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Amplifying control RNA for RT-PCR applications by nucleic acid sequence based amplification (NASBA)

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Abstract

Control RNA for RT-PCR applications was amplified by nucleic acid sequence based amplification (NASBA) using the NucliSens® Basic Kit. This method was used to construct positive control RNA for enterovirus, insulin, and G-protein RT-PCR, and for interferon-α real-time RT-PCR. The primers were designed to amplify identical RNA from RNA templates, which differs from the usual NASBA procedure, where opposite strand RNA is amplified from the target. This "inverse NASBA" method is easy to use and it does not require any expensive special equipment. The amplification reaction is done using a water bath and detection of amplified product by agarose gel electrophoresis. Generated RNA fragments were 195–714 bases long, of positive polarity and the amount of RNA was sufficient for thousands of RT-PCR reactions depending on the sensitivity of the RT-PCR.

Keywords: RNA amplification; NASBA; RT-PCR; Control RNA

1. Introduction

Nucleic acid sequence based amplification (NASBA) is an isothermal amplification method for RNA and it is designed to produce complementary RNA to the target (Compton, 1991). It is used mostly for diagnostic purposes in human and veterinary medicine and quality control of food products. Most of the applications are for detecting infective agents, like viruses and bacteria, for example HIV (de Baar et al., 2001; Kievits et al., 1991), CMV (Blok et al., 1998; Mengoli et al., 2004), West Nile virus (Lanciotti and Kerst, 2001), rabies (Sugiyama et al., 2003; Wacharapluesadee and Hemachudha, 2001), *Mycoplasma pneumoniae* (Loens et al., 2003a,b), *Salmonella enterica* and *Listeria monocytogenes* (Cook, 2003; D'Souza and Jaykus, 2003). It does not amplify DNA, because there is no denaturation step in the amplification. Therefore it is optimal for amplifying intronless genes like interferon-β (Heim et al., 1998). However, in the amplification process small amounts of dsDNA and DNA:RNA hybrids are produced as by-products.

Amplification methods like RT-PCR, real-time RT-PCR and NASBA need specific positive control RNA, which is not always easily available. Especially in cases where the target is a tightly regulated gene product, which needs to be induced first, or viruses might be difficult to propagate. Also the use of a RNA fragment is safer than an infective virus like HIV, although for the first amplification the virus RNA is needed.

In this paper, we describe how the NASBA method can be used to produce specific positive control RNA for RT-PCR applications. Four control RNA fragments were amplified: enterovirus, interferon-α21 (IFN-α21), insulin and G-protein. Primers for these amplifications were designed to produce positive strand RNA in contrast to the usual NASBA procedure, where opposite strand RNA is amplified from the target RNA. Therefore, we call this method "inverse NASBA". To achieve this, the T7 RNA polymerase promoter sequence was added to the forward primer (P2) instead of the reverse primer (P1).

2. Materials and methods

2.1. Starting material and RNA isolation

Enterovirus was amplified from purified Coxsackie virus B3 (CBV3) RNA (30 ng) obtained from cell culture supernatant

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Table 1
Primers used for inverse NASBA amplification of control RNA

Application/primer name	Primer sequence
Insulin	
P1: SINSR	TTCCATCTCTCTCGGTGCAGGA
P2: SINSF	AATTCTAATACGACTCACTATAGGGAGAAACATCACTGTCCTTCTGCCA
Interferon-α21	
P1: SIFA4R	CATGGTCATCTGTAAAGGACTA
P2: SIFA3F	AATTCTAATACGACTCACTATAGGGGGGAGGTTGTCAGAGCAGAAATCATGAGA
G-protein	
P1: SGPROTR	GGTGGGTGATTAACTGCTTGTACA
P2: SGPROTF	AATTCTAATACGACTCACTATAGGGAGGCTGACTATGTGCCGAGCGATCA
Enterovirus CBV3	
P1: SCBV3R	ATTGTCACCATAAGCAGCCA
P2: SCBV3F	AATTCTAATACGACTCACTATAGGGAGACATGGTGCGAAGAGTCTA

of infected green monkey kidney cells (GMK). RNA isolation was done by QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The IFN-α21 was amplified from RNA (40 ng) purified from human leukocytes. RNA isolation was done using the PAXgene™ Blood RNA System (Qiagen) according to manufacturer's instructions. The insulin and G-protein RNA amplification was done using the commercial human pancreas BD Premium Poly-A^+ RNA (5 ng) (BD Biosciences, Palo Alto, CA) as starting material.

2.2. Inverse NASBA primer design

Primer design was modified to produce positive strand RNA. Also the complementary region for the detection probe (ECL probe) was left out, which was not needed for this application. Primer sequences are presented in Table 1. P1 primer (reverse primer): the T7 RNA polymerase promoter sequence was not attached to this primer as usually is done for NASBA. The primer was 20–30 nucleotides long, complementary to the target RNA and it had an A at the 3′-end. P2 primer (forward primer): the ECL detection complementary sequence was not added to this primer, instead the T7 RNA polymerase promoter sequence (AATTCTAATACGACTCACTATAGGG) was added to the primer sequence at its 5′-end. The promoter sequence was followed by a 20–30 nucleotides long region identical to the target RNA, it had an A at the 3′-end, and the first 10 nucleotides after the promoter sequence were purine-rich (A or G). The purine-rich sequence could also be inserted between the promoter sequence and the target RNA sequence. Neither capture nor detection probe was needed.

2.3. Amplification of control RNA

RNA amplification was done by inverse NASBA using the NucliSens® Basic Kit (Biomerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, 5 μl of extracted RNA (5–40 ng) was mixed with 10 μl of reagent mixture containing the Basic Kit reagent mixture, 140 mM KCl and 1.7 μM of each primers (Table 1). This mixture was incubated for 5 min at 65 °C and for 5 min at 41 °C. The Basic Kit enzyme mixture (5 μl) was added and incubation at 41 °C was continued for a further 2–3 h.

The amplified RNA was detected from an 1.5% agarose gel: 1.5 μl inverse NASBA reaction mixture, 2 μl 5× loading buffer (instructions in PAXgene Blood RNA Kit Handbook, Appendix E) and 6.5 μl RNase free water were mixed, heated at 70 °C for 10 min and loaded on the gel. The RNA ladder, low range (Fermentas, Burlington, Canada) was heat treated in the same way before loading on the gel.

The amplified RNA was treated with RNase-Free DNase (Qiagen) to digest DNA generated in the NASBA reaction. After amplification, 5 μl RDD buffer and 1.3 μl DNaseI stock solution (2.7 Kuniz units/μl) were added to the inverse NASBA reaction mixture. RNase free water was added to make the total volume 50 μl. This mixture was incubated at RT for 45–60 min. After the DNase treatment, the RNA fragment was purified using the NucleoSpin® RNA Clean-Up Kit (Macherey-Nagel GmbH & Co., Düren, Germany) or from an 1.5% agarose gel using the QIAquick Gel Extraction Kit (Qiagen) according to instructions.

2.4. RT-PCR reactions

The ultra-sensitive enterovirus specific RT-PCR has been described earlier (Lonnrot et al., 1999). The amplified CBV3 control RNA was added in a volume of 10 μl (0.29 pg) to the RT reaction mixture (total volume 40 μl) containing the M-MLV reverse transcriptase (Moloney murine leukaemia virus reverse transcriptase, Promega, Madison, WI, USA) and the reverse primer (4−) GAAACACGGACACCCAAAGTA. The mixture was incubated for 1 h at 37 °C. An amount of 10 μl of the RT mixture was added to the PCR reaction (total volume 100 μl) containing the forward (636+) CGGCCCCTGAATGCGGCTAA and reverse (4−) primers and DyNAzyme™ DNA polymerase (Finnzymes, Espoo, Finland). PCR products were analysed from an 1.5% agarose gel and by the hybridisation protocol described earlier.

Insulin and G-protein specific RT-PCR has been described previously (Ritz-Laser et al., 2002). The RT reaction for insulin was done in a total volume of 25 μl using 10 μl of amplified insulin control RNA (0.1–1 ng), random hexamers