A rapid method for detecting specific amplified PCR fragments in microtiter plates

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ABSTRACT

A simple method is presented to circumvent laborious and time-consuming electrophoretic separations of specific PCR amplification products. Specific target DNA is amplified using nucleotides labelled with DIG-dUTP or biotin-dCTP. The labelled PCR products are separated from un-incorporated nucleotides or oligonucleotides by using a positively charged DEAE cellulose matrix. Amplification products are visualized directly in the matrix using immunoenzymatic methods or streptavidin-conjugated enzymes. The detection process can be carried out within 2 h, allows the processing of large sample sizes and can potentially be automated.

PCR products are usually visualized after electrophoresis on agarose or polyacrylamide gels by staining them with ethidium bromide or silver nitrate. This process is time consuming, laborious and difficult to automate. To circumvent electrophoretic separations of specific amplification products, several attempts were made to allow the processing of large sample sizes.

In one method for viral RNA detection, immunocapturing of viruses was combined with RT–PCR and the amplified products were quantified after adding a fluorescent dye using a fluorometer (1). However, this sensitive technique may generate considerable background noise if DNA other than the target is present in the sample. Other approaches involve hybridizations of PCR products to homologous probes. Avidin beads have also been used to collect biotinylated PCR products (3). The amplification products were separated from unincorporated nucleotides or oligonucleotides by using a positively charged DEAE cellulose matrix. Amplification products are visualized directly in the matrix using immunoenzymatic methods or streptavidin-conjugated enzymes for detection. The general validity of the method is demonstrated with three examples including the detection of viral RNA from PVY in potato leaves after RT–PCR, the detection of a mycoplasma-like organism (MLO) in coconut tissues and the detection of inserts in a genomic library of Prunus domestica L.

Specific primers for DNA amplification were employed for PVY detection (7) following immunocapturing and RT–PCR (1) and for the amplification of the 16S rDNA of MLO (8). The genomic Prunus library in Bluescript KS+ was screened for inserts using primers derived from T3 and T7 promoter sequences (9). PCR reactions were performed in microtiter plates (Thermowell, Costar) using the amplification conditions given by the authors mentioned above (1,8,9), except that the nucleotide mixtures contained, in addition to 100 µM of the other dNTPs, 90 µM dTTP and 2.5 µM DIG-dUTP or 90 µM dCTP and 2.5 µM biotin-14-dCTP, respectively, in order to label the corresponding PCR products. After amplification, 100 µl of DEAE Sephacel (Sigma, I 6505), equilibrated as described previously (9), was added to each well. The resin was kept in suspension for 10 min by gentle shaking of the plate in order to allow an efficient binding of larger DNA molecules to the matrix. Sedimentation of the resin occurs by gravity in a few minutes. The supernatant of each well was then carefully removed with a pipette, in order to avoid loss of resin and cross-contaminations. The resin was washed three times using the above procedure with 150 µl TE buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA) containing 0.3 M NaCl in the first two washes and 0.1 M NaCl in the last. Unspecific binding sites in the resin, as well as in the walls of the wells, were blocked by adding 1.5% skimmed milk suspension in TC buffer (TE buffer containing 0.1 M NaCl) and incubating for 30 min. After washing

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with TC, the resin was incubated for 30 min with 75 μl anti-DIG antibody solution (1:5000 v/v in TC) conjugated with alkaline phosphatase (Boehringer) or with a 75μl conjugate of streptavidin solution (1:1000 v/v in TC) and alkaline phosphatase (Tropix), keeping the resin in constant suspension. Afterwards the resin was washed three times with TC and once with TS buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2). Finally 75μl TS containing 0.033% NBT (nitro blue tetrazolium) and 0.017% BCIP (5-bromo 4-chloro 3-indolyl phosphate) were added and the microtiter plate was placed in the dark. Colorimetric reactions became visible after a few minutes and were stopped after 20 min by adding 50μl 0.1 M HCl (10%). Optical densities were recorded using a Spectrophotometer (Bausch & Lomb, Spectronic 2000) at 550 nm.

The whole detection process can be carried out within 2 h, and it is evident that all steps involved in this procedure including washing, suspending, sedimentation and decantation can potentially be automated. Compared with previous methods (5,6), no primer labelling or additional coated microtiter plates are necessary. Although the procedure shows a general applicability, one must ensure that non-specific amplifications are excluded or suppressed and only one unique DNA fragment is amplified during the PCR reaction. Therefore appropriate primers and highly selective amplification conditions must have been set up previously for each detection system.

**REFERENCES**