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Efficient amplification with NASBA® of hepatitis B virus, herpes simplex virus and methicillin resistant *Staphylococcus aureus* DNA

Birgit Deiman[a,*], Corinne Jay[b], Carine Zintilini[c], Saskia Vermeer[a], Dianne van Strijp[a], Fokke Venema[a], Paul van de Wiel[b]

[a] *bioMérieux bv, Boseind 15, P.O. Box 84, 5280 AB Boxtel, The Netherlands*
[b] *bioMérieux Molecular Biology Center, Christophe Mérieux Center, 5 Rue des Berges, 38 024 Grenoble Cedex 01, France*
[c] *bioMérieux SA, Chemin de l'Orme, 69280 Marcy l'Étoile, France*

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ABSTRACT

A new mechanism is described for DNA amplification using nucleic acid sequence-based amplification (NASBA®) including a restriction enzyme digestion and P1 primer binding directly upstream of the digestion. For hepatitis B virus (HBV), herpes simplex virus (HSV) and methicillin resistant *Staphylococcus aureus* (MRSA) DNA, which all show very poor amplification with normal NASBA®, assay sensitivity was improved by a factor 100–1000 when restriction enzyme digestion was performed prior to amplification. For the quantitative HBV assay, in combination with the NucliSENS® Extractor (bioMérieux, Boxtel, The Netherlands), a 95% target detection rate of 242 WHO IU/ml and 50% detection rate of 35 WHO IU/ml was achieved. The lowest detectable HBV concentration was 10 WHO IU/ml. HBV DNA could be quantified with an algorithm comparable to that used for RNA quantitation and by using a two step approach a dynamic range of 10^2–10^9 WHO IU/ml (>6 log) was shown to be quantifiable. For the qualitative HSV assay, in combination with the NucliSENS® miniMAG® (bioMérieux, Boxtel, The Netherlands), the 95% detection rate was determined to be 84 and 138 copies/isolation for HSV 1 and HSV 2, respectively, which corresponds to approximately 10 copies per amplification for both targets. For MRSA, the limit of detection was <10 equivalent CFU per amplification.

1. Introduction

Nucleic acid sequence-based amplification (NASBA®) was designed originally for the amplification of RNA targets (Guatelli et al., 1990; Compton, 1991; for review Deiman et al., 2002). For most DNA targets no amplification was observed under normal NASBA conditions (Heim et al., 1998; Simpkins et al., 2000). However, for some DNA targets amplification was shown, but this was only at very high input levels of DNA or in the absence of the accompanying RNA target and with very low assay sensitivity (Deiman et al., 2002). In NASBA, primer binding to the target nucleic acid takes place after the pre-incubation step, which is often performed at 65 °C. After this step, NASBA is isothermally performed at 41 °C and annealing of the second primer to the extended first primer takes place after cleavage of the target RNA from the RNA–DNA hybrid by RNase H. In case of DNA targets, the NASBA enzymes, AMV RT, T7 and RNaseH, are not able to cleave selectively the target DNA strand from the extended first primer. Annealing of the second primer is therefore inhibited, explaining that DNA targets are not efficiently amplified by NASBA. One model has been published (Sooknanan et al., 1995) describing a NASBA reaction that is optimized for DNA amplification including two denaturing steps and twice the addition of NASBA enzymes to solve the problem described above. However, this procedure for DNA NASBA amplification is not only labor intensive, but also costly and in addition, amplification is still not very efficient.

The present paper describes a DNA NASBA in which restriction enzyme digestion is incorporated into the NASBA reaction to allow controlled initiation of amplification (Fig. 1). In this model, restriction enzyme digestion of the target DNA takes place prior to amplification. The P1 primer is designed in such a way that the hybridizing part will interact with the target directly upstream of the digestion. After the addition of the NASBA® enzymes, AMV RT will extend the 3′ end of the DNA target at the digestion site using the hybridized P1 primer as template. As the P1 primer includes a 5′ T7 promoter sequence, this will become a functional double stranded T7 promoter site. Subsequently, T7 polymerase (POL) will start transcription, generating a single strand RNA amplicon to which the p2 primer can easily anneal as in RNA NASBA.

* Corresponding author. Tel.: +31 411 65 4154; fax: +31 411 65 4311.
E-mail address: birgit.deiman@eu.biomerieux.com (B. Deiman).

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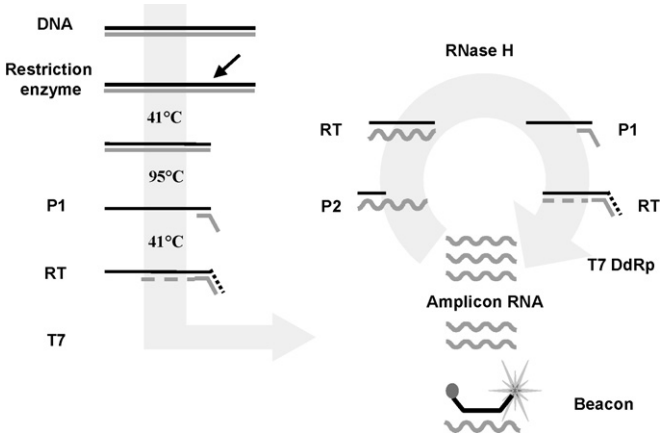

Fig. 1. Schematic representation of new mechanism for DNA NASBA®. The activities of restriction enzyme digestion (black arrow), extension of the primers and target DNA (dotted lines) by reverse transcriptase (RT), transcription by T7 polymerase (T7) and digestion of the RNA amplicon by RNaseH is indicated. Amplicons are detected by molecular beacon binding.

For the amplification of DNA targets, real-time PCR is currently the method used most commonly worldwide. In PCR, assay specificity is mainly determined by the binding of the two primers and probe and, although primer binding takes place at a defined temperature, non-specific amplification often occurs. Based on the mechanism described above, DNA amplification with NASBA requires a specific digestion of the DNA target with a selected restriction enzyme. Together with the specific binding of the primers and probe, this restriction enzyme digestion should result in a very well controlled initiation of amplification of the selected sequence, which is in favor of both assay specificity and assay sensitivity.

This paper shows the amplification of DNA targets with NASBA including restriction enzyme digestion of the target. The DNA NASBA is performed on the NucliSENS EasyQ® platform (bioMérieux, Boxtel, The Netherlands) without any adaptations of the instrumentation. The performance of a quantitative assay for detection of genomic hepatitis B virus (HBV) DNA, a qualitative assay for detection of genomic herpes simplex virus (HSV) DNA, and amplification and qualitative detection of the *mec*A gene present in methicillin resistant *Staphylococcus aureus* (MRSA) is presented.

2. Materials and methods

2.1. Alignments

The selection of restriction enzymes and accompanying primers and probes is based on sequence alignments of the different genotypes of the target of interest. For HBV the following accession numbers (Genbank) were aligned: X70185, S50225, X51970 (HBV A), D00330, D00329, D00331 (HBV B), X75665, X14193, M38636, V00867 (HBV C), X02496, X59795, X65258, X68292 (HBV D), X75657, X75664 (HBV E) and X75663, X69798 (HBV F).

For HSV the following accession numbers (Genbank) were aligned: AB070847, AB072389, AB070848, X04495, M10792, D10879, S68160, S79749, X03181, X04771, X14112 (HSV 1) and M16321, AY038366, AY038367, M14793, M16321, Z86099 (HSV 2).

For the *mec*A gene in MRSA the following accession numbers (Genbank) were used for alignment: E03736, X52593 and Y14051.

2.2. Primer and molecular beacon design

Selection of the primers and molecular beacon probes is dependent on the selected restriction site. The restriction site is selected in a highly conserved part of the sequence just downstream of the P1 binding region and the selected restriction site should not be present in the amplification area. For HBV, based on the sequence alignment, two conserved restriction sites, XbaI and BssSI, encoded in a highly conserved part of the surface (S) gene, nucleotides 244–285 according to the unique EcoRI site of X51970, were selected. Amplification primers and the detection probe were designed in the conserved region directly downstream of the digestion site. Two P1 primers, HBV p3.8-S (Table 1) and HBV p3.10-S (Table 1), are directed against nucleotides 251–269 and 258–278 of the minus strand of HBV DNA, respectively. The p2 primer (HBV p4.5-S, Table 1) is directed against nucleotides 422–443, resulting in an amplicon length of approximately 192 or 185 nt, dependent on the P1 used. The detection probe (HBV S-WT2, Table 1), labeled with FAM is directed against nucleotides 304–320. The genomic DNA of HBV consists of a partial single stranded DNA molecule in which part of the positive strand is missing, meaning that the selected part of the S-gene could be single stranded and thus cannot be digested by restriction enzymes. Therefore, an additional oligonucleotide (restriction primer, RP) complementary

Table 1
Primer and probe sequences

HBV	
HBV p3.8-S	5′- *AATTCTAATACGACTCACTATAGGG* A **GACTCGTGGTGGACTTCTCTCA** -3′
HBV p3.10-S	5′- *AATTCTAATACGACTCACTATAGGG* AGAA **GGTGGACTTCTCTCAATTTTC** -3′
HBV p4.5-S	5′- **GAACCAACAAGAAGATGAGGCA** -3′
HBV RP-2	5′- **CCTCACAATACCGCAGAGTCTAGAC** -*Dabsyl*-3′
HBV RP-3	5′- **AATACCGCAGAGTCTAGACTCGTGG** -*Dabsyl*-3′
HBV S-WT2	5′- *FAM*- CGATCG A**GGGACTGCGAATTTTggC C**GATCG -*Dabsyl*-3′
HBV S-Q1	5′- *ROX* -CGATCG A**ggAGGTCACGTTGTTAGC CGATC**G -*Dabsyl*-3′
HSV	
HSV pol p1.1	5′- *AATTCTAATACGACTCACTATAGGG* AGA **CCAGGGCCCTGGAGGTGCGG** -3′
HSV pol p2.2	5′- **ACGTTCACCAAGCTGCTGCT** -3′
HSV Gen WT2	5′- *FAM*- CGATCG **AAAAGTACATCGGCGTCATCTA** CGATCG -*Dabsyl*-3′
HSV 1 spec	5′- *FAM*- CTaTCCC **GTCATCTaCGGIGGTAAG** gggATag -*Dabsyl*-3′
HSV 2 spec	5′- *Cy5*- CGATCG **GTCATCTGCGGGGGCAAG CGATCG** -*Dabsyl*-3′
HSV IC	5′- *ROX*- CCCAAGC **GCAAAGTATCATCCCTCCAG** GCTTGGG -*Dabsyl*-3′
MRSA	
MRSA 83-P1	5′- *AATTCTAATACGACTCACTATAGGG* AGAG **TCCATTTGTTGTTGATATAGTC** -3′
MRSA 82-P2	5′- **CCGAAACAATGTGGAATTGGCCA** -3′
MRSA 91-MB	5′- *ROX*-CGTACGT **GAGATTAGGCATCGTTCCA A**CGTACG- *Dabsyl*-3′

Bold: target specific sequence; italics: T7 promoter sequence; g: 2-ome-G phosphoramidite; a: 2-ome-A phosphoramidite; I: inosine; RP is restriction primer. The molecular beacons are labeled with a fluorephore, FAM, ROX or Cy5 and quencher, dabsyl.