Phytopathol. Mediterr. (2004) 43, 221–227

Nucleic acid-based assay for the diagnosis of viral pathogens

Donato Gallitelli

Dipartimento di Protezione delle Piante e Microbiologia Applicata and Istituto di Virologia Vegetale del CNR, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy

Summary. A knowledge of the genetic structure and evolution of plant virus populations is crucial to predict the possible emergence of resistance-breaking pathotypes, as recently demonstrated with *Tomato spotted wilt virus*. The advent of analytical techniques for the fast identification of changes in nucleotide sequences, and the need to analyze the benefits and risks of new control strategies, such as those offered by virus-resistant transgenic plants, has led to an increasing interest in techniques exploiting the fine analysis of the virus genome. The choice of an analytical technique should depend on the goal of the analysis, as well as on the sensitivity and cost of the technique. Some techniques provide only qualitative data that can be used to identify variants, whereas others can be used to assess how different the identified variants are. Such estimates can be given by the amino acid composition of the viral proteins, or by restriction fragment length polymorphism (RFLP) analysis. DNA microarray technology is another promising tool which meets both the diagnostic and the detection needs of modern plant virology.

Key words: plant virus detection, nucleic acid hybridization, *in situ* hybridization, real-time PCR, DNA microarrays.

Introduction

Many methods have been developed and applied to diagnose plant viruses. Some of these methods are biological (e.g. indexing on a susceptible plant genotype), others are serological (enzyme-linked immunosorbent assay [ELISA] and lateral flow test carried out with either polyclonal antisera or monoclonal antibodies) or molecular (nucleic acid hybridization, PCR or real time PCR). Most of diagnostic methods are directed at the highly specific detection of an individual virus target that is known or presumed to occur (Bos, 1999). Therefore they could be more properly described as detection methods. True diagnostic methods find a causal virus and identify it as such by the application of Koch's rules. Detection establishes the occurrence of a predetermined target virus, with a special emphasis on symptomless plants, while diagnosis tries to establish the nature and cause of a disease, and thus mainly concerns plants already showing symptoms.

Why to use nucleic acid techniques to detect plant viruses?

In biological assays the virus that is to be detected is transmitted to a suitable herbaceous or woody indicator plant (indexing), where it is identified by reading off the indicator plant's reactions. Although bioassays can be very sensitive and reliable, the procedures are costly, time-consuming and require expensive facilities (glasshouses,

To correspond with the author:
Fax: +39 80 5442911
E-mail: gallitel@agr.uniba.it.

screenhouses, field plots etc.). Moreover, the symptoms induced by a virus on indicator plants may vary according to the virus strain, the age of the plant, and growing conditions. Moreover, different plant species frequently react to different viruses with the same symptoms. All these factors mean that biological assays are not always the certain indicators of a given viral presence. Finally bioassays do not detect viruses that remain latent also in the indicator plant. This is now being recognized as an emerging problem as e.g. with a strain of *Plum pox virus* (PPV) from Slovakia. Glasa *et al*. (2001) reported that unlike what happened with other PPV isolates, when the indicator *Prunus persica* cv. GF 305 was infected with BOR-3, a natural recombinant isolate of PPV, the infection produced was either symptomless or symptoms were very weak. This makes GF305 questionable as an indicator.

Serology explores the different immunological properties of the viral coat protein, which represents only a small part of the coding capacity of the viral genome. Although serology is mostly sufficient for the proper identification of plant viruses, it has serious limitations when applied to: (i) unstable or poorly immunogenic viruses, (ii) viruses requiring laborious purification procedures, (iii) detection of non encapsidated double-stranded or single-stranded RNAs, (iv) detection of defective virus particles, defective interfering RNAs or satellite RNAs, (v) detection of viroids (e.g. infective agents deprived of coat protein), (vi) the differentiation of very closely related virus strains. Nevertheless, serology is still widely used and ELISA remains among the major developments that have taken place in plant virus detection over the past 25 years. This is not only because of the sensitivity of this method but also because of the number of ELISA analyses carried out in different laboratories all over the world each year. The specificity and robustness of detection has improved greatly with the use of monoclonal antibodies which make it possible to select specific target epitopes and thus to avoid or dramatically reduce false positives. Finally, serology offers the most immediate answer to the increasing demand for simple commercial methods for the rapid on-site-testing of large numbers of samples. Tissue print-ELISA and lateral flow devices (Danks and Barker, 2000) fit these purposes quite well. Specificity of these technique is high when using the appropriate monoclonal antibodies but sensitivity is relatively low making them more appropriate for screening plants that already have symptoms.

Owens and Diener (1981) were the first to show that nucleic acid-based assays could be used to detect plant pathogenic viruses and viroids, thus introducing a technique that has gained great popularity with many applications ranging from pure research to large-scale and routine testing. Molecular applications are highly sensitive, reliable, space- and labour-saving and possess enough flexibility to be carried out in parallel, as in the case of microarray technology (Schena, 2000).

Hybridization formats

The theoretical basis of nucleic acid hybridization is that, under suitable conditions of temperature and salt concentration, complementary sequences of single-stranded molecules will anneal to form stable double-stranded structures (hybrids). When applied as a diagnostic tool, the method requires the target nucleic acid to be fixed, usually to a solid support, and the complementary nucleotide sequence (probe) carries a label that provides the necessary signal whereby hybridization is recognized. Nucleic acid probes can be DNA or RNA single-stranded or double-stranded and labelled with either a radioactive (e.g. ^{32}P) or a non-radioactive (e.g. digoxigenin) reporter group . Unless required for specific purposes, the trend is to prepare and use RNA instead of DNA probes because: (i) the majority of plant viruses have RNA genomes; (ii) single-stranded probes are less likely to self-anneal than double stranded probes; (iii) RNA:RNA hybrids are more stable than RNA:DNA or DNA:DNA hybrids. Stability of RNA:RNA hybrids enables the use of highly stringent hybridization conditions, enhancing probe specificity and reducing background problems due to the interference of plant sap. Various refinements and modifications of the hybridization techniques have been proposed, aimed at improving sensitivity and reducing background interference, which with certain types of plant material is unacceptably high. Initially proposed as a detection technique in plant virology, nucleic acid hybridization is now widely used to study plant viral genome organization and virus-host interactions.

Mixed-phase formats

Dot blot or spot hybridization

This hybridization format simply answers the question of whether a plant is or is not infected by a given virus. Dot blotting does not distinguish between the number and size of hybridized molecules since the hybridization signal is the sum of all sequences recognized by the probe. However, the technique is rapid and versatile identifying specific nucleic acid sequences in samples ranging from crude plant sap to highly purified preparations. This makes it suitable for routine and large-scale testing in, for example, sanitary certification schemes that require processing of many samples in a short time. To save time and reduce cost and labor, the simultaneous use of the six riboprobes in a hybridization reaction was proposed by our laboratory for the sanitary certification of tomato seedlings in the nursery (Saldarelli *et al.,* 1996). Dot blot hybridization is also widely used in breeding programs to screen for resistance and to detect viruses in their vector.

Southern and Northern blot hybridization

This hybridization format gives more qualitative results than dot blot since it precisely identifies the molecule recognized by the probe. Total nucleic acid preparations or viral nucleic acid extracted from purified virions are suitable material for this type of analysis. Nucleic acid samples must be subjected to gel electrophoresis, transferred to a membrane by capillarity and subjected to hybridization. This technique is useful mainly in basic research and includes determination of the pattern of the viral nucleic acid, detection of the non-encapsidated nucleic acid (e.g. subgenomic RNAs, defective-interfering nucleic acids [DI], satellite RNAs, small-interfering RNAs [siRNAs] in RNA silencing studies) and detection of virus-related transgenic inserts for basic research and for regulatory issues.

In situ *hybridization.*

In situ hybridization (ISH) is used to detect either specific viral sequences or proteins; it combines microscopy observation and hybridization. Its application to the detection of plant viruses is quite recent and parallels the renewed interest in virus-host interactions as output of the post-genomic era. *In situ* hybridization gives information on the distribution of the target nucleic acid within a cell or tissue and is routinely applied to the localization of specific viral sequences involved in replication (see, for example, Cillo *et al*., 2002) and movement, and to detect the integration of viral sequences in the plant chromosome (e.g. *Banana streak virus* in *Musa* sp.) (Harper *et al.,* 1999).

Liquid-liquid hybridization

Polymerase chain reaction

Increasing understanding of the nucleotide sequence of viral genomes is making it possible to design specific oligonucleotides that can be used as primers for the selective amplification of a target nucleic acid from a pool of complex template by a polymerase chain reaction (PCR) driven by a thermostable polymerase (e.g. *Taq*). The amplified fragment (amplicon) is detected by gel electrophoresis or characterized by nucleic acid hybridization, sequencing and restriction enzyme digestion. A major advantage of PCR is that it can be used to increase the concentration of pathogen-related sequences which in naturally infected hosts are below detection level either because they occur in too low amounts, are localized in certain tissues (i.e. phloem-limited viruses), or are erratically distributed.

Since *Taq* polymerase recognizes only DNA templates, to be applied to RNA viruses, PCR requires first a reverse-transcription step (RT-PCR). In the RT-PCR protocol, antisense primers for the synthesis of first-strand cDNA are a crucial factor for specific amplification. To ensure strain specificity, these primers must be selected within those regions of the genome that are highly variable among members of the same group. Sense primers are usually chosen from the more conserved regions, as the selection has already been made by primers for first-strand synthesis of cDNA. Designing a multiplex PCR saves time and reagent costs, and colorimetric detection of PCR products (e.g. with primers labelled with different fluorophores) facilitates interpretation of the results if the technique is used in routine analyses (Bertolini *et al.,* 2001). Since templates of different viruses can be co-amplified in a single reaction using a set of specific primers it is possible to simultaneously detect and identify unrelated viruses in the case of mixed infections. This may be useful to detect viral diseases affecting vegetatively propagated species, in which a

great array of viruses have accumulated over time as, for example, in woody crops. Although PCR can be very sensitive and specific, its introduction for routine detection has been hampered by its lack of robustness and by the complexity of the post-amplification analysis required. PCR sometimes fails to correctly diagnose both infected and non-infected plant material since carry-over contamination of amplicons may lead to false-positive results, and inhibitor components in sample extracts may yield false negatives.

The sensitivity of PCR amplification can be enhanced in a number of ways. The template chosen can be a viral nucleic acid released from viral particles that were first trapped on a solid support by a specific antiserum. This system, named immunocapture-PCR (IC-PCR) (Wetzel *et al.,* 1992), makes it possible to use sample volumes 200–250 times greater than those utilized in standard PCR, and has been used with plant extracts or with immobilized targets on paper (print/squash-capture [PC/SC] RT-PCR) (Olmos *et al.,* 1996) allowing viral detection from plant material or insect vectors without extract preparation. A nested (or heminested) PCR can be helpful when the amount of the target sequence is below the detection limit of standard PCR or when the plant sap contains inhibitors. However, nested-PCR requires two rounds of amplification in different tubes, increasing the risk of contamination. In order to avoid this problem, several alternatives with single closed tubes have been developed (Yourno, 1992). Lastly, a technique known as co-operational PCR (Co-PCR) has recently been described. Co-PCR is based on the simultaneous action of four or three primers. The reaction process consists of the simultaneous reverse transcription of two different fragments from the same target, one internal to the other, the production of four amplicons by the combination of the two pairs of primers, one pair external to the other, and the cooperational action of amplicons for the production of the largest fragment (Lopez *et al.,* 2003). Coupled with colorimetric detection, the sensitivity observed is at least 100 times greater than that achieved with RT-PCR, and is similar to that of nested RT-PCR.

Other applications of the PCR technology include: (i) direct production of probes by incorporating nucleotides labelled with a reporter group into the reaction mix; (ii) direct cloning of the amplicon (e.g. pGEM-T [Promega] system or primers incorporate a suitable restriction site); (iii) direct transcription of the amplicon (a primer incorporates an RNA-polymerase promoter); (iv) production of overlapping amplicons to obtain the complete nucleotide sequence of large fragments. A particular format of PCR, PCR-RFLP (RT-PCR-RFLP for RNA viruses), is a powerful tool to study plant virus evolution, a subject that, viewed from a molecular standpoint, is known as molecular epidemiology (reviewed by García-Arenal *et al.,* 2001). PCR-RFLP can carry to typify a great number of isolates and characters using hot spots provided by sequence data to detect variations in the resident virus population and can predict the emergence of resistance-breaking pathotypes as in recently reported cases of *Tomato spotted wilt virus* (Hoffman *et al.,* 2001; Aramburu *et al.,* 2002; Finetti-Sialer *et al.,* 2002). It can also be used to assess the risks of new control strategies such as those involving the use of virus-resistant transgenic plants. Finally, *in situ* PCR (ISPCR) allows specific nucleic acid sequences to be detected in intact cells and tissues. This technique is based on a reaction performed on fixed whole cells or tissue sections, to identify amplicons at the site where they are produced (Nuovo, 1992). When applied to plant viruses, this technique is an excellent means to localize virus and virus-related sequences in infected cells. Similar results can in principle be obtained with the ISH described above. However, ISH not only requires hundreds of target molecules per cell for a reliable signal, but detection of the hybrid molecule often involves autoradiography or immunodetection. Although ISH has been successfully applied to plant viruses, there are cases in which virus and virus-related sequences are below detection level either because they occur in extremely low amounts, or are restricted to certain tissues (e.g. phloem) or are erratically distributed. This problem can be circumvented with a two-step protocol using *in situ* PCR to increase the amount of the target molecules, and ISH to detect the amplicon.

Real time PCR

Most of the limitations of conventional PCR mentioned above can be overcame using a real-time PCR detection system. Real-time PCR, which detects PCR products while the reaction is going on,

has been available for 7–8 years, but it has shown a dramatic increase in use in the last 3–4 years. A Medline search with the keyword "real-time PCR" will generate thousands of hits, showing how this technique is emerging into the mainstream of qYny scientific disciplines. Examples of real-time PCR as applied to the detection of plant viruses can be found in Schoen *et al.* (1996), Eun *et al.* (2000), Finetti-Sialer *et al.* (2000), Roberts *et al.* (2000), Boonham *et al.* (2002), Korimbocus *et al.* (2002). Real-time PCR monitoring with specific instruments and fluorescent probes offers the advantage of combining the amplification, detection and quantification of the target molecule in a single step. The chemistries (http://www.eurogentec.com/code/en/catalogues.htm#top) most commonly used with real-time PCR can be divided in non-specific and specific. Non-specific methods use a dye (e.g. SYBR green I [Morrison *et al.,* 1998]) emitting fluorescent light when intercalated into double-stranded DNA (dsDNA). In solution, unbound dye exhibits very little fluorescence but when the dye is bound to DNA, fluorescence is greatly enhanced and is proportional to the amount of total dsDNA in the reaction. Since these dyes do not discriminate between the different dsDNA molecules, synthesis of non-specific amplicons, as well as of dimers, must be prevented by accurate primer design and condition optimization. Specific methods are based on the use of oligonucleotide probes labeled with a donor fluorophore and an acceptor dye (quencher) (Whitcombe *et al.,* 1999) that generate a light signal according to fluorescence resonance energy transfer (FRET) chemistry.

The advantage of fluorogenic probes over DNA-binding dyes is that specific hybridization between the probe and the target DNA sequence is required to generate a fluorescent signal; so that non-specific amplifications do not generate a signal. Furthermore, fluorogenic probes can be labeled with different distinguishable reporter dyes to amplify and detect two or more distinct sequences in a single PCR reaction tube, without melting curve analysis (multiplex PCR). The specific method includes TaqMan (Livak *et al.,* 1995), molecular beacons (Tyagi and Kramer, 1996), and scorpion PCR (Whitcombe *et al.,* 1999). The fluorescent probes and the instrumentation required are still very expensive, although there is a less expensive alternative: portable rapid cycling real-time PCR platforms (e.g, Smart Cycler, International Laboratory, which allow multiple sample analysis and can be used for on-site (field) detection.

A reverse format of mixed-phase hybridization: DNA microarrays

DNA microarrays or biochips are the most recent tool developed for plant virus detection. A DNA chip allows the simultaneous interrogation of hundreds to thousands of cDNAs arrayed on a small surface (a microscope slide in the simplest format) approximately 1 cm^2 in size. Each cDNA is located at a specific address on the surface, called a spot or a feature. Interrogation is carried out by reverse mixed-phase hybridization format, in which target molecules extracted from the sample are cDNAs labeled with a specific fluorophore and maintained in solution, while probes are cDNAs or short oligonucleotides (50 bp) obtained from sequence data of the pathogen to be detected and arrayed on the support. The detection system uses one or more fluorophores, which are read with laser technology, while nucleic acids are extracted from the sample, labelled and hybridized using standard laboratory techniques. Chip technology can be used to monitor gene expression in different plant-pathogen combinations (see for example Itaya *et al.,* 2002; Park *et al.,* 2004) and has been applied for the detection and differentiation of four cucurbit-infecting Tobamoviruses (Lee *et al.,* 2003) and four potato viruses (Boonham *et al.,* 2003). Microarray methodology was examined in those two papers for its potential in viral diagnostics. Since this method is completely generic, it can be used to detect all viruses whose sequence is currently available, but its cost is very high. Consequently, it is still far from common in routine detection, but it is being increasingly used in functional genomics studies.

Concluding remarks

The array of techniques available for the rapid, specific, and sensitive detection of plant pathogenic viruses has much improved in the last few years and signal amplification through chemical, molecular or electronic methods has increased, becoming more and more independent from visible disease symptoms. As new genomic and proteomic data become available, techniques with increased sensitivity and specificity will probably be devel-

oped and adapted for the simultaneous and real-time detection of viruses and other plant pathogens using hot spots in their genetic profile. These new approaches, coupled with more appropriate sampling methods, will provide a more complete picture of the life-cycle of plant pathogens by improved detection of latent infections, pathogen reservoirs and by a better understanding of the structure and function of pathogen communities. These techniques will advance the understanding and prevention of plant diseases, but will at the same time also demand a proportionally increasing amount of economic and expert human resources.

Literature cited

Aramburu J., A. Crescenti and M. Martì, 2002. Primera detecciòn de un aislado del TSWV en tomate que sobrepasa la resistancia conferida por el gen sw-5. *XI Congreso de la Sociedad Espanola de Fitopatologia*, 14–18 Octubre, 2002, Almeria, Spain.

Bertolini E., A. Olmos, M.C. Martınez, M.T. Gorris and M. Cambra, 2001. Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA viruses in olive trees. *Journal of Virological Methods* 96, 33–41.

Boonham N., P. Smith, K. Walsh, J. Tame, J. Morris, N. Spence, J. Bennison and I. Barker, 2002. The detection of *Tomato spotted wilt virus* (TSWV) in individual thrips using real time fluorescent RT-PCR (TaqMan). *Journal of Virological Methods* 101, 37–48.

Boonham N., K. Walsh, P. Smith, K. Madagan, I. Graham and I. Barker, 2003. Detection of potato viruses using microarray technology: towards a generic method for plant viral disease diagnosis. *Journal of Virological Methods* 108, 181–187.

Bos L. (ed.), 1999. Disease diagnosis and routine virus detection. In: *Plant Viruses, Unique and Intriguing Pathogens*. Backhuys Publishers, Leiden, Netherlands, 193 pp.

Cillo F., I.M. Roberts and P. Palukaitis, 2002. *In situ* localization and tissue distribution of the replication-associated proteins of *Cucumber Mosaic Virus* in tobacco and cucumber. *Journal of Virology* 76, 10654–10664.

Danks C. and I. Barker 2000. On-site detection of plant pathogens using lateral-flow devices. *EPPO Bulletin* 30**,** 421–426.

Eun A.J.C., M.L. Seoh and S.M. Wong 2000. Simultaneous quantification of two orchid viruses by the TaqMan® real-time RT-PCR. *Journal of Virological Methods* 87, 151–160.

Finetti-Sialer M., A. Ciancio and D. Gallitelli, 2000. Use of fluorogenic scorpions for fast and sensitive detection of plant viruses. *EPPO Bulletin* 30, 437–440.

Finetti-Sialer M., C. Lanave, M. Padula, C. Vovlas and D. Gallitelli, 2002. Occurrence of two distinct *Tomato spotted wilt virus* (TSWV) subgroups in Southern Italy. *Journal of Plant Pathology* 84(3), 145–152.

García-Arenal F., A. Fraile and J. Malpica 2001. Variability and genetic structure of plant virus populations. *Annual Review of Phytopathology* 39, 157–186.

Glasa M., R. Kúdela, V. Marie-Jeanne and J.B. Quiot, 2001. Evidence of a naturally occurring recombinant isolate of *Plum pox virus* from Slovakia. *Plant Disease* 85, 920.

Harper G., J. Osuji, J.S. Heslop-Harrison and R. Hull 1999. Integration of banana streak badnavirus into the Musa genome: molecular and cytogenetic evidence. *Virology* 255, 207–213.

Hoffman K., W.P. Qiu and J.W. Moyer 2001. Overcoming host and pathogen-mediated resistance in tomato and tobacco maps to the M-RNA of tomato spotted wilt virus. *Molecular Plant-Microbe Interactions* 14, 242–249.

Itaya A., Y. Matsuda, R.A. Gonzales, R.S. Nelson, and B. Ding 2002. *Potato spindle tuber viroid* strains of different pathogenicity induce and suppress expression of common and unique genes in infected tomato. *Molecular Plant-Microbe Interactions* 15, 990–999.

Korimbocus J., D. Coates, I. Barker and N. Boonham, 2002. Improved detection of Sugarcane yellow leaf virus using a real-time fluorescent (TaqMan) RT-PCR assay. *Journal of Virological Methods* 103, 109–120.

Lee G.P., B.E. Min, C.S. Kim, S.H. Choi, C.H. Harn, S.U. Kim and K.H. Ryu 2003. Plant virus cDNA chip hybridization for detection and differentiation of four cucurbit-infecting Tobamoviruses. *Journal of Virological Methods* 110, 19–24.

Livak K.J., S.J.A. Flood, J. Marmaro, W. Giusti and K. Deetz, 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* 4, 357–362.

Lopez M.M., E. Bertolini, A. Olmos, P. Caruso, M.T. Gorris, P. Llop, R. Penyalver and M. Cambra, 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology* 6(4), 233–43.

Morrison T.M,, J.J. Weis and C.T. Wittwer, 1998. Quantification of low-copy transcripts by continuous SYBR green I monitoring during amplification. *BioTechniques* 24, 954–962.

Nuovo G.J., 1992. *PCR in situ hybridization: protocols and applications*. Raven Press, New York, NY, USA, 237 pp.

Olmos A., M.A. Dasi, T. Candresse and M. Cambra 1996. Printcapture PCR: a simple and highly sensitive method for the detection of plum pox virus (PPV) in plant tissues. *Nucleic Acids Research* 24, 2192–2193.

Owens R.A. and T.O. Diener 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. *Science* 213, 670–672.

Park C.J., K.J. Kim, R. Shin, J.M. Park, Y.C. Shin and K.H. Paek 2004. Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. *Plant Journal* 37, 186–198.

Roberts C.A., R.G. Dietzgen, L.A. Heelan and D.J. Ma-

clean 2000. Real-time RT-PCR fluorescent detection of tomato spotted wilt virus. *Journal of Virological Methods* 88, 1–8.

Saldarelli P., L. Barbarossa, F. Grieco and D. Gallitelli 1996. Digoxigenin-labelled riboprobes applied to phytosanitary certification of tomato in Italy. *Plant Disease* 80, 1343–1346.

Schena M., 2000. *Microarray biochip technology*. BioTechniques books publication, Eaton Publishing, Natick, MA, USA, 297 pp.

Schoen C.D., D. Knorr and G. Leone 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 F.R. Kramer 1996. Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnology* 14, 303–308.

Wetzel T., T. Candresse, G. Macquaire, M. Ravelonandro and J. Dunez 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods* 39, 27–37.

Whitcombe D., J. Theaker, S.P. Guy, T. Brown and S. Little, 1999. Detection of PCR products using self probing amplicons and fluorescence. *Nature Biotechnology* 17, 804–807.

Yourno J., 1992. A method for nested PCR with single closed reaction tubes. *PCR Methods Applied* 2, 60–65.

Accepted for publication: June 14, 2004