Highly sensitive detection of gene expression of an intronless gene: amplification of mRNA, but not genomic DNA by nucleic acid sequence based amplification (NASBA)

Albert Heim¹, Isabella Maria Grumbach², Stefanie Zeuke and Bert Top²

Institut für Virologie, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany, ¹University of Illinois at Chicago, Department of Medicine, Section of Hematology/Oncology, 900 South Ashland Avenue, Chicago, IL 68607-7173, USA and ²Organon Teknika, NASBA Development Unit, PO Box 84, 5280 AB Boxtel, The Netherlands

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ABSTRACT

NASBA is an isothermal nucleic acid amplification reaction that amplifies mRNA in a dsDNA background. Although similar to the sensitive reverse transcription/polymerase chain reaction (RT–PCR) in mRNA detection, NASBA is not prone to give false positive results caused by genomic dsDNA. Therefore, NASBA is unique for sensitive detection of transcription of intronless genes, which preclude strategies such as intron spanning primer pairs to control false positive results in RT–PCR. Using NASBA, mRNA of the intronless human interferon-β gene was demonstrated with a sensitivity of 10 copies, whereas 100 ng genomic DNA gave a negative result.

Nucleic acid hybridizations as for example northern hybridization or the RNase protection assay are reliable techniques to detect a specific mRNA, but the sensitivity may not be sufficient to detect a low level gene expression. Therefore, reverse transcription followed by polymerase chain reaction (RT–PCR) is frequently used for highly sensitive detection of gene expression. However, RT–PCR studies on gene expression may give false positive results if the RNA sample contains traces of genomic DNA. A strategy to cope with this problem is the selection of primer pairs which span an intron, and thus result in a longer PCR product in case of a DNA contamination. However, this strategy is not possible for intronless genes of eukaryotes and gene expression studies in prokaryotes. Other strategies to control a contamination of the RT–PCR with genomic DNA are more complicated or not completely reliable, e.g., performing an additional PCR, but omitting the RT, or a DNase digestion prior to RT–PCR. As even the low level transcription of the intronless interferon-β (IFN-β) gene (1) is important in the interaction of cytokines (2), we searched for a procedure to amplify mRNA, but not the corresponding genomic dsDNA. NASBA is an isothermal nucleic acid amplification reaction with two specific oligonucleotide primers (one of these containing the T7 promoter), AMV reverse transcriptase, Escherichia coli RNase H and T7 RNA-polymerase (3,4) identical to the independently invented self-sustained sequence replication (3SR) (5). Similarly to PCR, NASBA has been used frequently in recent years for the sensitive detection of various pathogens in medical microbiology. In contrast to PCR, NASBA can directly amplify RNA and is selective for ssRNA, if the denaturation of the nucleic acids prior to the isothermal amplification is carried out at 65°C, thus avoiding denaturing dsDNA.

NASBA was performed as described previously (3,4) using premixed enzymes, buffers and nucleotides provided in the NASBA amplification kit (Organon Teknika, Turnhout, Belgium). After an initial denaturation of the template nucleic acids and the IFN-β specific primers at 65°C (5 min), the temperature was adjusted to 41°C, premixed enzymes (0.08 U E.coli RNase H, 32 U T7-RNA polymerase, 6.4 U AMV-RT) were added, and the reaction was carried out for 120 min at 41°C in a total volume of 20 μl. Final concentrations were 40 mM Tris–HCl pH 8.5, 12 mM MgCl₂, 85 mM KCl, 5 mM dithiotreitol, 1 mM of each dNTP, 2 mM of ATP, CTP and UTP, 1.5 mM GTP, 0.5 mM IPT and 0.2 μM of each IFN-β specific amplification primer. Primer sequences were aat ctc atc atc tac tgg gAG AGG CAC AGG CTA GGA GAT CA (primer 1, nucleotides 909–933 of the IFN-β gene; EMBL accession nos J00218, K00616, M11029; lower case letters indicate the T7 promotor) and AAA CTC ATG AGC AGT CTG CA (primer 2, nucleotides 763–783; all oligonucleotides custom synthesized by Eurogentec, Seraing, Belgium). An aliquot of 10 μl of the NASBA reaction products was analyzed on a non-denaturing 2% agarose gel stained with ethidium bromide. In addition, 10 μl of the reaction product were denatured, analyzed on a 2% agarose gel containing formaldehyde, blotted on a positively charged Nylon membrane (Boehringer, Mannheim, Germany), and hybridized to a biotinylated IFN-β specific oligonucleotide probe (5-GGC CAA GGA GTA CAG TCA CTA GTC CTG CAC GA-3, nucleotides 822–853 of the IFN-β gene) according to standard procedures (6). Stringent washing was performed at 71°C in 0.3× SSC (0.005 M sodium chloride, 0.0045 M sodium citrate), 0.1% SDS. Visualization was done by chemoluminescence with CSPD using the Seq-Light kit (Tropix, Bedford, MA).

In order to estimate the sensitivity IFN-β NASBA, a serial dilution of in vitro transcribed IFN-β RNA (10¹⁰–10 copies), and 100 ng total RNA from human fibroblast (FS4) cultures were
Figure 1. (A) NASBA reaction products (10 μl) analyzed on a non-denaturing agarose gel stained with ethidium bromide. Lanes 1–8, in vitro transcribed IFN-β RNA: lane 1, 10¹⁰ molecules; lane 2, 10⁸ molecules; lane 3, 10⁶ molecules; lane 4, 10⁴ molecules; lane 5, 10² molecules; lane 6, 10⁰ molecules; lane 7, 10⁻² molecules; lane 8, 10⁻⁴ molecules; lane 9, negative control (H₂O); lane 10, FS4 fibroblasts treated with the interferon inducing agent poly IC; lane 11, untreated fibroblasts; MW, molecular weight standard. Note: RNA bands appear a little bit ‘fuzzy’ on a non-denaturing gel, and additional bands may be present in samples containing total cellular RNA (lanes 10 and 11). (B–D) Northern hybridization of NASBA amplificates with a biotinated oligonucleotide probe. (B) Decreasing amounts of in vitro transcribed IFN-β RNA: lane 1, 10¹⁰ molecules; lane 2, 10⁸ molecules; lane 3, 10⁶ molecules; lane 4, 10⁴ molecules; lane 5, 10² molecules; lane 6, 10⁰ molecules; lane 7, 10⁻² molecules; lane 8, 10⁻⁴ molecules; lane 9, negative control (H₂O); MW, 100 bp ladder of biotinilated DNA. (C) Lanes 1 and 2, RNA from human foreskin fibroblasts (FS4) 1 and 3 h after washing with PBS and change of culture medium, respectively; lane 3, negative control (H₂O); lane 4, RNA from FS4 cells treated with poly IC. (D) Lane 1, 100 ng human genomic DNA; lane 2, 100 ng human genomic DNA plus 10⁴ copies IFN-β RNA; lane 3, 100 ng human genomic DNA plus 10⁶ copies IFN-β RNA; lane 4, negative control, 100 ng total RNA from Vero (monkey kidney) cells; lane 5, negative control (H₂O).

used as templates. The specificity of NASBA for ssRNA, but not dsDNA, was investigated with 100 ng human genomic DNA. Briefly, DNA was prepared from cultures of human fibroblasts using the Qiagen RNeasy kit. For in vitro transcription of IFN-β RNA, the plasmid pCR-IFB was used. pCR-IFB consists of the pCR II plasmid (Invitrogen, Carlsbad, CA) with a human IFN-β insert (nucleotides 584–1133 of the human IFN-β gene) in the multiple cloning site between the SP6 promoter and the T7 promoter. The plasmid pCR-IFB was linearized with Xhol, and IFN-β plus strand RNA was transcribed with SP6 RNA polymerase. The reaction product was digested with RNase free DNase I (Boehringer), and purified using the Qiagen RNeasy kit. The transcript was quantified by photometry at 260 and 280 nm and checked for purity and correct size on a denaturing agarose gel.

IFN-β NASBA resulted in a very high yield of amplified RNA from as few as 100 molecules of IFN-β RNA. Thus, it was feasible to visualize the main reaction product, negative strand RNA (3,5,7), directly on an ethidium bromide stained, non-denaturing agarose gel (Fig. 1A). Both the RNA band on the agarose gel, and the hybridization signal on the northern blot had the presumed size of ~170 nucleotides (Fig. 1A and B). Northern hybridization of the NASBA products with a biotinilated oligonucleotide probe enhanced the sensitivity of IFN-β RNA detection ~10-fold to 10 molecules (Fig. 1B). In addition, the sensitivity of NASBA was demonstrated by detecting the low level, basal IFN-β transcription of FS4 fibroblasts (Fig. 1C). Previously, a basal IFN-β expression in FS4 cells had been suggested from results on complex cytokine interactions, and detected so far only using RT–PCR, but not by any other, less sensitive method (2). As presumed, NASBA for IFN-β mRNA detection did not result in false positive results with up to 100 ng of genomic DNA (Fig. 1D). Therefore, it was feasible to amplify IFN-β mRNA selectively in a genomic dsDNA background (Fig. 1D). However, care has to be taken during nucleic acid sample preparation to avoid conditions that denature genomic dsDNA, as NASBA can amplify specific ssDNA sequences (3).

In conclusion, NASBA proved to be a fast, ‘one-pot’ reaction, which permitted a highly sensitive and specific amplification of IFN-β mRNA. Moreover, the NASBA technique may be useful for the highly sensitive detection of mRNA of other human intronless genes, for example the Jun protooncogene (8), the beta-adrenenergo receptor genes (9,10), the H1 histamine receptor gene (11) and the angiotensin II receptor genes (12,13). As NASBA can be modified to a quantitative assay by the use of internal standards (7), both quantitative and highly sensitive assays for gene expression of intronless genes seem to be feasible.

REFERENCES