Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA

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ABSTRACT

Molecular beacon probes can be employed in a NASBA amplicon detection system to generate a specific fluorescent signal concomitantly with amplification. A molecular beacon, designed to hybridize within the target sequence, was introduced into NASBA reactions that amplify the genomic RNA of potato leafroll virus (PLRV). During amplification, the probe anneals to the antisense RNA amplicon generated by NASBA, producing a specific fluorescent signal that can be monitored in real-time. The assay is rapid, sensitive and specific. As RNA amplification and detection can be carried out in unopened vessels, it minimizes the risk of carry-over contaminations. Robustness has been verified on real-world samples. This homogeneous assay, called AmpliDet RNA, is a significant improvement over current detection methods for NASBA amplicons and is suitable for one-tube applications ranging from high-throughput diagnostics to *in vivo* studies of biological activities.

INTRODUCTION

The isothermal amplification method for nucleic acids, NASBA, is a technology with the potential for broad applications in the field of RNA amplification and detection (1). With respect to other amplification systems such as the PCR technique, the ability of NASBA to homogenously and isothermally amplify RNA analytes (e.g., viral genomic RNA, mRNA or rRNA) extends its application range from viral diagnostics to the indication of biological activities such as gene expression and cell viability (2). A NASBA reaction is based on the concurrent activity of AMV reverse transcriptase (RT), RNase H and T7 RNA polymerase, together with two primers to produce amplification (3). This process occurs at one temperature (41°C) without the need of adding intermediate reagents and results in the exponential amplification of RNA and DNA products within 90 min, producing as the major amplification product antisense, single-stranded RNA.

The characteristics of NASBA make this method potentially suitable for high-throughput sample analysis and the development of completely automated workstations (2). Post-NASBA product detection, however, can still be a labor-intensive procedure, normally involving ethidium bromide (EtBr)-stained agarose gel electrophoresis and various probing and/or blotting techniques. Since it was first described (3), the post-NASBA detection step has undergone significant improvements, such as the use of an enzyme-linked gel assay (ELGA; 4), enzymatic bead-based detection and electrochemiluminescent (ECL) detection (5), and fluorescent correlation spectroscopy (6). However, as these methodologies are heterogeneous or they require some handling of sample or robotic devices that are currently not cost-effective, they still have a relatively low impact for high-throughput applications in routine laboratories. A homogeneous procedure in which product detection is concurrent with target amplification by the generation of a target-specific signal would facilitate large-scale screenings and full automation. Moreover, the procedure could take place in unopened reaction vessels, avoiding the risk of carry-over contamination in the post-amplification processing steps.

Homogeneous, fluorescence-based assays, in which amplification and detection occur without the need to separate reaction components, have recently been described for PCR. Higuchi *et al.* (7) reported the monitoring of EtBr fluorescence during the PCR process. Livak *et al.* (8) described the use of fluorescent-labelled oligonucleotide probes coupled to the endogenous 5′ exonuclease activity of *Taq* DNA polymerase as a means of cleaving a quenched fluorescent moiety from the probe. Both methods, however, introduce limitations in specificity of the assay (7) or in the reaction components (8), rendering them impracticable for NASBA reactions.

Very recently, a novel nucleic acid detection technology, based on probes (molecular beacons) that fluoresce only upon hybridization with their target, has been introduced (9). Molecular beacons are single-stranded oligonucleotides having a stem–loop structure. The loop portion contains the sequence complementary to the target nucleic acid, whereas the stem is unrelated to the target and has a double-stranded structure. One arm of the stem is labelled with a fluorescent dye, and the other arm is labelled with a non-fluorescent quencher. In this state the probe does not produce

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fluorescence because the energy is transferred to the quencher and released as heat (10). When the molecular beacon hybridizes to its target it undergoes a conformational change that separates the fluorophore and the quencher, and the bound probe fluoresces brightly.

This paper describes the coupling of RNA amplification by NASBA with amplicon detection by molecular beacons technology to produce a truly homogeneous RNA assay, called AmpliDet RNA. We report how molecular beacons improve NASBA, enabling a one-tube assay suitable for high-throughput applications without compromising specificity and sensitivity. In addition, the novel assay permits real-time monitoring of the NASBA amplification reaction, endowing the determination of the effect of different NASBA conditions, and enabling future applications for kinetic and *in vivo* studies of biological activities.

MATERIALS AND METHODS

Plant material and virus

Healthy and PLRV-infected tubers were produced as previously described (11) and stored at 4°C until necessary. Purified, intact PLRV was a gift from Dick Maat at IPO-DLO. RNA was extracted from PLRV virions by repeated phenol/chloroform extractions in the presence of 0.5% SDS.

Selection of amplification primers and probe

Amplification of PLRV was sustained by oligonucleotides PD415 or PD416 (antisense) and PD417 (sense; 11) and designed from the coat protein open reading frame (ORF) of the virus (12). Preparation of oligonucleotides was as previously described (11). As detection probe, the 20mer oligonucleotide PRO1 (5′-GCAAAGTATCATCCCTCCAG-3′) was chosen. It binds to nucleotides 4003–4022 of the coat protein ORF of PLRV.

Synthesis of the molecular beacons

A molecular beacon (sw75-Fl) was synthesized, containing the same probe sequence as oligonucleotide PRO1, embedded within 6 nucleotide long arm sequences (underlined in the text), resulting in the sequence 5′-<u>CCAAGC</u>GCAAAGTATCATCCCTCC-AG<u>GCTTGG</u>-3′. Coupling of amino-reactive DABCYL to the 3′ end of the oligonucleotide and of fluorescein at its 5′ end was as described by Tyagi and Kramer (9). A detailed protocol is available on the worldwide web at htpp://www.phri.nyu.edu/molecular_beacons

NASBA

A premix for a number of reactions was prepared. Each reaction contained 6 µl of sterile water, 4 µl of 5× NASBA buffer and 4 µl of 5× primer mix. When working with the molecular beacons, the premix contained 4 µl of water, 1 µl of molecular beacon dilution (see Results), and 1 µl of a 20 pmol/µl solution of ROX [5-(and-6)-carboxy-X-rhodamine; Molecular Probes]. The 5× NASBA buffer consisted of 200 mM Tris–HCl, pH 8.5, 60 mM MgCl$_2$, 350 mM KCl, 2.5 mM DTT, 5 mM of each dNTP (Pharmacia), 10 mM each of ATP, UTP and CTP, 7.5 mM GTP (Pharmacia) and 2.5 mM ITP (Boehringer). The 5× primer mix consisted of 75% DMSO and 1 µM each of antisense and sense primers. The premix was divided into portions of 14 µl in

microtubes. Then, 1 µl of input material (purified PLRV RNA or purified virus) was added. With immunocapture (IC)-NASBA or with water controls, 1 µl of water was added to the premix. The immunocapture step was essentially performed as described previously (11). The reaction mixtures were incubated at 65°C for 5 min and, after cooling to 41°C for 5 min, 5 µl of enzyme mix was added. This mix consisted of (per reaction) 375 mM sorbitol, 2.1 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase. All enzymes were purchased from Pharmacia, except AMV-reverse transcriptase (Seigakaku). Reactions were then incubated at 41°C for 90 min and amplificates were stored at –20°C for further use.

Post-NASBA analysis

NASBA amplification products were analyzed at the end of the amplification step, by electrophoresis of 5 µl of reaction on a 2% pronarose (Sphaero Q) gel containing 0.5 µg/ml EtBr. Gels were run at 150 V for 20 min in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Bands were visualized by UV excitation and photographed.

The specificity of the amplification products was analyzed further by northern blotting using oligonucleotide probe PRO1 carrying a biotin label at its 5′ end for subsequent chemiluminescent, non-radioactive detection. Gels were blotted in 2× SSC (1× SSC: 0.15 M NaCl and 15 mM Na-citrate) onto a Zeta-Probe nylon membrane (BioRad) for 1 h, using a vacuumblot apparatus (VacuGel XL; Pharmacia). Nucleic acids were cross-linked to the membrane with exposure to UV light for 2 min or by baking at 80°C for 1 h. Hybridization with 5 µl, 3 µM biotinylated probe PRO1 occurred at 50°C for 30 min in 5× SSC, 7% SDS, 20 mM Na-phosphate, pH 6.7, and 10× Denhardts solution (100× Denhardts: 20 g/l PVP, 20 g/l BSA, 20 g/l Ficoll 400). The blots were washed twice for 5 min in 3× SSC, 1% SDS at 50°C and incubated for 30 min with (1 vol) streptavidine/peroxidase conjugate (Boehringer) in (5000 vol) 5× SSPE (1× SSPE: 10 mM Na$_2$HPO$_4$, 0.18 M NaCl and 1 mM EDTA), 0.5% SDS at room temperature. All subsequent steps were carried out at room temperature. Blots were washed twice for 1 min with 2× SSPE, 0.1% SDS, once more for 10 min in the same solution, and thereafter twice for 2 min in 2× SSPE. Blots were then incubated for 60 s in a reagent solution for chemiluminescent detection (Amersham Life Science) and exposed for 15, 30 or 60 s to X-ray films (Kodak).

Post-NASBA detection by molecular beacon was performed by titrating aliquots of NASBA reactions into solutions of molecular beacons (see Results) in a microtiter plate, warming up the solutions to 70°C for 5 min and monitoring the fluorescence spectra in a Perkin-Elmer LS-50B luminescence spectrometer at room temperature. The 20 µl reactions were excited at 488 nm and fluorescence was measured at 520 nm.

Real-time monitoring of NASBA reactions and thermal denaturation profiles

NASBA amplification of PLRV genomic RNA was assessed in real-time by adding molecular beacons to the reaction mix as described above. After the addition of the enzyme mix to the reactions, microtubes were transferred to an Applied Biosystems ABI Prism™ 7700 Sequence Detector. Development of fluorescence was followed in closed tubes for 90 min at 41°C. All readings taken were relative to the fluorescence of a reference
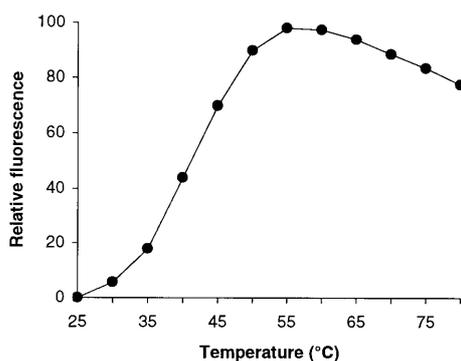
Figure 1. Thermal transition profile of molecular beacon sw75-Fl.

Figure 2. Fluorescence emission by molecular beacon sw75-Fl upon hybridization to its target NASBA amplicon present in different volumes of the same NASBA reaction mixture.

fluorophore (ROX). To obtain curves starting from the same point, fluorescence values were normalized according to the following formula: $F_{t=x} - F_{t=0}$, where $F_{t=x}$ is the fluorescence of molecular beacon at time x of the NASBA reaction and $F_{t=0}$ is its fluorescence at the start of the NASBA reaction.

After completion of NASBA amplification, the thermal denaturation profile of the molecular beacons was followed in all reactions by measuring the fluorescence after raising the temperature to 80°C and then decreasing the temperature to 25°C at a rate of 5°C/20 s. Fluorescence values were normalized according to the following formula: $1 - (F_{80°C} - F_{x°C})$, where $F_{80°C}$ is the fluorescence of the molecular beacon at 80°C and $F_{x°C}$ is its fluorescence at temperature x.

The secondary structure of RNA amplicons was predicted using the computer program STAR (13), distributed by the authors.

One-tube detection of PLRV RNA in potato tubers

For the homogeneous, direct detection of PLRV in potato tubers, potato extracts were prepared and the virus was immunocaptured in microtubes or microplates as described by Leone *et al.* (11). NASBA and real-time monitoring of reactions were then performed as described above.

RESULTS

Working conditions for using molecular beacons with NASBA

Molecular beacon sw75-Fl was tested for its performance in the NASBA reaction mix. A thermal transition profile was generated by following the fluorescence intensity of a 20 µl NASBA reaction mix without template as a function of temperature (Fig. 1). We found that the components of the NASBA reaction mix had sufficient stabilizing effect on the molecular beacon to obtain a melting curve consistent with the stem–loop conformation of the probe. At low temperatures, the arms of the stem were closed, causing fluorescence of the fluorescein reporter to be quenched by DABCYL. At high temperatures, thermal denaturation occurred, and the random-coil conformation caused the fluorophore and the quencher to be apart, giving rise to fluorescence emission. The fluorescence that occurred in the range of temperatures above 55°C was slightly lower than that at 55°C, where fluorescence reached its maximum. This might have been due to a decrease in the intrinsic fluorescence of fluorescein at the higher temperatures.
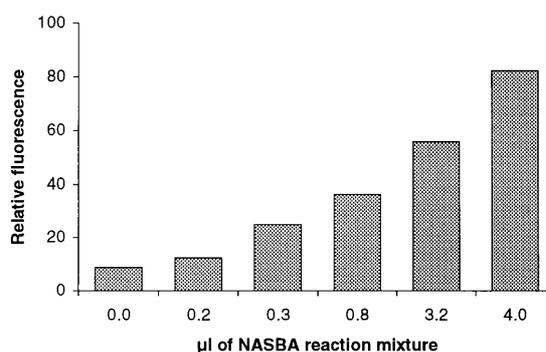
A NASBA amplification with 1 ng PLRV input was performed and analyzed by gel electrophoresis. Aliquots of the reaction mixture containing the amplicon were brought to a final concentration of 0.28 pmol (3 ng) molecular beacon. The final volume of these solutions was 20 µl. Figure 2 shows that at room temperature the fluorescence of the reactions increased in a concentration-dependent manner, following the increase of NASBA amplification mix, therefore of amplicon, in the solution. In this test, the fluorescence increase was only due to the hybridization of molecular beacon to its target.

Optimization of real-time monitoring of NASBA amplification reactions

For real-time monitoring of NASBA amplification, the molecular beacons need to be present in the reaction mix from the beginning of the reaction and have to produce a detectable fluorescent signal concurrently with target amplification. During a typical NASBA reaction, antisense RNA is synthesized at 41°C by the activity of T7 RNA polymerase. This RNA amplicon also acts as the substrate for subsequent amplification cycles. During a NASBA reaction in the presence of molecular beacons, the probe might compete with the amplification by hybridizing to the amplicon, making it unavailable for following RNA synthesis. In order to optimize real-time monitoring of NASBA, we evaluated the effect on fluorescence yield and NASBA sensitivity of different amounts of molecular beacon in the reaction and of two different primer sets/probe combinations.

The sensitivity of NASBA in the presence of molecular beacon sw75-Fl, expressed in number of input target RNA molecules that can still be detected, is depicted in Figures 3 and 4. The reactions were performed with primer set PD415–PD417 and a 10-fold dilution series of PLRV RNA, ranging from 0 to 10^6 copies per reaction, was used as input for the amplifications. Different amounts of molecular beacon (3, 6, 9 and 12 ng per reaction) were included in the reactions. The fluorescence signal was measured after 90 min of incubation of the amplification reactions (Fig. 3). There was no significant increase in fluorescent signal when increasing amounts of target RNA was used. This is caused by the fact that the amount of molecular beacon used in these experiments is lower than the amount of amplicon RNA that is synthesized in the amplification reaction. As a result, the maximum signal is defined by the amount of molecular beacons that is used and is independent of the amount of input target RNA.
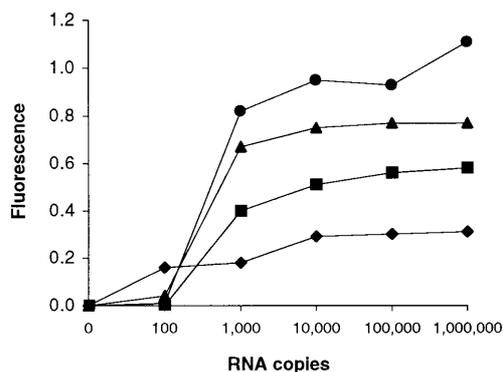
Figure 3. Optimization of the detection of amplicon by molecular beacon sw75-Fl during NASBA amplification. Fluorescence was measured after 90 min incubation of the NASBA reactions. The reactions contained PLRV RNA (from 0 to 10^6 copies per reaction), primer set PD415–PD417 and 3 ng (◆), 6 ng (■), 9 ng (▲) or 12 ng (●) of molecular beacon sw75-Fl.
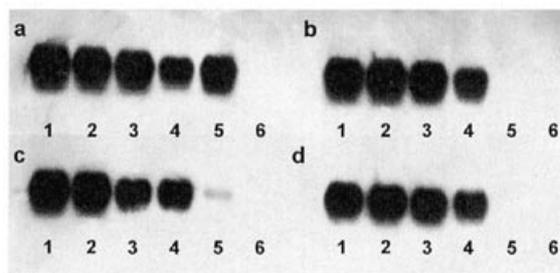
Figure 4. Northern blot analysis of the NASBA reactions of Figure 3, showing that detection by chemiluminescence and by molecular beacon sw75-Fl is equally sensitive. (**a**) Three ng of molecular beacon; (**b**) 6 ng of molecular beacon; (**c**) 9 ng of molecular beacon and (**d**) 12 ng of molecular beacon were included in NASBA reactions started with 10^6 (lane 1), 10^5 (lane 2), 10^4 (lane 3), 10^3 (lane 4), 10^2 (lane 5) or 0 (lane 6) copies of PLRV RNA.

The reactions with 6, 9 or 12 ng molecular beacon had a sensitivity of 10^3 RNA copies, while the reactions with 3 ng molecular beacons had a sensitivity of 10^2 RNA molecules. The results obtained with fluorescence measurements were confirmed by northern blotting analysis of the amplicon RNA (Fig. 4).

A similar experiment was set up using primer set PD416–PD417. In this case fluorescence increased in a concentration-dependent way only when 3 ng of molecular beacon were present in the NASBA reaction, while with 9 ng of molecular beacon, fluorescence could be measured only at the highest amount of input RNA (results not shown). Northern blot analysis of the amplification products showed that with 9 ng of molecular beacon in the reaction the amplification was inhibited when NASBA was started with 10^2–10^4 copies of RNA (Fig. 5). Without molecular beacon or at 3 ng probe in the reaction, primer set PD416–PD417 performed in NASBA as efficiently as primer set PD415–PD417. To understand how the probe might interfere with amplification, we used a computer program to predict the secondary structure of the RNA amplicons, formed by both primer sets, to determine the target sites for molecular beacon sw75-Fl. Figure 6 shows that the target site of the amplicon relative to primer set PD416–PD417 is much more exposed to hybridization with the molecular beacon than that of the amplicon
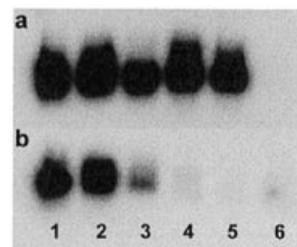
Figure 5. Northern blot analysis of NASBA reactions started with primer set PD416–PD417 in the presence of molecular beacon sw75-Fl at 3 ng (**a**) or at 9 ng (**b**). Lane 1, 10^6 copies PLRV RNA; lane 2, 10^5 copies PLRV RNA; lane 3, 10^4 copies PLRV RNA; lane 4, 10^3 copies PLRV RNA; lane 5, 10^2 copies PLRV RNA; lane 6, no-template control.

relative to primer set PD415–PD417. The decrease of NASBA amplification efficiency with primer set PD416–PD417 is therefore probably caused by a high hybridization efficiency of the molecular beacon for the amplicon synthesized. In this way, RNA is subtracted as substrate for the reverse transcription steps and further RNA synthesis by T7 RNA polymerase. This competition was not significant when there was 3 ng of molecular beacon in the NASBA reactions or, in the case of 9 ng molecular beacon, this inhibition was apparently overcome when the reactions were initiated with a high numbers of copies of input RNA.

Homogeneous, real-time detection of NASBA RNA amplicon

The effectiveness of combining NASBA and molecular beacons as a one-tube RNA assay for routine sample analyses was assessed by applying the previously optimized procedure for the detection of encapsidated PLRV RNA under different sampling conditions. Results obtained by NASBA amplification and molecular beacon fluorescence were validated by a thermal transition profile of each reaction and gel electrophoresis followed by northern blot analyses with chemiluminescent detection.

A series of 10-fold dilutions of intact PLRV virions, ranging from 100 pg to 10 fg, was amplified by NASBA in the presence of 9 ng molecular beacon sw75-Fl and primer set PD415–PD417. Real-time monitoring at 41°C of the fluorescence of the probe during NASBA amplification enabled detection of PLRV RNA at all the target concentrations tested (Fig. 7a). Validation of the results by thermal transition profile determinations and northern blot analysis of the products synthesized in the NASBA reactions confirmed that the fluorescence produced by the molecular beacons was specific for its target (Fig. 7b and c). Furthermore, the intensity of the fluorescent signal was in complete agreement with the level of detection obtained by northern blot analysis, indicating that all detection methods were equally sensitive. The real-time measurements showed typical exponential curves, with the usual tendency to reach a plateau. This is consistent with a repeated cycling mechanism for the NASBA reaction, in which the products function as templates, followed by a phase in which depletion of reaction components occurs. The difference in the thermal transition profile determinations, between the signals of the reaction started with 10 fg PLRV and the reactions started with no template, became more evident as the temperature at which measurements took place decreased from 40 to 25°C. This was due to some background fluorescence in the no template control reaction caused by the presence of some unfolded probe at 40°C.
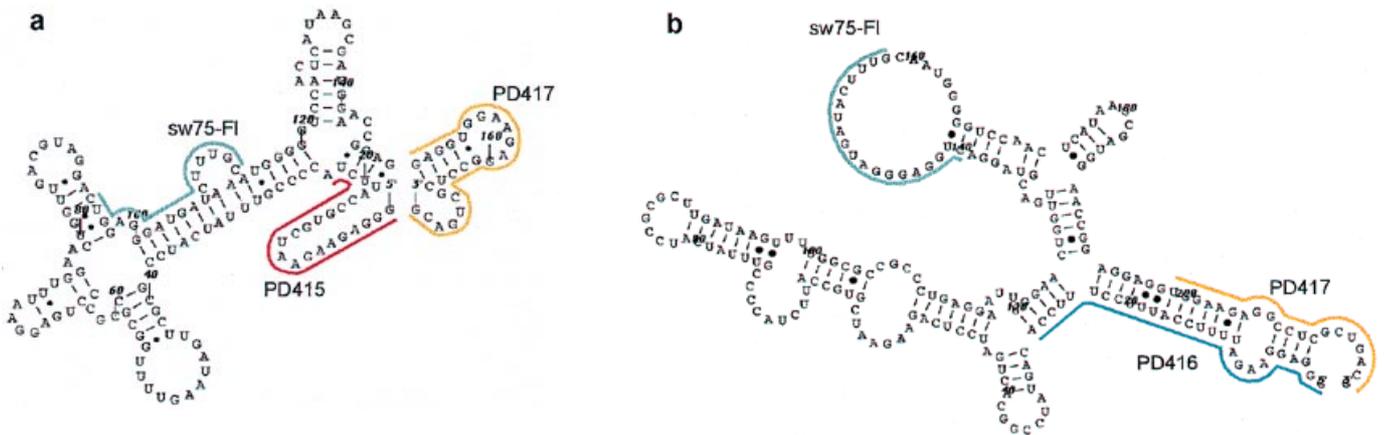
Figure 6. Secondary structure models of NASBA amplicons produced by primer set PD415–PD417 (172 nt) (**a**) or PD416–PD417 (218 nt) (**b**) and binding sites for molecular beacon sw75-Fl and primers. Each amplicon also contains at its 5′ end the transcription initiation sequence from primer PD415 (5′-GGGA-3′) or primer PD416 (5′-GGGAGG-3′) for the T7 RNA polymerase.
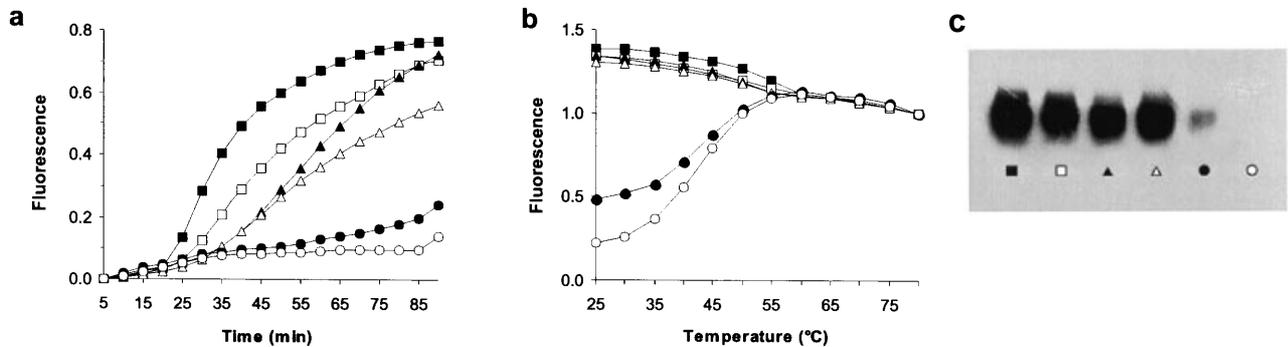
Figure 7. Homogeneous detection of RNA from a series of 10-fold dilutions of intact PLRV virions during NASBA amplification including 9 ng molecular beacon sw75-Fl and primer set PD415–PD417. (**a**) Real-time monitoring of reactions; (**b**) thermal transition profiles of terminated reactions and; (**c**) northern blot analysis of terminated reactions. (■) 100 pg PLRV; (□) 10 pg PLRV; (▲) 1 pg PLRV; (△) 100 fg PLRV; (●) 10 fg PLRV; (○) no template control.

This background fluorescence, acceptable in our experiments in relation to the generation of specific signal, might be reduced by designing a different stem structure for the molecular beacon.

The feasibility of the novel assay to amplify and detect RNA in a one-tube format under real-world conditions was tested in an experiment using uninfected and PLRV-infected potato tubers. To detect PLRV RNA directly in potato tuber sap without the need of chemical nucleic acid extractions (11), virions were immuno-captured in wells coated with specific antibodies against PLRV. As a control for the procedure, an uninfected tuber was spiked with 1 ng purified PLRV prior to the immunocapture step. Figure 8 shows that the tubers infected with PLRV could be readily and unambiguously distinguished from uninfected tubers, confirming the feasibility of the assay for real-time and homogeneous detection of RNA in real-world applications.

DISCUSSION

This paper presents a convenient and effective method to amplify and detect RNA homogeneously and in real-time. This novel assay, called AmpliDet RNA, is based on the use of molecular beacon probes in NASBA reactions to generate a fluorescent signal for direct amplicon detection during the amplification process. RNA amplification and generation of a target-specific fluorescent signal are accomplished simultaneously in a one-tube system, and measurements can take place in a fluorometer. The technology described is specific, sensitive and feasible for high-throughput sample analysis. This has been tested using as a model system PLRV, one of the major viral pathogens of potato able to cause relevant economic losses world-wide, and therefore requiring a strict control of its incidence during disease management (11).

We have demonstrated that a molecular beacon included in a NASBA reaction does not inhibit amplification of specific product. However, we observed that the relationship between hybridization of the molecular beacon to the RNA amplicon and subsequent NASBA amplicon formation can vary somewhat among different primer sets. An important requirement for this detection method seems to be that probe hybridization to target must not remove antisense RNA intermediates from subsequent amplification steps. NASBA amplification is based on the concurrent activity of the enzymes AMV-RT, RNase H and T7 RNA polymerase, following kinetics described by Kievits *et al.* (3) and Sooknanan *et al.* (1). DNA molecular beacon probes might interfere with the early NASBA amplification steps by binding to the first antisense RNA amplicons produced by the T7
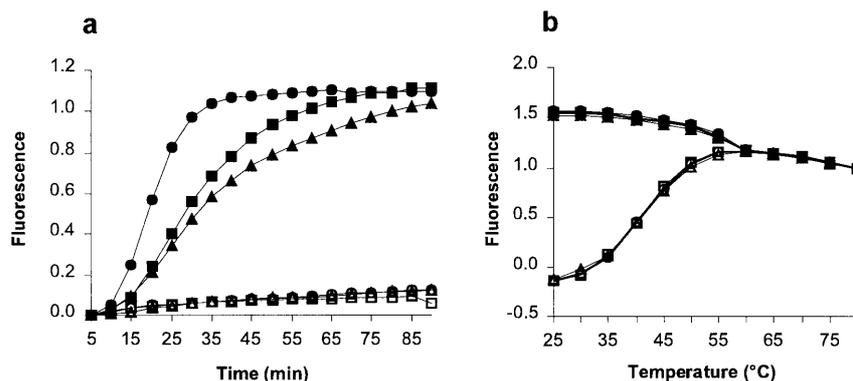
Figure 8. One-tube detection of PLRV RNA in infected and uninfected potato tubers by NASBA reactions containing 9 ng of molecular beacon sw75-Fl after immunocapture of virus from tuber extracts. (**a**) Real-time monitoring of reactions; (**b**) thermal transition profiles of reactions. (●) Uninfected tuber spiked with 1 ng PLRV; (■,▲) infected tubers; (□,Δ,○) uninfected tubers.

RNA transcription step. In that case, RNA:DNA hybrids are formed which are prone to RNase H degradation, eliminating RNA substrates from further cDNA synthesis and T7 RNA polymerase transcriptions in the NASBA cyclic phase. However, this may easily be overcome by a good selection of the primer sets, by varying the amounts of molecular beacon in the reaction, by manipulating the sequence and length of the probe or by designing molecular beacons carrying an RNA complementary sequence in the loop.

Real-time monitoring of NASBA reactions gave fluorescence plots with typical initial exponential rates followed by a plateau phase. The initial exponential rate is consistent with a cyclic phase of the NASBA reaction in which the products of amplification function as templates (3). The plateau phase is consistent with the stage of NASBA amplification when the amplicon stops accumulating exponentially, due to depletion of reaction components. In this study, reaction conditions (initial copy number, probe design and concentrations) were not optimized for kinetic studies. Therefore, the relationship between probe hybridization, i.e. fluorescent signal, and product formation may not be linear. This may cause each of the two variables to enter their own plateau phase at different stages of the NASBA reaction. Nevertheless it seems evident that monitoring NASBA reactions in real-time with molecular beacons will also facilitate kinetic studies.

In conclusion, the novel technology presented in this report offers a truly homogeneous assay in which amplification and detection of RNA occur in one-tube. Compared to current RNA probing and/or blotting methods, the use of molecular beacons to detect NASBA amplicons, retains the same level of specificity and sensitivity, is easy to perform and timesaving, due to a reduction of handling steps. The risk of carry-over contamination is minimized by the advantage of performing the entire method in unopened vessels. Furthermore the assay is sensitive and robust, as demonstrated by working with very complex samples such as potato tuber extracts. This shows that AmpliDet RNA has the potential to be used in routine settings for high-throughput sample analysis. However, it is conceivable that this system may be employed for many other applications, including also the indication of biological activities, i.e. gene expression and cell

viability (2). Other developments for this technology will result by the introduction into the NASBA reaction of more probes with different fluorescent labels (14), allowing the simultaneous detection of more target sequences, useful in multiplex and/or quantitative NASBA systems.

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