academy of Science (USA) 88: 7276–7280

Meng B, Johnson R, Peressini S, Forsline PL and Gonsalves D (1999) Rupestris stem pitting-associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. European Journal of Plant Pathology 105: 191–199

Nolasco G, Sequeira Z, Santos MT, Sequeira JC and Sequeira OA (1997) IC/RT-PCR coupled to exonuclease fluorescent assay. Early-spring detection of GLRaV-3 in leaf petioles. In: Extended Abstracts, 12th Meeting ICVG, Lisbon Portugal, 29 September–2 October. p 91

Nolasco G, Sequeira Z, Sabino J, Febres VJ, Cevik B, Lee RF and Niblett CL (1999) PCR based detection and strain typing of *citrus tristeza virus*. Petria 9: 131–135

Nolasco G, Mansinho A, Santos MT, Soares C, Sequeira Z, Sequeira JC, Correia PK and Sequeira OA (2000) Large scale evaluation of primers for diagnosis of Rupestris stem pitting-associated virus 1. European Journal of Plant Pathology 106: 311–318

Rowhani A, Biardi L, Routh G, Daubert SD and Golino D (1998) Development of a sensitive colorimetric-PCR assay for detection of viruses in woody plants. Plant Disease 82: 880–884

Schoen CD, Knorr D and Leone G (1996) Detection of potato leafroll virus in dormant potato tubers by immunocapture and a fluorogenic 5′-nuclease RT-PCR assay. Phytopathology 86: 993–999

Tyagi S and Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridisation. Nature Biotechnology 14: 303–308