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New device and method for capture, reverse transcription and nested PCR in a single closed-tube

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ABSTRACT

A device and improved method based on the use of a compartmentalized Eppendorf tube that allows capture, reverse transcription and nested-PCR in a single closed-tube has been developed and patented. The main advantages of the system are the high sensitivity obtained, the simplicity, the low risk of contamination and the easy establishment of adequate conditions for nested-PCR. The method has been successfully applied to the detection and characterization of citrus tristeza closterovirus and plum pox potyvirus isolates in plant tissues and single aphids squashed on paper. This device and methodology could be easily adapted to the detection of other targets.

Detection of plant RNA viruses is needed in sanitary improvement programs in which the purpose is the exclusive propagation of plant virus-free material. The most useful techniques for routine plant virus diagnosis are based on the use of serology (ELISA) with specific monoclonal antibodies. However, these techniques are not sensitive enough for detection of viral RNA targets in some woody plant tissues and vectors due to the low viral titre. In recent years, more sensitive techniques have been assayed to overcome the diagnosis problem. Different RT–PCR variants including heminested and nested-PCR have been proposed in plant pathology using immunocapture (IC) (with plant extracts) (1–4) or print/squash–capture (with immobilized targets on paper) (5,6). Several reports have demonstrated the potential of nested-PCR in other fields (7–9). However, the use of two rounds of amplification in different tubes enhances high risk of contamination, especially when the method is used in large scale. To avoid this, some authors proposed single tube nested-PCR protocols (10–12) in which the main disadvantage is the need to accurately establish the ratio between primers. In this article we describe a simple device and improved method based on the use of a compartmentalized Eppendorf tube that allows capture (if necessary), reverse transcription and nested-PCR in a single closed tube. In order to compartmentalize the PCR tube a number of systems have been assayed but the simplest one consisted of the use of the end (~2.7 cm) of a standard 200 µl plastic pipette tip. The small cone was introduced into a PCR tube allowing the possibility of physically separating two different PCR cocktails in the same Eppendorf tube (Spanish patent P9801642 of July 31, 1998 and Fig. 1). The first RT–PCR reaction mix was added to the bottom of the Eppendorf tube and the PCR reaction mix for the second amplification (nested) was added into the cone where it remained due to capillarity. After the first round of PCR the Eppendorf tube was centrifuged and consequently the second PCR cocktail mixed with the products of the first reaction. Accidental flow of the second PCR mix from the tip device might be caused by an incorrect manipulation of the device or the use of pipette tips wider than standard. This problem was rarely observed following our protocol and can be solved by closing (heating) the tip end. In this case, after the first round of PCR it is necessary to inverse the tube and vortex to mix the reagents. After the second round of PCR the tube was finally opened to analyze the amplicons. The final result was an amplification of at least 100 times more sensitive than a conventional IC RT–PCR (Fig. 2). The high sensitivity obtained with this method makes possible the detection of citrus tristeza closterovirus (CTV) and plum pox potyvirus (PPV) in plant tissues from several hosts or the amplification of PCR targets of non-persistent (PPV) and semipersistent (CTV) viruses from squashed aphids on paper. The cocktail for reverse transcription and external amplification was a mixture of 30 µl containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25°C), 0.3% Triton X-100 (w/v), 3 mM $MgCl_2$, 250 µM dNTPs, 0.5 µM of external primer 1, 0.5 µM of external primer 2, 1.2 U of AMV-RT (Promega) and 0.6 U of *Taq* DNA polymerase (Promega). The cocktail for the second (internal) amplification was a mixture of 10 µl containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25°C), 8 µM of internal primer 1 and 8 µM of internal primer 2. Primers for CTV detection were designed from conserved 3′UTR region, allowing the amplification of all isolates tested from the IVIA collection. External primers were: PEX1 (5′-TAAACAACACACTCTAAGG-3′) and PEX2 (5′-CATC-TGATTGAAGTGGAC-3′), and internal primers were: PIN1 (5′-GGTTCACGCATACGTTAAGCCTCACTT-3′) and PIN2 (5′-TATCACTAGACAATAACCGGATGGGTA-3′). Primers for PPV detection were designed for differentiation between non-aphid transmissible (NAT) and transmissible (AT) isolates. The external primers were: DEL1 (5′-GTTGGGTTCTTGAACAAGC-3′) and DEL2 (5′-CTAGGTGATGCATCCTCAT-3′) and the internal were: DEL3 (5′-CCCCGTACATTGCGGAGACAGC-3′) and DEL 4 (5′-CCGTAGTCCGGGGTGCAGGC 3′). RT–PCR was carried out in a Techne PHC3 cycler at 42°C for 30 min followed

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Figure 1. Simple and reliable device compartmentalizing an Eppendorf tube that allows capture, reverse transcription and nested-PCR in a single closed tube. Cocktail A corresponding to the reverse transcription and first PCR reaction mix is added to the bottom of the tube. Cocktail B, the PCR reaction mix for the second amplification, is added into the pipette tip cone.

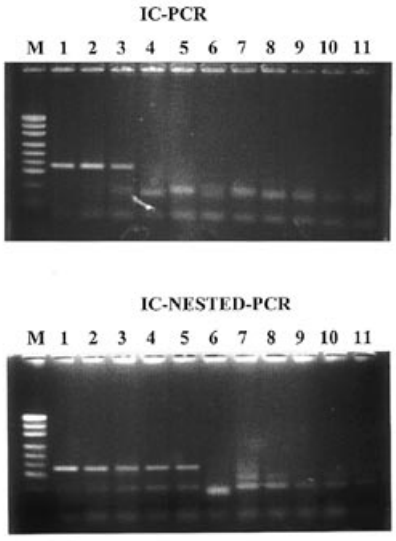

Figure 2. Comparison of the respective sensitivities of IC-PCR and IC-nested-PCR in single tube procedures for the detection of CTV. Ten-fold serial dilutions of an extract of infected Washington Navel sweet orange were prepared in healthy control extract (lanes 1–9). Amplification products (131 bp) obtained using IC-PCR (with internal primers of IC-nested-PCR) were detected (lanes 1–3) up to the $1:10^2$ (corresponding to a 1:3000 plant material w/v dilution factor). IC-nested-PCR products (131 bp) were detected (lanes 1–5) up to the $1:10^4$ (corresponding to a 1:300 000 plant material w/v dilution factor). No amplification products were detected in healthy control (lane 10) and cocktail controls (lane 11). Lane M, pUC19DNA/*Msp*I(*Hpa*II) Marker, 23 (MBI Fermentas).

by a denaturation at 94°C for 2 min and 20 cycles of amplification (92°C for 40 s, 45°C for 40 s and 72°C for 1 min 20 s) for CTV, and (92°C for 30 s, 54°C for 30 s and 72°C for 1 min) for PPV. After RT–PCR tubes were vortexed and centrifuged (6000 *g* for 2 s). Nested-PCR began with a denaturation phase of 2 min at 94°C, followed by 40 cycles of amplification, with a temperature profile of 30 s at 92°C, 30 s at 60°C and 1 min at 72°C (for CTV) and 30 s at 92°C, 30 s at 62°C and 1 min at 72°C (for PPV). Products of amplification were analyzed by 3% agarose gel electrophoresis in TAE buffer, stained by ethidium bromide and visualized under UV light. The expected bands obtained were a 131 bp product for CTV, 261 bp for NAT-PPV isolates and 216 bp for AT-PPV isolates.

Several aphid species (*Toxoptera citricida, Toxoptera auranti, Aphis gossypii, Aphis spiraecola, Aphis nerii, Myzus persicae* and *Hyalopterus pruni*) were analyzed by squash capture (6) nested PCR after feeding in CTV and PPV infected plants. The results show that PCR targets from both viruses were acquired by all assayed species independently of their ability to transmit the virus. Both AT and NAT-PPV were acquired also by aphids and amplified (data not shown). The main advantages of this method are the high sensitivity obtained without risk of contamination and the possibility of using external primers with the low annealing temperature and internal primers with the highest, in contrast with previously described protocols. This procedure saves time in establishing the optimal primers ratio because external primers do not interfere in the second amplification. The targets fixed in the plastic surface of the tube constitute enough material for the reaction without the need of using antibodies or other proteins in the capture. This method coupled with a simple preparation of printed samples on paper (5), instead of extract preparation, constitutes a useful tool for routine detection of RNA viruses. The high sensitivity of nested-PCR allows the use of immobilized targets on paper without the need of previous capture. In addition, this device and methodology should be easily adapted to the detection of other targets. The method has been successfully employed for sensitive amplification of single chain Fv (scFv) genes in transformed *Nicotiana tabacum* plants expressing 3DF1-scFv specific for CTV. This method allowed for the first time the capture, RT–PCR and nested in a single closed tube by a simple and reliable manner.

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