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NASBA™ isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection

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Summary

Isothermal nucleic acid amplification of target RNA or DNA sequences is accomplished by the simultaneous enzymatic activity of AMV reverse transcriptase, T7 RNA polymerase and RNase H. Amplification factors of the nucleic acid sequence based amplification (NASBA™) method range from 2×10^6 to 5×10^7 after 2.5 h incubation at 41°C. During NASBA™ there is a major accumulation of specific single stranded RNA. RNA:DNA hybrid and double stranded DNA are also synthesized, although to a minor extent.

The system is optimized for the detection of HIV-1 sequences in in vitro infected cells, blood and plasma. Detection levels are 10 molecules of HIV-1 in a model system with in vitro generated HIV-1 RNA as input and 5 infected cells on a background of 5×10^4 non-infected cells. Blood and plasma can also be used as the source of nucleic acid for detection of HIV-1 sequences using a specifically developed sample preparation method.

Using NASBA™ it is possible to amplify specifically RNA or DNA from a pool of total nucleic acid, which permits the investigation of the expression of specific genes involved in pathogenesis of infectious agents. The combination of NASBA™ with a rapid and user-friendly nucleic acid extraction method makes the whole procedure suitable for large scale diagnosis of infectious agents (e.g. HIV-1)

Amplification; NASBA; HIV-1; Viral RNA

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Introduction

Diagnostic procedures for detection of nucleic acids of viral pathogens or mutations in sequences showing allelic variations are commonly used now because of the high sensitivity and because they allow detection in the absence of a measurable immunological response. Direct detection of nucleic acid by Southern or Northern blot is not sufficiently sensitive if there are low levels of viral pathogens present in blood, i.e. HIV-1 (Shaw et al., 1984). In these cases detection is facilitated by a nucleic acid amplification step by which the sensitivity can be increased more than a million-fold.The polymerase chain reaction (PCR; Mullis and Faloona, 1987; Saiki et al., 1988) was the first nucleic acid amplification technique to be applied on a large scale for this purpose. PCR DNA amplification has been used as a diagnostic tool for the detection of, among others, proviral HIV-1 (Ou et al., 1988; Locke and Mack, 1988), HTLV-1 (Bhagawatie et al., 1988) and HPV (Shibata et al., 1988). For the direct detection of RNA sequences the PCR method requires a preceding reverse transcriptase (RT) step, 'translating' the RNA into a DNA sequence which can serve as a template for PCR amplification. Although this RT step complicates the procedure considerably, RT-PCR has been used successfully for the detection of RNA sequences of enteroviruses (Rotbart, 1990; Xu et al., 1990) and HCV (Kubo et al., 1989; Okamoto et al., 1990). Recently, new nucleic acid amplification techniques, more suited for the amplification of RNA target sequences than PCR, have been described (Davis et al., 1990; Guatelli et al., 1990; Kwoh et al., 1989; Chu et al., 1986; Lizardi et al., 1986). These techniques are based mostly on an RNA synthesis step from an RNA or DNA template. Davis et al. (1990) applied their TAS method for the detection of HIV-1 in peripheral blood mononuclear cells of AIDS patients.

We now report the development and optimization of HIV-1 detection by the NASBA™ amplification technique in which nucleic acid amplification is based on the simultaneous activity of 3 enzymes without the necessity of thermal cycling or the intermediate addition of enzymes. While this work was in progress Guatelli et al. (1990) published the development of their 3SR amplification technique, that resembles NASBA™. However, it is shown here that there are striking differences between the two, especially concerning specificity and product profile.

Materials and Methods

Plasmids and RNA synthesis

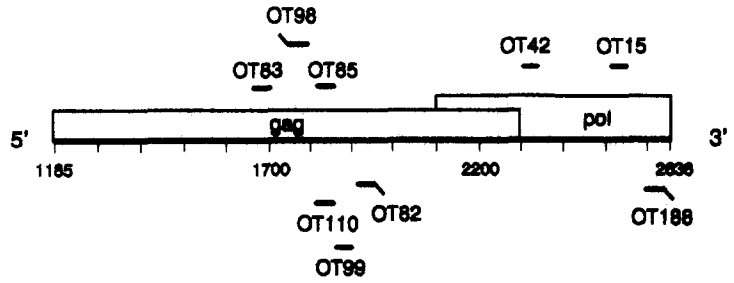
Genetic and recombinant DNA techniques followed standard procedures (Sambrook et al., 1989). Plasmids used for in vitro RNA systhesis contained a 1416 bp fragment of the HIV sequence resulting from a partial Fok 1 restriction enzyme digest (nucleotides 1186–2638 of the HIV-1hxb2 sequence; (Ratner et al., 1987) cloned in pGEM3 or pGEM4 (Promega).

In vitro RNA was generated from these constructs either by T7 RNA polymerase or SP6 RNA polymerase (Sambrook et al., 1989). Reaction mixtures were treated with DNase to remove plasmid DNA. After phenol extraction and ethanol precipitation the recovered RNA was quantitated on ethidium bromide-stained agarose gels by comparison to a calibration series of known amounts of ribosomal RNA.

The RNA solutions were diluted to the desired concentrations and used as input for amplification by NASBA™.

Oligonucleotides

Oligonucleotides were synthesized using the solid phase phosphoramidite method on an Applied Biosystems PCR-MATE DNA synthesizer model 391. Elution from the column was with 25% NH_4OH followed by overnight incubation at 55°C for deprotection. The oligonucleotides were purified by gel electrophoresis on a 20% acrylamide gel (Sambrook et al., 1989) and eluted from the ethidium bromide-stained band containing the molecules of the required size. After ethanol precipitation the oligonucleotides were redissolved in water and their concentrations were determined spectrophotometrically. Sequences of the primers and annealing sites are presented in Fig. 1.



Oligo	Sequence	Function
OT188	*AATTCTAATACGACTCACTATAGGG*CCTGGCTTTAATTTTACTGGTA	P1
OT42	ACAGGAGCAGATGATACAGTATTAG	P2
OT15	TGGAAGAAATCTGTTGACTCAGATTGGTTGC	Probe
OT83	*AATTCTAATACGACTCACTATAGGG*ATTGCCTCTCTGCATCATTA	P1
OT82	GTAGACCGGTTCTATA	P2
OT85	TAGAAGAAATGATGACAGCATGTCAGGGA	Probe
OT98	*AATTCTAATACGACTCACTATAGGG*AGCAAGCTTCACAGGAGGTA	nested P1
OT99	CCAAAACTCTTGCCTTA	nested P2
OT110	TCCCTGACATGCTGTCATCATTTCTTCTA	probe

Fig. 1. Schematic presentation of in vitro RNA used as target (nucleotides 1186 to 2638 of HIV-1 hxb2 sequence) comprising part of the gag and pol genes and the primer sequences used in this study. The T7 promoter part in primer 1 sequences is given in italics.

Cell lines and virus stock

H9 T-cells and U937 cells were infected in vitro with HIV-1 at the Central Laboratory of the Blood Transfusion Service, Amsterdam. The supernatant of the cultured H9 cells was used as virus stock solution (2500 $TCID_{50}$/ml). The infected H9 and U937 cells were collected by centrifugation, washed with PBS, counted and used for experiments.

Nucleic acid isolation

Nucleic acids from cells, blood or plasma were isolated essentially according to Boom et al. (1990). One hundred-μl samples were lysed in 1 ml lysis buffer (50 mM Tris, pH 6.4; 20 mM EDTA, 1.3% w/v Triton X-100, 5.25 M GuSCN). Nucleic acid released in the lysate was bound to activated silica (50 μl silica, Fluka, 1 g/ml suspension in 0.1 N HCl) which was added to the lysis mixture. The silica particles carrying adsorbed nucleic acid were washed twice with 1 ml GuSCN containing washing buffer (50 mM Tris, pH 6.4, 5.25 M GuSCN), twice with ethanol and once with acetone. After drying the silica at 56°C, the nucleic acid was eluted with 50 μl H_2O and stored at -20°C.

Nucleic acid amplification and characterization

All enzymes used and the RNA guard were purchased from Pharmacia, except AMV-reverse transcriptase, that was purchased from Seikagaku. BSA was purchased from Boehringer.

As shown in Fig. 2 one of the two oligonucleotide primers used in NASBA™ contains a T7 promoter sequence (P1). Starting with an RNA molecule as input, RNase H degrades the RNA strand in the RNA:DNA hybrid molecule that results from AMV-reverse transcriptase (AMV-RT) enzyme activity (Fig. 2, top left). Subsequently, primer 2 (P2) can anneal to the resulting single stranded cDNA and the second DNA strand is synthesized by the DNA-dependent DNA polymerase activity of the AMV-RT, yielding a double stranded DNA molecule including a T7 RNA polymerase promoter sequence. The T7 RNA polymerase gives a 100–1000-fold increase in specific RNA (Milligan et al., 1987). When DNA is used as input 2 heat denaturing steps (Fig. 2, top right) are compulsory to obtain single stranded DNA intermediates, available for primer annealing (P1 and P2). In the cyclic phase the events are the same (as for RNA input), but P1 and P2 are incorporated in reverse order.

Typical NASBA reactions were performed in a 25-μl reaction mixture containing 40 mM Tris, pH 8.3, 20 mM $MgCl_2$, 40 mM KCl, 5 mM DTT, 10–15% DMSO (depending on the primerset used), 1 mM each dNTP, 4.1 mM each NTP, 0.1 μg/μl BSA, 12 U RNA Guard, 20 U T7 RNA polymerase, 4 U AMV-reverse transcriptase, 0.2 U RNase H, 0.2 μM Primer 2, 0.2 μM Primer 1 and target RNA. After addition of the enzymes the contents of the tubes were

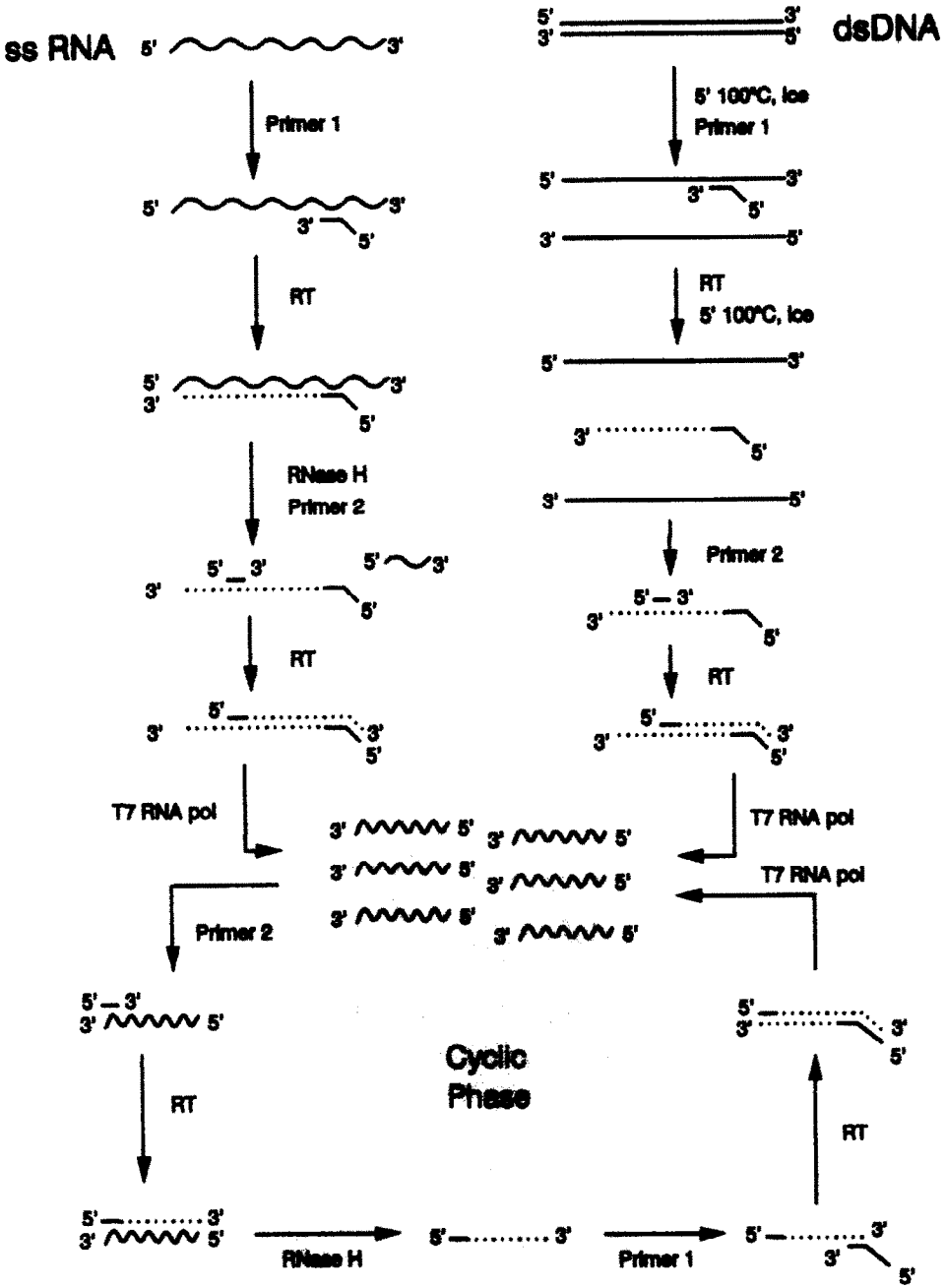


Fig. 2. Schematic presentation of the NASBA™ method applied to ssRNA and dsDNA. The method is based on extension of Primer 1 (T7-containing) by AMV-reverse transcriptase on a RNA/DNA template, degradation of the RNA strand by RNase H (or heat denaturation for dsDNA), synthesis of second DNA strand by AMV-RT and RNA synthesis by T7 RNA polymerase. With RNA synthesis the system enters the cyclic phase, that is based on the principles described above. R.T. = AMV reverse transcriptase. Solid lines represent target DNA, broken lines newly synthesized DNA and wavy lines represent RNA. For further explanation see text.

mixed by gentle tapping. The reactions were incubated at 41°C for 3 h following the addition of target RNA and stopped by placing them on ice or at −20°C. After amplification the products to be detected are antisense single stranded RNA, RNA:DNA hybrid and double stranded DNA.

From the amplified material 1–5 μl of a 100-fold diluted solution can be used for a second round of amplification using nested primers (see Fig. 1). For detection 10 μl of each reaction is run on a 3% Nusieve, 1% agarose gel (Sambrook et al., 1989) and the nucleic acid stained with ethidium bromide. The nucleic acid is blotted onto Zeta-Probe (BioRad) filters using a vacuum-blot apparatus (Pharmacia) and hybridized with a ^{32}P-labelled oligonucleotide (Fig. 1, probes; Zeta Probe protocol. Sambrook et al., 1989). Exposure times of the blots to X-ray films (Kodak) ranged from 30 min. to 3 days. When appropriate, films were scanned with a LKB Ultroscan XL densitometer. Amplified RNA was used directly for sequence analysis by the dideoxynucleotide method using 5′ kinased primers.

Results

Optimization of NASBA™

Optimization of the NASBA™ technology was achieved by varying the concentration of all ingredients in the reaction mixture. The results obtained by

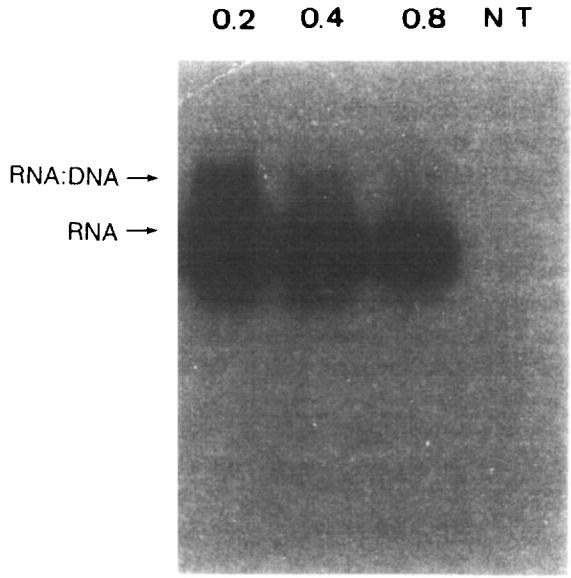


Fig. 3. Autoradiograph showing NASBA™ amplified products of reaction mixtures in which the RNase H concentration was varied. Primers used were OT188/OT42 generating an RNA molecule of 250 nt. RNAse H concentrations used were 0.2, 0.4 and 0.8 units. As input 10^7 molecules of in vitro generated HIV-1 RNA was used. Exposure time was overnight. NT = no template.

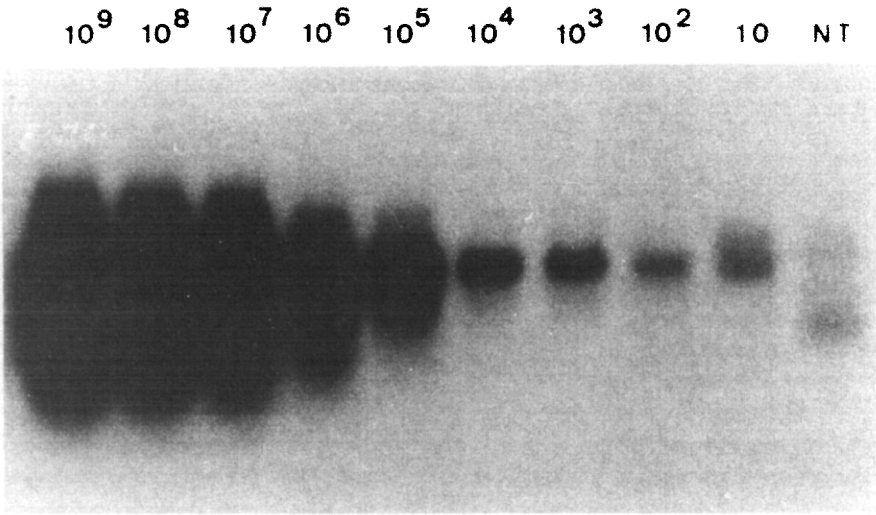


Fig. 4. Auroradiograph showing sensitivity of NASBA on in vitro generated HIV-1 RNA molecules with primer set OT188/OT42. Ten μl of every reaction mixture was loaded on the gel. Above the lanes are the number of input molecules stated. Exposure time was overnight. NT = no template.

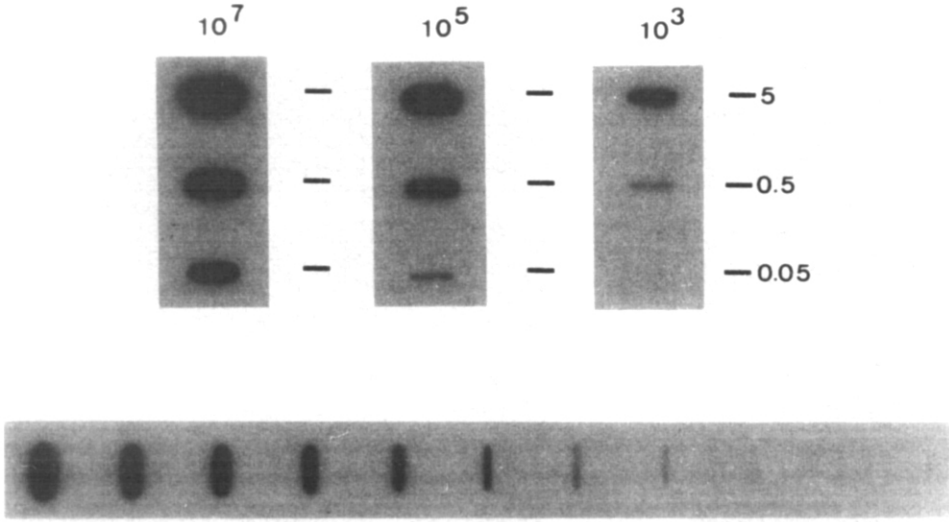


Fig. 5. Autoradiograph of a slot-blot showing quantitation of NASBA signal and determination of amplification factors. Samples containing 10^7, 10^5 and 10^3 RNA molecules as input were slot blotted (5, 0.5 and 0.05 μl) and hybridized with a ^{32}P-labelled probe. Also hybridized was a calibration series of two-fold dilutions of RNA ranging from 10^{11} to 9×10^7 molecules. After autoradiography all the bands were scanned and peak areas calculated. The calibration series was used to make a calibration curve. Amplification factors were 2×10^6, 10^7 and 5×10^7 for 10^7, 10^5 and 10^3 input molecules, respectively.

varying the concentration of RNase H are shown in Fig. 3. Identification of amplified products was achieved by RNase A and DNase I treatment and strand specific oligonucleotide hybridization. Low concentrations of RNase H result in the accumulation of RNA:DNA hybrid and high yield of single stranded RNA. Increasing RNase H concentrations accelerates the turnover of the RNA:DNA hybrid so fast that the overall yield of single stranded RNA decreases (0.8 U RNase H).

Using optimized NASBA™ the detection level in a model system with in vitro generated RNA as input was as little as 10 molecules (Fig. 4). A further increase in sensitivity (1 molecule giving a clearly detectable signal) can be achieved by using the amplified material for a second round of amplification with a nested primer set (Fig. 2, primer sets 82/83 and 98/99). NASBA™ amplified reaction mixtures with an initial input of 10^7, 10^5 and 10^3 molecules were slot-blotted and hybridized with a labelled detection probe. After autoradiography the films were scanned for quantitation of the signal in the bands (Fig. 5). A comparison with the signals of a calibration series showed that the amplification factors were 2×10^6, 10^7 and 5×10^7 for 10^7, 10^5 and

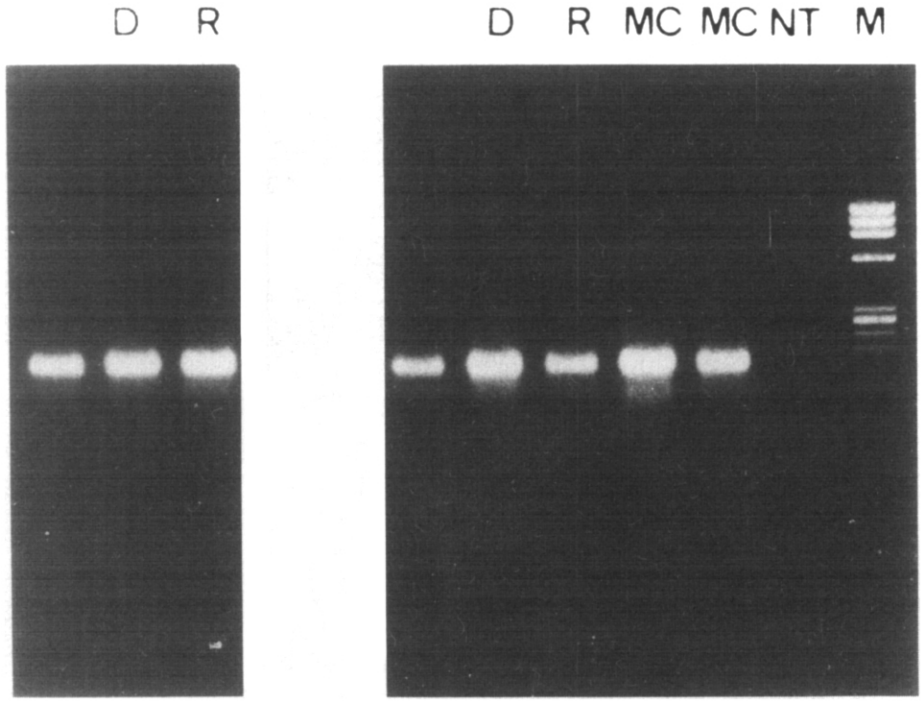


Fig. 6. Ethidium bromide-stained agarose gel showing specificity of NASBA with primer set OT188/OT42. The left panel is direct amplification of in vitro RNA; the right panel shows amplification products after sample preparation treatment [19] of the in vitro RNA. Input was in all lanes 10^8 molecules of in vitro generated HIV-1 RNA, except for the NT and M lane. D, 100 ng human placenta DNA added; R, 300 ng total cellular RNA added; MC, 10^5 in vitro-cultured cells added. NT, No template. M, marker.

10^3 molecules of input, respectively. Kinetic studies indicated that, as early as 15 min after the start of amplification, a signal can be detected for an input of 10^8 molecules. For low input (10^4 molecules) approximately 45 to 60 min are required to develop a clear signal (data not shown). The fidelity of the amplification was confirmed by the direct sequencing of amplified RNA.

Suitability of NASBA™ for biological samples

Addition of either non-specific RNA or DNA had little or no effect on NASBA™ amplification factors or specificity of the reaction at relatively high inputs of target sequences (Fig. 6). Combining the nucleic acid extraction method described in the Methods and Materials section with NASBA™ resulted in specific amplification of target without loss of yield. Addition of non-specific RNA, DNA or myeloma cells had little or no effect on the amplification efficiency (Fig. 6).

In the process of evaluating the applicability of NASBA™ to clinical samples an important step is the performance of NASBA™ on nucleic acid isolated from HIV-1 infected cells in vitro. Experiments of this nature (Fig. 7A) showed specific amplification of HIV-1 target sequences from nucleic acid isolated from infected cells, compared to the absence of signal when non-infected cells were used. The positive signal in the supernatant of infected cells may either be due to virus particles or nucleic acid released from lysed cells. However, the strength of NASBA™ lies in its ability to detect 50 infected cells in a background of 5×10^4 non-infected cells. Combining a standard primer set with a nested primer set lowers the detection level to 5 infected cells in a background of 5×10^4 non-infected cells (Fig. 7B).

The final step in the feasibility study was the mimicking of clinical samples by adding in vitro infected cells and HIV-1 virus particles to both blood and plasma in decreasing concentrations. These 'clinical' samples were treated according to the standard sample preparation protocol and the isolated nucleic acid was used as input for NASBA™ amplification and detection (Fig. 8). It was demonstrated that both blood and plasma are sources for nucleic acid to be amplified by NASBA™ using the described sample preparation method.

The level of detection in these samples was about 100 infected cells in 100 µl blood (10 infected cells in 100 µl plasma, a non-physiological sample) and 0.001 µl of a virus stock solution (2500 TCID 50/ml) in 100 µl blood or plasma. These detection levels, however, can be lowered by using a 'nested' primer set (data not shown).

Discussion

Diagnosis based on a nucleic acid amplification technology, is especially suited for detection of low levels of infectious agents in blood or plasma. The number of HIV-1 infected cells in blood samples can be as little as 10 infected

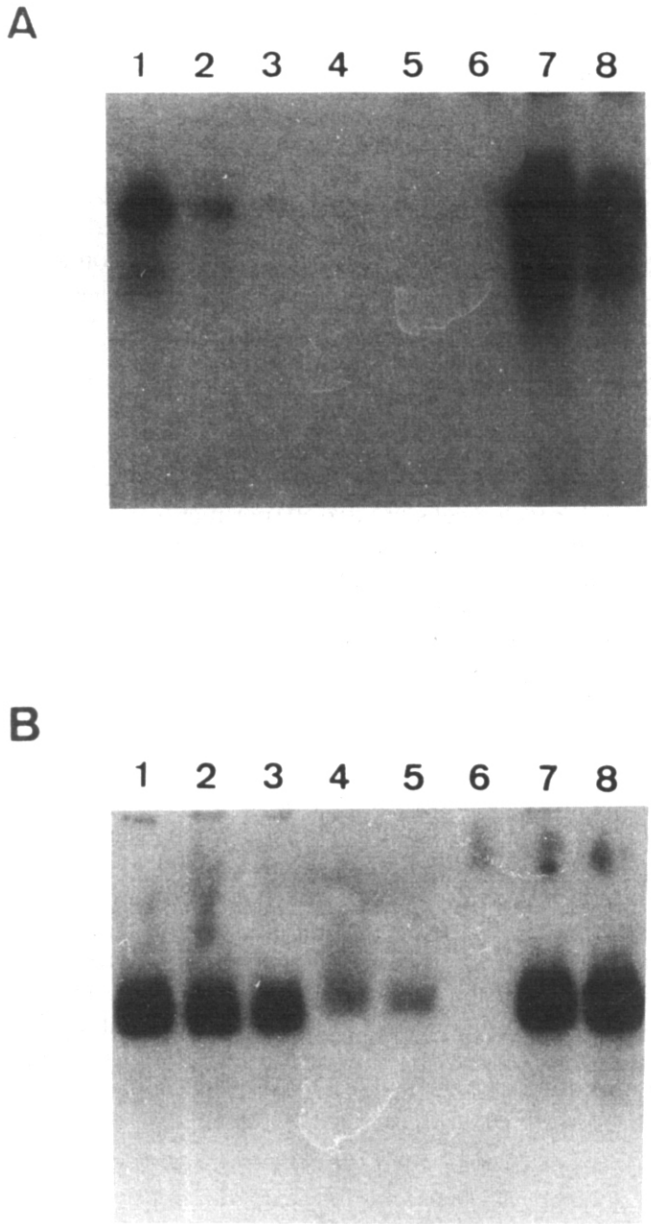


Fig. 7. Autoradiographs showing NASBA™ amplification products of nucleic acid isolated from HIV-1 infected U937 cells with primer set OT82/OT83 (panel A) and nested primer set OT98/OT99 (panel B). For amplification with the nested set the amplification products of OT82/OT83 were used. (1 μl of a 100-fold diluted solution). Number of infected cells (lanes 1–5: 500, 50, 5, 0.5, 0.05, respectively) in a background of 5×10^4 non-infected cells. Control lanes: 6, 5×10^4 non-infected cells; 7, 5×10^4 infected cells; 8, supernatant of infected cells..

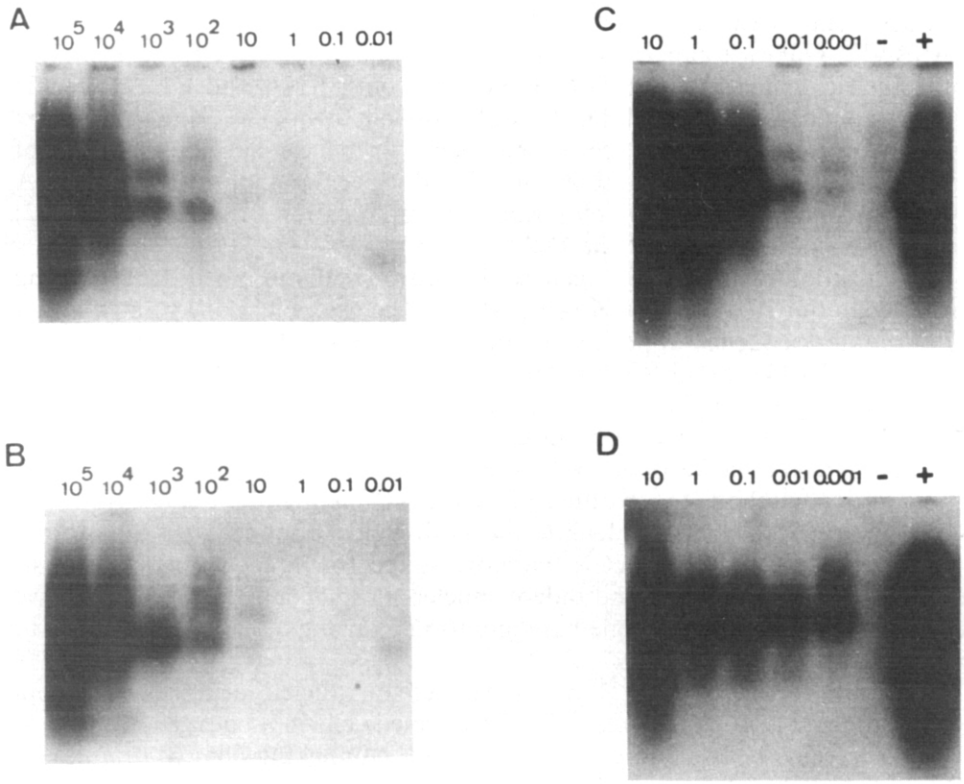


Fig. 8. Autoradiographs showing the detection of in vitro HIV-1 infected H9 cells and HIV-1 virus particles in blood and plasma with primer set 184/185. Panel A: HIV-1 infected cells in blood; panel B: HIV-1 infected cells in plasma; panel C: HIV-1 virus particles in blood; panel D: HIV-1 virus particles in plasma. The 10-fold dilution series of in vitro infected cells ranged from 1×10^5 to 0.05 cells per 100 μl blood or plasma. Of the virus stock solution (2500 TCID 50/ml) a 10-fold dilution series (10 μl to 0.001 μl) was added to 100 μl blood or plasma. +, positive control (10^8 molecules HIV-1 RNA); −, negative control.

cells in 1 ml of blood (Guatelli et al., 1989). The simplicity of NASBA™ in combination with the described sample preparation method (Boom et al., 1990) is likely to make feasible nucleic acid-based diagnosis on a routine scale.

By choosing RNA instead of DNA a higher number of molecules will be available to serve as targets. Even in low expressing cells the number of RNA copies will exceed the number of DNA copies. An exception to this rule is cells with HIV-1 integrated virus which is not transcribed and can only be detected after DNA amplification or by direct hybridization. In the application of PCR for amplification of RNA target sequences it must be preceded by a reverse transcriptase step. This is a time-consuming step that considerably complicates the whole procedure, also in terms of carry-over risks. It is shown here that for RNA detection other amplification techniques are easier to use than RT-PCR. NASBA™ is a technique that not only utilises RNA as input material for amplification, but also single stranded antisense RNA is the main amplification

product. The necessity of heat denaturation for DNA amplification by NASBA™ makes it capable of specifically amplifying RNA from a pool of total nucleic acid, whereas RT-PCR amplifies both RNA and DNA. Specific RNA amplification using RT-PCR is only possible by not choosing the primers within a single intron sequence. Using such a primer set specific detection of spliced messenger RNA can be accomplished. Specific amplification of DNA by NASBA™ is achieved by choosing the amplification primers (P1 and P2) on the non-coding strand of the DNA.

Optimization of NASBA™ as described here results in a protocol enabling the detection of 10 molecules of target RNA. The use of just one primer with a T7 promoter sequence appended to the 5′ end (Fig. 1) resulted in a drastic reduction of background RNA formed during amplification. Compared to the 3SR amplification method (Guatelli et al., 1990) addition of DMSO in the NASBA™ protocol, with optimized concentrations for every primer set, results in reduced background and increased specificity. Compared to the 3SR amplification method, the product of NASBA™ is mainly a single stranded RNA molecule of defined length on both sides bounded by the primer sequences. The RNA product is therefore suited for direct sequence analysis using reverse transcriptase and dideoxynucleotides. This approach may prove useful for the mapping of single base mutations causing disease or resistance to treatment.

Performance of NASBA™ was measured on nucleic acid isolated from HIV-1 infected cells. As little as 5 HIV-1 infected cells in a background of 5×10^4 non-infected cells could easily be detected. Mimicking clinical samples by adding infected cells or purified virus particles to blood and plasma led to detection levels that are probably significant in clinical diagnosis (Fig. 8). Detection levels can be improved further by using nested primer sets and concentrating the nucleic acid isolated from the sample (i.e., blood or plasma).

The specificity of the amplification technique is determined by the sequence and annealing sites of the primers. For that reason it is necessary to have enough sequence data available on the conserved regions in target sequences.

In the case of HIV it is possible to design primer sets that are specific for HIV-1 and also primer sets that will detect both HIV-1 and HIV-2 (Willey et al., 1986 and Hahn et al., 1985). Clinical studies on HIV-1 infection using NASBA™ are performed with several primer sets located in different regions of the HIV-1 genome (manuscript in preparation). In our studies it was found that both the HIV-1 RNA packaged in virus particles and HIV-1 in infected cells were stable enough to be amplified from frozen samples. This makes it possible to use NASBA™ for retrospective studies on frozen plasma, mononuclear cells or blood samples from HIV-1 infected individuals and determine the first presence of HIV-1 virus sequences, before seroconversion. By combining the high efficiency of amplification with a non-radioactive detection technique we are planning to develop NASBA™ further to permit its use in any setting without the need of special demands on laboratory equipment.

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