



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

ARTICLE INFO

Article history:

Received 14 August 2007

Received in revised form 19 March 2008

Accepted 1 April 2008

Available online 2 June 2008

Keywords:

NASBA

DNA

Amplification

HBV

HSV

MRSA

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 10²–10⁹ 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.

© 2008 Elsevier B.V. All rights reserved.

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).

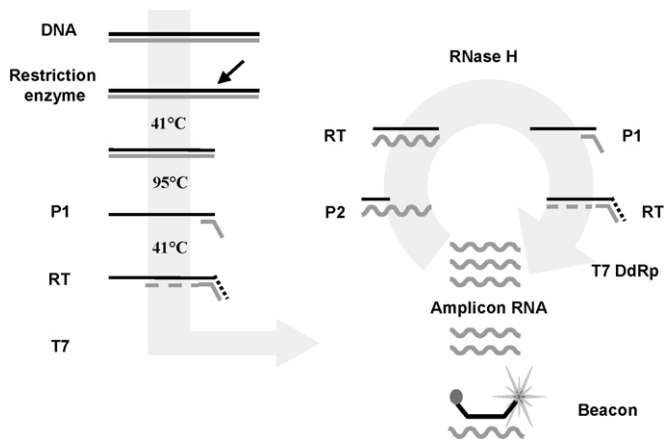


Fig. 1. Schematic representation of new mechanism for DNA NASBA®. The activities of restriction enzyme digestion (black arrow), extension of the primers and target DNA (dotted lines) by reverse transcriptase (RT), transcription by T7 polymerase (T7) and digestion of the RNA amplicon by RNaseH is indicated. 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 *mecA* 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 *mecA* 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 AGGGACTGCGAATTTggC CGATCG -Dabsyl-3'
HBV S-Q1	5'- ROX -CGATCG AggAGGTCACGTTGTTAGC CGATCG -Dabsyl-3'
HSV	
HSV pol p1.1	5'- AATTCTAATACGACTCACTATAGGG AGA CCAGGGCCCTGGAGGTGCCG -3'
HSV pol p2.2	5'- ACGTTCAACAAGCTGCTGCT -3'
HSV Gen WT2	5'- FAM- CGATCG AAAAGTACATCGCGTCACTA CGATCG -Dabsyl-3'
HSV 1 spec	5'- FAM- CTA TCCC GTCATCTaCGGIGGTAAG gggATag -Dabsyl-3'
HSV 2 spec	5'- Cy5- CGATCG GTCATCTCGGGGGCAAG CGATCG -Dabsyl-3'
HSV IC	5'- ROX- CCCAAGC GCAAAGTATATCCCTCCAG GCTTGGG -Dabsyl-3'
MRSA	
MRSA 83-P1	5'- AATTCTAATACGACTCACTATAGGG AGAG TCCATTGTGTGATATAGTC -3'
MRSA 82-P2	5'- CCGAAACAATGTGGAATTGGCCA -3'
MRSA 91-MB	5'- ROX-CGTACGT GAGATTAGGCATCGTTC CA ACGTACG- Dabsyl-3'

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

to the region including the restriction site sequences is included to create a double stranded restriction site for all genomic DNA present. RP is blocked by a dabsyl group at the 3' end and thus cannot be extended during amplification. The functionality of RP was determined by hybridization to a complementary oligonucleotide and digestion of the hybrid with both XbaI and BssSI under NASBA[®] conditions. The digestion products were analyzed by gel analysis (results not shown). The restriction primer HBV RP-2 (Table 1), directed against nucleotides 229–253, is used in combination with XbaI digestion and HBV RP-3 (Table 1), directed against nucleotides 235–262, is used in combination with BssSI digestion. As RP-3 includes both the XbaI and BssI restriction site, this primer is used in double digestions.

Based on the alignment of the HSV genomic sequences, two restriction sites Apal and Sall, encoded in a highly conserved part of the polymerase gene, nucleotides 2912–2925 of accession number AB070847, were selected. The P1 primer (HSV pol p1.1, Table 1) is directed against nucleotides 2900–2920 of the plus strand of HSV DNA. The p2 primer (HSV pol p2.2, Table 1) is directed against nucleotides 2785–2805, resulting in an amplicon length of approximately 135 nt. The detection probe (HSV Gen WT2, Table 1) is labeled with FAM and is directed against nucleotides 2817–2839. In addition a FAM-labeled HSV 1 specific molecular beacon probe (HSV 1 spec, Table 1) and Cy5-labeled HSV 2 specific beacon (HSV 2 spec, Table 1) were designed, directed against nucleotides 2832–2849.

Based on an alignment of the *mecA* sequences, one conserved Sau3A-I restriction site was selected. The P1 primer (MRSA 83-P1, Table 1) was designed directly upstream of the restriction site and is directed against nucleotides 583–604 of the plus strand of accession number E03736 (Genbank). The P2 primer (MRSA 82-P2, Table 1) is directed against nucleotides 468–487, resulting in an amplicon length of approximately 137 nt. The detection probe (MRSA 91-MB, Table 1), labeled with ROX is directed against nucleotides 507–526.

2.3. Construction of the HBV internal calibrator and HSV internal control (IC)

As the HBV assay is a quantitative assay, an internal calibrator (Q) was constructed. This Q is co-isolated and co-amplifies with wild type (WT) HBV DNA using the same primer set. For the construction of Q, first a HBV WT construct was prepared. The HBV WT construct is a plasmid including part of the HBV S/P-gene. This HBV S/P sequence (serotype adw2, Genbank accession X02763) is derived by PCR from the plasmid pSV BX 24H (Ono, 1983). The PCR fragment (nt 1599–2285 of X02763) is cloned in the pGEM-3X-based vector (Promega) after digestion with PvuII (New England BioLabs Inc., Beverly, MA, USA) and Csp45I (Promega Biosciences Inc., San Luis Obispo, CA, USA). In this step also the T7 promoter sequence of the pGEM-3X-based vector is deleted to prevent interference with NASBA[®] amplification. Subsequently, part of the HBV and vector sequence is deleted by BsrGI (New England BioLabs Inc., Beverly, MA, USA) digestion in order to obtain a HBV WT construct of ~3.1 kb which is comparable to the size of genomic HBV DNA (3.2 kb). For the construction of Q, the molecular beacon binding position for WT detection of this HBV WT construct is replaced by the binding sequence for the Q molecular beacon probe, using overlapping PCR. The nucleotide sequence of the Q construct was checked by sequence analysis (Baseclear, Leiden, The Netherlands). The concentration and purity of the WT and Q construct was determined by OD260/OD280 measurement. A ROX-labeled molecular beacon was designed to specifically detect the Q (HBV S-Q1, Table 1).

For the qualitative HSV NASBA an internal control was designed. This IC is a modified version of the HSV WT construct. The HSV WT construct is a plasmid including part of the HSV pol-gene. Nucleic

acid isolated from cultured HSV 1 (strain SC16) has served as template in PCR for the HSV pol-gene sequence. The PCR fragment was cloned in the pGEM-3X-based plasmid (Promega Biosciences Inc., San Luis Obispo, CA, USA) after digestion with EcoRI (New England BioLabs Inc., Beverly, MA, USA) and Csp45 (Promega Biosciences Inc., San Luis Obispo, CA, USA). In this step also the T7 promoter sequence of the pGEM-3X-based vector is deleted to prevent interference with NASBA amplification. For the construction of the IC, the molecular beacon binding position for WT detection (both for generic and specific detection) of this HSV WT construct is replaced by the binding sequence for the IC molecular beacon probe, using overlapping PCR. The nucleotide sequence of the IC was checked by sequence analysis (Baseclear, The Netherlands). The concentration and purity of the WT and IC construct was determined by OD260/OD280 measurement. A ROX-labeled molecular beacon was designed to specifically detect the Q (HSV IC, Table 1).

For the qualitative MRSA *MecA* assay, no IC was designed.

2.4. Strains and DNA isolation

HBV DNA was isolated from a dilution series of plasma infected with HBV genotype A of 3×10^9 Europhep copies/ml (VQC, CLB-Sanguine) or the World Health Organization (WHO) International standard (NIBSC) or a reference sample calibrated against the WHO international standard. The NucliSENS[®] Extractor (bioMérieux, Boxtel, The Netherlands) and the NucliSENS[®] Isolation kit (bioMérieux, Boxtel, The Netherlands) were used to isolate the HBV DNA according to the manufacturer's generic protocol. For samples with high viral loads, 50 μ l sample was mixed with 900 μ l of NucliSENS[®] Lysis buffer. For samples with low viral loads, 1 ml sample was mixed with 9 ml of NucliSENS[®] Lysis buffer. The mixture was incubated at room temperature for 10 min. For quantitation, the calibrator DNA, 10^4 copies for low viral load samples and 10^5 copies for high viral load samples, was added prior to isolation.

HSV DNA was isolated from tissue culture infected with strain SC16 type (HSV 1). Initially, automated extraction was performed using the NucliSENS[®] Extractor (bioMérieux, Boxtel, The Netherlands) and the NucliSENS[®] Isolation kit (bioMérieux, Boxtel, The Netherlands). Of the HSV infected tissue culture, 50 μ l was added to 450 μ l Lysis buffer and the extraction was performed according to the 1 ml plasma-protocol described by the manufacturer and finally nucleic acids were eluted in 50 μ l elution buffer. During development of the assay the semi-automated extraction with NucliSENS[®] miniMAG[®] (bioMérieux, Boxtel, The Netherlands) in combination with the NucliSENS[®] magnetic extraction reagents (bioMérieux, Boxtel, The Netherlands) and NucliSENS[®] Lysis buffer (bioMérieux, Boxtel, The Netherlands) was used to isolate the HSV DNA according to the standard manufacturer's protocol. For isolation and amplification control, 1500 copies of the IC were added prior to isolation.

For MRSA, total nucleic acids were released from two different strains, type MREJ i and type MREJ iv, which were kindly provided by Dr. Van Leeuwen of the Erasmus Medical Center in Rotterdam, The Netherlands. Nucleic acid extraction was performed using a mechanical lysis technique (Cleuziat et al., 2003). Bacteria obtained from an overnight culture on trypticase soy agar plate (bioMérieux, Marcy l'Étoile, France) were suspended in sterile water to reach a 0.5 McFarland suspension as determined with the Densimat[®] (bioMérieux, Marcy l'Étoile, France) instrument. After mechanical lysis of this suspension, 1 ml of lysate was submitted to semi-automated extraction on the NucliSENS[®] miniMAG[®] (bioMérieux, Boxtel, The Netherlands) instrument and the standard isolation protocol as described by the manufacturer. Nucleic acids were eluted in 100 μ l of elution buffer. The amount of MRSA genomic DNA in the eluate is defined as equivalent colony-forming unit

(CFU) and was extrapolated from the original McFarland input used in extraction, knowing that 0.5 McFarland corresponds to 1.5×10^8 CFU/ml (Grisold et al., 2002) and based on 100% lysis and extraction yields.

2.5. Restriction enzyme digestion, amplification and real-time detection

For HBV amplification, an amplification mixture was prepared, using NucliSENS EasyQ[®] Basic kit V.2 reagents (bioMérieux, Boxtel, The Netherlands), including 40 mM Tris pH 8.5, 12 mM MgCl₂, 5 mM DTT, 1 mM of each dNTP, 2 mM ATP, 2 mM UTP, 2 mM CTP, 1.5 mM GTP, 0.5 mM ITP, 15% DMSO and 70 mM KCl and 0.2 μM P1 primer (HBV p3.8-S for XbaI, HBV p3.10-S for BssSI, and HBV p3.8-S for XbaI + BssI, Fig. 2 and Table 1), 0.2 μM P2 primer (HBV p4.5-S, Fig. 2 and Table 1), 0.1 μM FAM-labeled WT molecular beacon probe (HBV S-WT2, Fig. 2 and Table 1), 0.1 μM ROX-labeled Q molecular beacon probe (HBV S-Q1, Table 1) and 0.2 units per reaction of restriction enzyme BssSI (New England BioLabs Inc., Beverly, MA, USA) or 3.0 units per reaction of restriction enzyme XbaI (New England BioLabs Inc., Beverly, MA, USA) or both in case of double digestion. Of the RP (HBV RP-2 for XbaI, HBV RP-3 for BssSI and HBV RP-3 for XbaI + BssI, Table 1), 0.17 μM was added to the amplification mixture. Of the HBV extract, 5 μl was mixed with 10 μl amplification mixture. After restriction enzyme digestion of 15 min at 41 °C in the NucliSENS EasyQ[®] Incubator (bioMérieux, Boxtel, The Nether-

lands), the restriction enzymes were heat-inactivated and the DNA template was denatured at 95 °C for 5 min. Hybridization of the primers occurred during cooling down to 41 °C for 3 min. Subsequently, 5 μl of NASBA[®] enzymes, 0.08 units of RNaseH, 32 units of T7 and 6.4 units of AMV RT, were added, the reaction mixture was mixed by gently tapping and short centrifugation after which the amplification and real-time detection was started. The reaction mixture was incubated at 41 °C in the NucliSENS EasyQ[®] Analyzer (bioMérieux, Boxtel, The Netherlands) for 60 or 120 min with fluorescence monitoring every 30 s or 1 min, respectively, for each independent reaction.

For HSV, 5 μl of the extract is added directly to 10 μl of amplification mixture including the NucliSENS EasyQ[®] Basic kit V.2 reagents (bioMérieux, Boxtel, The Netherlands) and 70 mM KCl, 0.1 μM of each primer (HSV pol p1.1 and HSV pol p2.2, Table 1) and 0.1 μM of FAM-labeled WT molecular beacon (HSV Gen-WT2, Table 1) or a combination of 0.1 μM of FAM-labeled HSV 1 specific molecular beacon (HSV 1 spec, Table 1) and 0.1 μM of Cy5-labeled HSV 2 specific beacon (HSV 2 spec, Table 1), and 0.1 μM of ROX-labeled IC molecular beacon (HSV IC, Table 1) and 1 unit per reaction of Sall (New England Biolabs Inc., Beverly, MA, USA) and initially also 1 unit per reaction of ApaI (New England BioLabs Inc., Beverly, MA, USA). After an incubation of 15 min at 41 °C to digest the template DNA in the NucliSENS EasyQ[®] Incubator (bioMérieux, Boxtel, The Netherlands), the samples were heated at 95 °C for 5 min to denature the template DNA and inactivate the restriction enzymes.

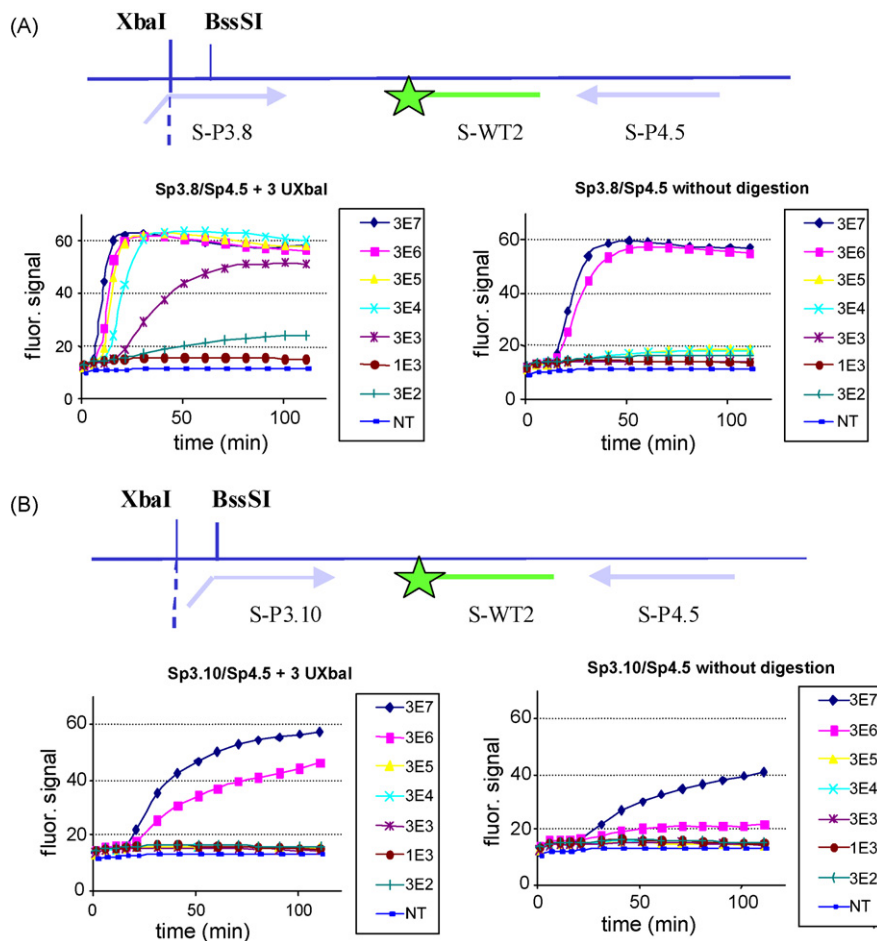


Fig. 2. Amplification of HBV DNA with and without XbaI digestion. Amplification was performed with (A) primer p3.8-S or (B) primer p3.10-S. After digestion, primer p3.8-S can be used as template for the extension of the target DNA at the digestion to obtain a double stranded T7 promoter. Primer p3.10-S cannot be used as template. Primer p4.5-S is used as P2 primer and S-WT2 as molecular beacon probe. HBV DNA of genotype A is used as target. Viral loads are defined as geq per ml used in isolation. Of the extract 10% was used in amplification. A sample without template (NT) is used as negative control.

Subsequently, the primers were hybridized to the template during 3 min incubation at 41 °C. After addition of 5 µl of enzyme mixture, the samples were amplified at 41 °C for 60 or 90 min in the NucliSENS EasyQ® Analyzer (bioMérieux, Boxtel, The Netherlands). Fluorescence was measured every 45 s.

For MRSA, 5 µl of extracted nucleic acid is added to 10 µl of amplification mixture including the NucliSENS EasyQ® Basic Kit v.2 reagents (bioMérieux, Boxtel, the Netherlands) and 90 mM KCl, 0.2 µM for each primer (MRSA 83-p1 and MRSA 82-p2, Table 1), 0.1 µM ROX-labeled WT beacon (MRSA 91-MB, Table 1), and for the tests with restriction enzyme, 1 unit per reaction of Sau3A-I (New England Biolabs Inc., Beverly, MA, USA). After an incubation of 15 min at 41 °C to digest the template DNA, the samples were heated for 5 min at 95 °C to denature the template DNA and inactivate the restriction enzyme. Subsequently the primers were hybridized to the template during 3 min incubation at 41 °C. After addition of 5 µl of NASBA® enzyme solution the samples were amplified at 41 °C for 90 min in the NucliSENS EasyQ® Analyzer. Fluorescence was measured every 30 s.

2.6. Quantitation of HBV DNA

An algorithm for the quantitation of the HBV DNA was designed, based on the algorithm used for quantifying HIV 1 RNA with real-time NASBA (Van Beuningen et al., 2001; Weusten et al., 2002). To determine the quantitative performance of the HBV NASBA, a dilution series of a proficiency panel calibrated against the WHO standard was isolated in the presence of Q, 10⁴ copies/isolation for the low end of the dynamic range (DR-low) and 10⁵ copies/isolation for the high end of the dynamic range (DR-high). The Q input for DR-low was selected based on the highest input of Q that did not influence assay sensitivity and the Q input for DR-high was selected based on the highest input of Q that permitted a good overlap between DR-low and DR-high. The different dilutions were tested, i.e. isolated and amplified, in 8, 10, 16 or 18 replicates; more replicates were tested for concentrations close to the upper or lower end of the dynamic range and for some dilutions additional testing was performed.

2.7. Assay sensitivity

To determine assay sensitivity of the HBV NASBA®, a dilution series of a proficiency panel calibrated against the WHO standard was isolated in the presence of Q, 10⁴ copies/isolation for the low end of the dynamic range and 10⁵ copies/isolation for high end of the dynamic range. The different dilutions were tested, i.e. both isolated and amplified in 12 or 16 replicates for the high concentrations and 24 or 32 replicates for the low concentrations. Probit analysis was performed using the SAS software package Version 6.12.

To determine the assay sensitivity of the HSV NASBA, dilution series of WT HSV 1 and WT HSV 2 constructs were isolated in the presence of 1500 copies/isolation of IC. All dilutions were tested, i.e. isolated and amplified, in 16 replicates. For detection, the HSV 1 and HSV 2 specific molecular beacon probes (HSV 1 spec and HSV 2 spec, Table 1) were used. Probit analysis was performed using the SAS software package Version 8.0. In addition, 1 ml of the different samples of a quality control for molecular diagnostics (QCMD) performance panel (2006) for HSV was isolated in the presence of 1500 copies of IC and the extracts were tested for the detection of HSV.

To determine assay sensitivity of the MRSA *mecA* NASBA, a dilution series of the purified nucleic acids obtained from bacterial lysates using the NucliSENS® miniMAG® (bioMérieux, Boxtel, The Netherlands), was tested. Limit of detection corresponds to the low-

est dilution that gives a positive NASBA signal. As described above (Section 2.4) the amount of MRSA genomic nucleic acids in the eluate was extrapolated from the original McFarland input used in extraction as defined in CFU, and therefore assay sensitivity is defined as equivalent CFU per NASBA.

3. Results

3.1. Amplification of HBV DNA

Based on an alignment of 20 HBV DNA sequences including genotypes A to F (Stuyver et al., 2000), two conserved restriction sites, XbaI and BssSI, encoded in a highly conserved part of the surface (S) gene were selected and primers and a detection probe were designed directly downstream of these restriction sites. A NASBA® with and without the treatment with the restriction enzyme XbaI was performed using the primers S-p3.8 and S-p4.5 and molecular beacon S-WT2. After digestion, the hybridizing part of S-p3.8 is complementary to the very 3' end of the target DNA strand at the XbaI restriction site. Real-time detection of amplification showed that without XbaI digestion amplicons were detectable only at high input levels of at least 3 × 10⁶ Eurohep copies/ml (Fig. 2A). After digestion with XbaI, however, the assay sensitivity was improved to 3 × 10³ Eurohep copies/ml, meaning a 1000-fold increase in assay sensitivity (Fig. 2A). In addition, without XbaI digestion the time to signal detection (i.e. time to positivity (TTP)) was about 16 min while after XbaI digestion a TTP of about 6 min was observed, meaning a decrease in TTP of about 10 min (Fig. 2A) during the 60 min of real-time detection. This decrease in TTP supports an increase in amplification rate. The increase in assay sensitivity and amplification rate is a clear indication for an improved amplification reaction.

To determine whether the digestion alone or the combination of the digestion and the selected P1 primer was the basis for the improved results, the XbaI digestion was repeated and primer S-p3.10 was used for amplification instead of S-p3.8. The primer binding site of S-p3.10 is situated 7 nt upstream of the XbaI restriction site, so S-p3.10 can hybridize to the target DNA stand but AMV RT cannot use S-p3.10 as template to extend the 3' end of the target sequence after digestion with XbaI. As shown in Fig. 2B, only a slight increase in assay kinetics but not in assay sensitivity and a small decrease in TTP (about 5 min: from 21 to 16 min) is obtained after digestion with XbaI in combination with S-p3.10, suggesting strongly that the extension of the 3' end of the target strand at the restriction site is important to improve assay sensitivity.

Subsequently, a NASBA® reaction with and without treatment with the restriction enzyme BssSI was performed with the same HBV DNA extract and comparable reaction conditions as described above. S-p3.10 is used as P1 primer, Sp4.5 as p2 primer and S-WT2 as probe (Fig. 3A). The hybridizing part of S-p3.10 is complementary to the very 3' end of the target DNA strand at the BssSI restriction site. Without restriction enzyme digestion an assay sensitivity of only 3 × 10⁷ Eurohep copies/ml is obtained while after digestion the sensitivity is 3 × 10⁴ Eurohep copies/ml (Fig. 3A), meaning again a 1000-fold increase in sensitivity. In addition, without BssSI digestion the TTP is about 21 min while after BssSI digestion a TTP of about 11 min was observed, meaning a decrease in TTP of about 10 min. These results indicate yet again that performing a restriction enzyme digestion prior to the NASBA reaction improved considerably the amplification of HBV DNA.

Finally, the BssSI digestion was repeated and primer S-p3.8 was used in amplification instead of S-p3.10. The primer-binding site of S-p3.8 is situated -7 to +15 nt compared to the BssSI restriction site of the target DNA strand, meaning that S-p3.8 can only

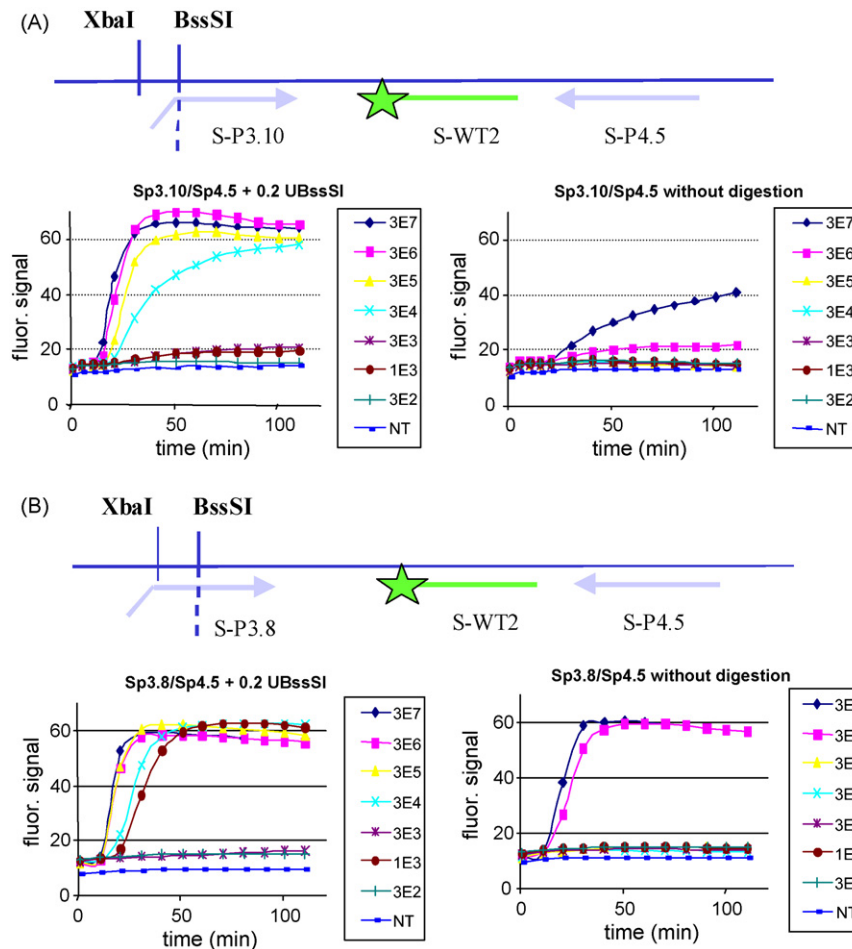


Fig. 3. Amplification of HBV DNA with and without BssSI digestion. Amplification was performed with (A) primer p3.10-S or (B) primer p3.8-S. After digestion, both primers can be used as template for the extension of the target DNA at the digestion although p3.8-S requires more target extension than p3.10-S to obtain a double stranded T7 promoter. Primer p4.5-S is used as P2 primer and S-WT2 as molecular beacon probe. HBV DNA of genotype A is used as target. Viral loads are defined as geq per ml used in isolation. Of the extract 10% was used in amplification. A sample without template (NT) is used as negative control.

hybridize to the target DNA strand with 15 nucleotides instead of 22 nucleotides. If binding does take place, however, AMV RT can use S-p3.8 as template to extend the 3' end of the target DNA after digestion with BssSI. As shown in Fig. 3B, a 100–1000-fold increase in sensitivity was also obtained with S-p3.8 after digestion with BssSI, again showing the added value of the possibility to extend the 3' end of the target strand immediately after primer binding.

Because of the importance of restriction enzyme activity on the sensitivity of the test, sequence conservation of the restriction site is required. For HBV, the conservation of both restriction sites was determined in more detail. An alignment of 454 GENBANK sequences was performed and the number of mismatches in the restriction sites was calculated. In 2.9% of the sequences a mutation is present in the XbaI restriction site and in ~1% of the sequences a mutation is present in the BssSI restriction site. However, in none of the sequences a mutation was found in both restriction sites. Therefore, it was decided to include both restriction enzymes in the HBV DNA NASBA[®]. As both XbaI and BssSI can be combined with S-p3.8, this primer was selected as P1 primer. Amplification efficiency was shown to be comparable to that of the single digestion NASBAs. Results obtained with the HBV DNA assay shown below are all obtained with the final format including the double digestion in combination with S-p3.8.

3.2. Amplification of HSV DNA

To test if the use of restriction enzymes with accompanying P1 primers is a generic way to improve the sensitivity of DNA amplification, amplification of HSV genomic DNA was performed. Based on an alignment of genomic sequences of HSV 1 and HSV 2 two restriction sites Apal and Sall, encoded in a highly conserved part of the polymerase gene were selected and primers and detection probes were designed in the conserved region directly upstream of the digestion sites. As both restriction enzymes could be combined with one and the same P1 primer (HSV P1.1) they were both included in the NASBA[®] reaction. A NASBA with and without the treatment with the restriction enzyme Apal and Sall was performed on a dilution series of a tissue culture derived sample of HSV 1 (Strain SC16) of unknown viral load. Real-time detection was performed using the generic detection probe (HSV Gen WT2). Without restriction enzyme digestion, amplification of HSV DNA showed good assay kinetics up to the 10⁻³-fold dilution (Fig. 4). However, after digestion with Apal and Sall good kinetics is obtained up to the 10⁻⁶-fold dilution, meaning a 1000-fold increase in assay sensitivity (Fig. 4). In addition, without digestion the TTP is about 13 min (17 cycles of 45 s) while after digestion a TTP of about 9 min (12 cycles) was observed, meaning a decrease in TTP of approximately 4 min. Both results are indications for an improved amplification reaction due to restriction enzyme treatment of the DNA.

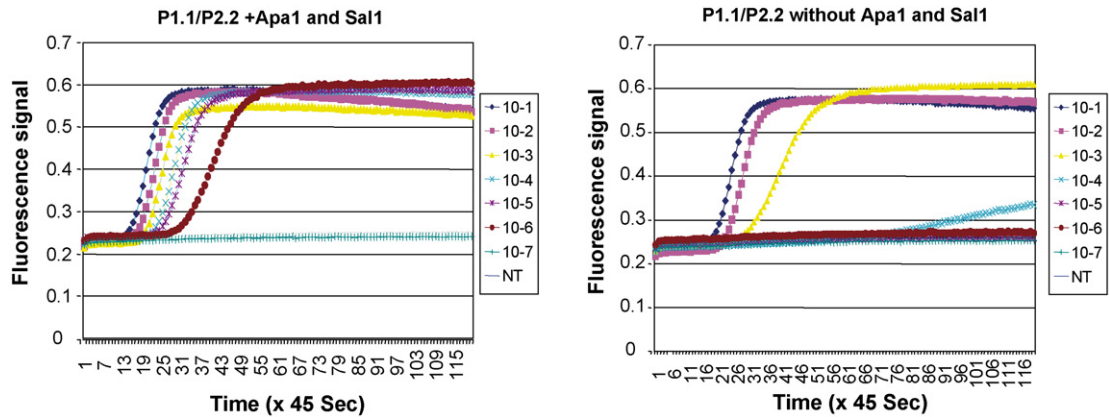


Fig. 4. Amplification of HSV DNA with and without Apal and Sall digestion. Amplification was performed with primer P1.1 and P2.2 and molecular beacon probe HSV GEN WT2 was used for real-time detection. After digestion, P1.1 can be used as template for the extension of the target DNA at the digestion to obtain a double stranded T7 promoter. A dilution series of tissue culture derived HSV 1 (strain SC16) of unknown viral load was used as input. Of the extract 20% was used in amplification. A sample without template (NT) is used as negative control.

3.3. Amplification of *mecA* in MRSA

To investigate if the use of restriction enzyme digestion also improves the amplification of genomic bacterial DNA, amplification of the *mecA* gene of MRSA was performed. Based on the alignment of MRSA derived *mecA* sequences, one conserved Sau3A-I restriction site was selected and primers and probes were designed directly upstream of this restriction site. A NASBA with and without the treatment with the restriction enzyme Sau3A-I was performed on a dilution series of purified nucleic acids obtained from two different MRSA strains, strain 1 (type MREJ I) and strain 2 (type

MREJ iv). Concentrations ranging from 10 to 1000 equivalent colony-forming units per NASBA were tested. For strain 1, a clear improvement in assay sensitivity is shown as a result of Sau3A-I digestion included in NASBA® (Fig. 5), as 1000 CFU/NASBA were not detectable without restriction enzyme digestion while even 10 CFU/NASBA were detectable when restriction enzyme digestion was performed prior to amplification. For strain 2, however, the use of Sau3A-I only led to a minor improvement of the limit of detection, since 50 CFU/NASBA were already detectable without restriction enzyme digestion while 10 CFU/NASBA were only detectable when restriction enzyme digestion was performed.

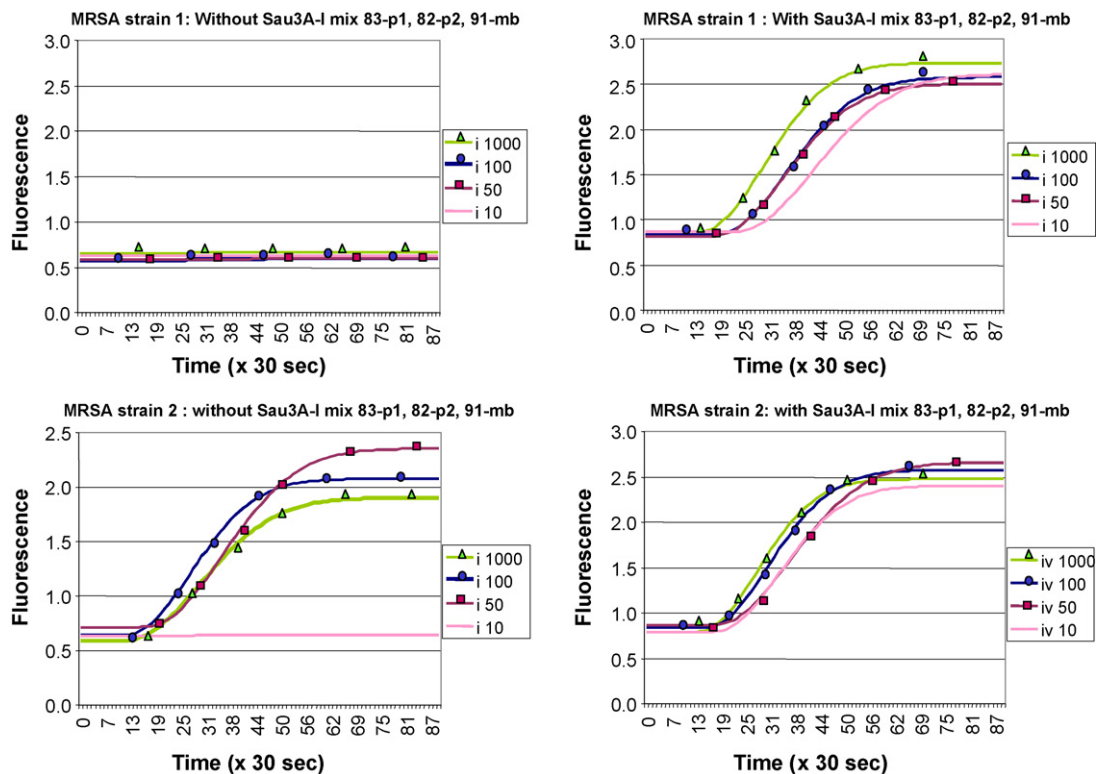


Fig. 5. Amplification of MRSA *mecA* with and without Sau3A digestion. Amplification was performed with primer MRSA 83-p1 and MRSA 82-p2 and molecular beacon probe MRSA 91-mb was used for real-time detection. A dilution series of culture derived bacteria of two different MRSA strain: strain 1 (type MREJ I) and strain 2 (type MREJ 2) of 10–1000 CFU/NASBA was used as input.

Table 2A
Quantitative performance of the HBV DNA NASBA (DR-low)

Input (log)	Number of samples			Mean (log)	S.D. (log)	Min (log)	Max (log)
	Invalid	Negative	Positive				
1.70	0	1	7	1.43	0.23	1.01	1.71
2.00	0	3	5	1.52	0.25	1.22	1.83
2.48	0	0	8	2.22	0.24	1.86	2.49
3.00	0	0	8	2.94	0.15	2.65	3.06
4.00	0	0	8	3.90	0.06	3.79	3.97
5.00	0	0	8	4.89	0.16	4.72	5.15
6.00	0	0	8	6.08	0.21	5.82	6.43
6.48	0	0	16	6.45	0.15	6.15	6.64
6.70	0	0	16	6.70	0.21	6.42	7.15
7.00	2	0	14	7.09	0.18	6.66	7.29

Input: log IU used in isolation; Mean: mean value of the calculated input values; S.D.: standard deviation on calculated mean value; Min: lowest calculated input value; Max: highest calculated input value.

3.4. Quantitation of HBV DNA

To show that HBV DNA amplified with DNA NASBA including restriction enzyme digestion with XbaI and BssSI, can be quantified on the NucliSENS[®] system (bioMérieux, Boxtel, The Netherlands), an internal calibrator (Q) was included in the HBV assay.

To cover the broad dynamic range of HBV in infected patients, two Q concentrations had to be selected. A series of dilutions of a reference sample with a high viral load was used to select the Q concentrations. The first Q concentration was selected based on the best assay sensitivity and was determined to be 10^4 copies per test. HBV DNA quantitation was performed using an HBV specific algorithm. Using this Q concentration, a linear dynamic range (DR-low) of approximately 10^2 – 10^7 IU/ml was quantifiable (Table 2A and Fig. 6A). The selection of the second Q concentration is based on quantitation of high viral loads and was determined to be 10^5 copies per test resulting in a linear dynamic range (DR-high) of 10^4 – 10^9 IU/ml (Table 2B and Fig. 6B). Combined, a dynamic range of more than 7 log₁₀ was quantifiable and a good overlap (~ 3 log₁₀) between the dynamic ranges existed. Precision estimates (standard deviation of log transformed copies) for DR-low, and DR-high were below 0.2 log₁₀ (Table 2). At the low end of the dynamic range using the DR-low protocol, the precision estimates were below 0.25 log₁₀ (Table 2).

3.5. Assay sensitivity of HBV NASBA[®]

To determine assay sensitivity of the HBV NASBA, a proficiency panel calibrated against the WHO standard was tested and a probit analysis was performed. The 95% detection rate (LOQ) of the lower and upper part of both dynamic ranges, DR-low and DR-high, was determined. The lower LOQ of DR-low was shown to be 242 IU/ml (Fig. 7A) and DR-high 3630 IU/ml (Fig. 7B). The upper LOQ of DR-low was determined to be 5×10^6 IU/ml and DR-high 5×10^8 IU/ml. This indicates that viral loads from 242 IU/ml to 5×10^8 IU/ml can be detected with a detection rate of 95%. A 50% detection rate of 35 IU/ml was determined for DR-low. The lowest detectable concentration (cut-off value) was determined to be 10 WHO IU/ml.

3.6. Assay sensitivity of HSV NASBA

For the qualitative HSV NASBA a dilution series of a WT HSV 1 construct and a dilution series of a WT HSV 2 construct were tested in the presence of the IC. For this experiment the detection of the amplicons was performed with a combination of HSV 1 and HSV 2 specific molecular beacon probes (HSV 1 spec and HSV 2 spec, Table 1). As the genomic DNA of HSV at the position of the

restriction sites is highly conserved, it was decided to only include the Sal I restriction enzyme in amplification. A dilution series of both HSV 1 and HSV 2 DNA was tested (Table 3) and probit analyses were performed. For HSV 1 the 95% detection rate was determined to be 84 copies in isolation and the 50% detection rate was 28 copies in isolation. For HSV 2, the 95% detection rate was determined to be 138 copies in isolation and the 50% detection rate was 23 copies in isolation. By testing a dilution series of WT HSV construct directly in

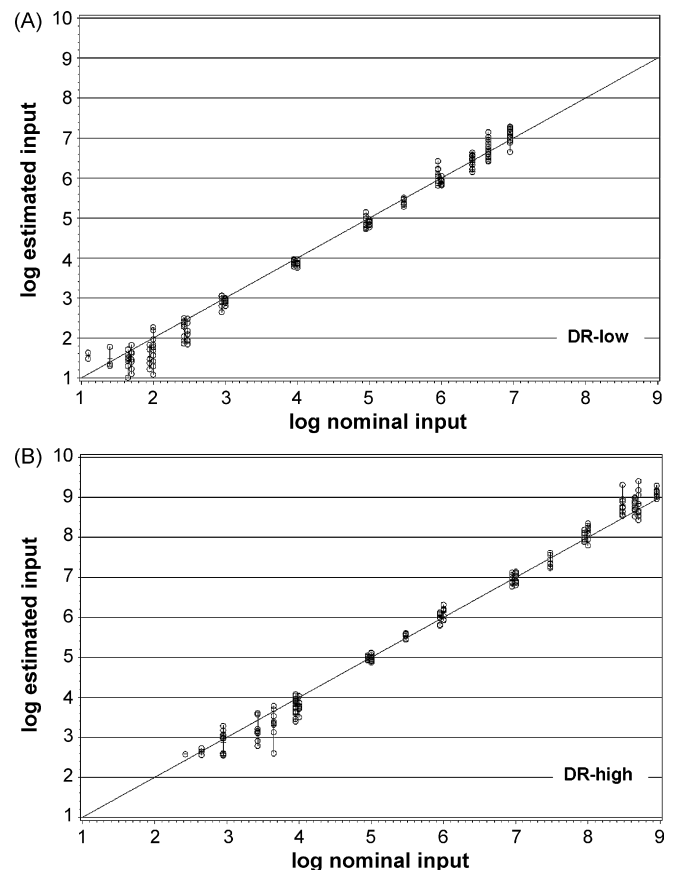


Fig. 6. Linearity of the HBV NASBA[®]. A two step approach, meaning two different internal calibrator inputs, 10^4 and 10^5 copies/isolation, and two different sample input volumes, 1 ml and 50 μ l, respectively, is used to broaden the dynamic range. The lower part (DR-low) (A) and upper part (DR-high) (B) of the dynamic range are presented. A proficiency panel calibrated against the WHO international standard was used as input and viral loads are indicated as log NASBA copies/ml which is equivalent to log WHO IU/ml as presented on the X-axis. The log of the estimated input levels is presented on the Y-axis.

Table 2B
Quantitative performance of the HBV DNA NASBA (DR-high)

Input (log)	Number of samples			Mean (log)	S.D. (log)	Min (log)	Max (log)
	Invalid	Negative	Positive				
3.48	0	1	9	3.17	0.27	2.79	3.60
3.70	0	0	9	3.35	0.35	2.60	3.78
4.00	0	0	18	3.77	0.19	3.40	4.07
5.00	0	0	8	4.99	0.04	4.93	5.05
6.00	0	0	8	6.01	0.13	5.80	6.13
7.00	0	0	8	6.93	0.12	6.77	7.12
8.00	0	0	8	8.00	0.12	7.89	8.19
8.70	0	0	8	8.81	0.16	8.53	9.00
9.00	0	0	8	9.11	0.11	8.97	9.30

Input: log IU used in isolation; Mean: mean value of the calculated input values; S.D.: standard deviation on calculated mean value. Min: lowest calculated input value; Max: highest calculated input value.

amplification, it was shown that this corresponds to a 95% detection rate of approximately 10 copies/amplification for both HSV 1 and HSV 2 (results not shown). This factor 10 is explicable because when using the NucliSENS® miniMAG® for isolation, only 1/5th of the extract is used in amplification and some nucleic acid can be lost in extraction.

In addition, to have an impression of the assay sensitivity on the level of the virus detection, a QCMD performance panel (2006) for HSV was isolated in the presence of 1500 copies of IC and the extracts were tested for the detection of HSV. HSV was detectable

Table 3
Limit of detection of the HSV DNA NASBA

Input Cp/isolation	Positive HSV 1	Hit rate HSV 1 (%)	Positive HSV 2	Hit rate HSV 2 (%)
10	2/16	12.5	1/16	6.3
25	5/16	31.3	9/16	56.3
50	14/16	87.5	13/16	81.3
75	14/16	87.5	15/16	93.8
100	16/16	100	15/16	93.8
125	16/16	100	16/16	100
250	16/16	100	16/16	100
500	16/16	100	16/16	100
1000	16/16	100	16/16	100
10000	16/16	100	16/16	100
NT	0/16	NA	0/16	NA
NS	0/48	NA	0/48	NA

Cp: copies; NT: no template (no IC and no WT target); NS: no sample (no WT target); NA: not applied.

Table 4
QCMD panel 2006 herpes simplex virus

Sample	Genotype viral load (IU/ml)	Result
QCMD2006-01	HSV 1 10E−6.5	HSV1
QCMD2006-02	HSV 1 10E−7	HSV1
QCMD2006-03	HSV 2 10E−2	HSV2
QCMD2006-04	MEDIUM	NEGATIVE
QCMD2006-05	HSV 1 10E−6	HSV1
QCMD2006-06	HSV 2 10E−5.5	HSV2
QCMD2006-07	HSV 1 10E−2	HSV1
QCMD2006-08	MEDIUM	NEGATIVE
QCMD2006-09	HSV 2 10E−6	HSV2
QCMD2006-10	HSV 1 10E−4	HSV1
QCMD2006-11	VZV	NEGATIVE
QCMD2006-12	HSV 2 10E−5	HSV2

in all HSV positive samples (Table 4). In addition, VZV was not detectable showing that the assay does not cross react with VZV.

3.7. Assay sensitivity of *mecA* NASBA®

For both MRSA strains, a dilution series of the eluates was tested, showing that the limit of detection with the restriction enzyme is less or equal to 10 equivalent CFU/NASBA (Fig. 5).

4. Discussion

The present paper shows that DNA can be amplified efficiently with NASBA® if the target DNA is digested during the initiation of amplification by a selected restriction enzyme and if it is amplified with a specific P1 primer designed to hybridize directly downstream of the digestion.

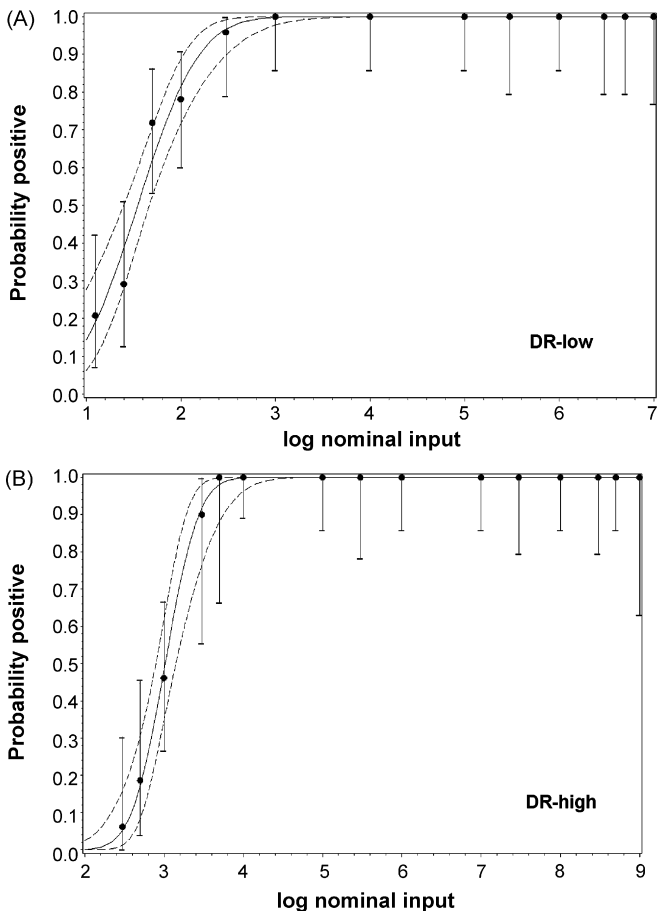


Fig. 7. Probit analysis of hit rates at different HBV DNA inputs. A proficiency panel calibrated against the WHO international standard was used as input and viral loads are indicated as log NASBA® copies/ml which is equivalent to log WHO IU/ml as presented on the X-axis. The probability of positive reactions is presented on the Y-axis. The 95% hit rate of the lower part of both dynamic ranges, DR-low and DR-high, was determined to be 242 and 3630 IU/ml, respectively. Error bars are indicated.

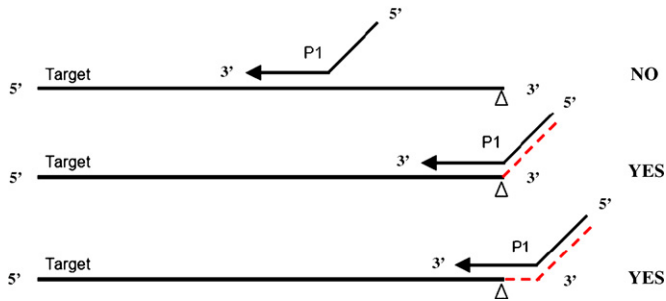


Fig. 8. Design of the P1 primer in DNA NASBA. Three different situations in which the p1 is bound to the DNA target are presented. The position of restriction enzyme digestion is indicated (Δ). The possibility to extend the DNA by AMV RT after restriction enzyme digestion is indicated with YES or NO. Extension of the DNA target strand by AMV RT after primer binding is shown by a dotted line.

For the amplification of HBV genomic DNA, two restriction sites, XbaI and BssSI, were selected and two accompanying P1 primers were designed: p3.8-S and p3.10-S, respectively. After digestion of the target DNA and hybridization of the primer, AMV-RT could extend the 3' end of the target strand, using the P1 as a template. Thereby a double stranded T7 promoter sequence is created that could directly be used by the T7 enzyme to start the production of amplicons. As expected, without restriction enzyme digestion amplicons were only detectable at high input levels. However, for both primers a 1000-fold increase in assay sensitivity was shown when performing the digestion with the accompanying restriction enzyme.

When exchanging the P1 primers, still a 100–1000-fold increase in assay sensitivity was obtained with HBV p3.8-S when BssSI was used instead of XbaI for digestion of the target DNA. Although the hybridizing part of HBV p3.8-S to the digested target DNA is smaller compared to that of HBV p3.10-S, hybridization is still possible under NASBA conditions and again the 3' of the target DNA could be extended and a double stranded T7 promoter could be created as described above. However, if HBV p3.10-S is used in combination with XbaI digestion instead of BssSI, extension of the 3' end of the target over the P1 primer is no longer possible. Indeed, no increase in assay sensitivity was observed for this combination. This proves that extension of the 3' end of the target DNA with the p1 primer exactly at the digestion site is fundamental for the increased assay sensitivity. The principle of target extension at the digestion site using the P1 primer as template is schematically presented in Fig. 8.

For detection of the genomic DNA of HSV, it is also shown that a 1000-fold increase in assay sensitivity is obtained when performing a restriction enzyme digestion with ApaI and Sall as part of the amplification reaction. In addition, it is shown that assay sensitivity of the amplification of MRSA targeting the *mecA* gene, can be improved by a factor ranging from 5 to 1000 when performing a Sau3A-I digestion as part of the amplification reaction. The level of improvement depends upon MRSA strains, presumably due to various levels of *mecA* encoded mRNA (Ryffel et al., 1992). If mRNA is highly expressed both mRNA and the genomic *mecA* DNA are amplified and the impact on the sensitivity of restriction enzyme addition is less prominent compared to a strain that does not express mRNA at high level.

For some targets it is preferred to make use of more than one restriction site to minimize loss of assay sensitivity due to mutations in the restriction site. For HBV DNA, it was determined that for some HBV genomic sequences mutations do occur in one of these restriction sites. The restriction sites of XbaI and BssSI are close together and HBV p3.8-S can be used as P1 primer in combination

with both restriction enzymes. Therefore, it was decided to include both enzymes in the HBV DNA NASBA[®].

As in RNA NASBA, the amplicons will be reused in amplification, suggesting that the main difference between RNA and DNA NASBA is the initiation of amplification. Therefore, it was assumed that DNA targets could be quantified in the same manner as RNA targets. When using a HBV related internal calibrator at two different input levels a linear dynamic range of 7 log 10 was obtained and a 95% detection rate of 242 IU/ml and an upper LOQ of 5×10^8 WHO IU/ml was determined. These results are in line with assay performances of some commonly used commercial HBV assays like the Cobas Amplicor HBV Monitor Test (Roche Molecular Systems, Pleasanton, CA, USA) or the Digene hybrid Capture II HBV DNA test (HCII HBV, Digene, Gaithersburg, MD, USA). For recent real-time HBV PCR assays detection down to 10 IU/ml has been reported (Hochberger et al., 2006; Thibault et al., 2007). It should be noted that the sensitivity of the total assay is determined by different factors like the specimen volume used, the recovery during extraction, the percentage of the extracted material used for amplification and the amplification efficiency. With the extraction system described in this study, only 10% of the extracted material was used for amplification and the system was not yet optimized for HBV DNA extraction. Preliminary data have shown that higher assay sensitivity can be obtained in combination with next generation extraction platforms, like the NucliSENS[®] miniMAG[®] (bioMérieux, Boxtel, The Netherlands) and the NucliSENS[®] easyMAG[®] (bioMérieux, Boxtel, The Netherlands).

For the qualitative HSV DNA NASBA[®] reaction a 95% detection rate of 84 and 138 copies of a HSV WT construct in isolation was obtained for HSV 1 and HSV 2, respectively. These results are in agreement with that obtained with WT transcripts in RNA NASBA reactions. In addition, by testing the QCMD panel, the clinical relevance of this HSV assay was shown, as all different HSV samples were detectable and a VZV sample was not detectable.

Previously, a model to amplify DNA with NASBA[®] was presented including two denaturing steps (Sooknanan et al., 1995). In practice, DNA amplification using this approach turned out to be very inefficient for most targets and requires extra hands-on time and reagents compared to RNA NASBA reactions. The results presented in this paper show that efficient amplification of DNA targets with a one-step NASBA is possible when using a new mechanism in which restriction enzyme digestion is included. More specifically, analytical assay sensitivity of the DNA NASBA is shown to be comparable to that of the RNA NASBAs like the NucliSENS EasyQ[®] HIV 1 v1.2 assay having a 95% detection rate of 250 IU/ml. Assay kinetics of the DNA NASBA is also comparable to that of the RNA NASBA reactions, allowing the use of the existing algorithm for DNA quantitation originally designed for RNA NASBA. The DNA NASBA including the restriction enzyme digestion is performed under amplification conditions that were originally designed for RNA NASBA and no extra hands-on time is needed. In addition, amplification is performed on the same NucliSENS[®] system as for RNA NASBAs without any adaptations of the instrumentation. Finally, the total reaction time of DNA NASBA reactions is comparable to that of RNA NASBA reactions, due to the restriction enzyme digestion, which requires a longer initiation time but decreases the amplification time considerably.

By performing a restriction enzyme digestion during initiation of NASBA, DNA targets are amplifiable with comparable assay sensitivity and assay kinetics as RNA targets. In addition, in the presence of an internal calibrator, DNA targets can be quantified using the same algorithm as designed for RNA targets. A broad linear dynamic range is also achieved, thus making DNA NASBA[®] quite an interesting alternative to PCR-based DNA amplification.

Acknowledgement

We would like to thank Dr. Van Leeuwen of the Erasmus Medical Center, Rotterdam, The Netherlands for providing the MRSA strains.

References

- Cleuziat, P., Jay, C., Incardona, S., 2003. Method for cellular lysis of prokaryotes or eukaryotes or simultaneous lysis of prokaryotes and eukaryotes. Patent PCT WO02103333.
- Compton, C.J., 1991. Nucleic acid sequence-based amplification. *Nature* 350, 91–92.
- Deiman, B.A.L.M., Van Aarle, P., Sillekens, P., 2002. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Mol. Biotechnol.* 20, 163–179.
- Grisold, A.J., Leitner, E., Mühlbauer, G., Marth, E., Kessler, H.H., 2002. Detection of methicillin-resistant *Staphylococcus aureus* and simultaneous confirmation by automated nucleic acid extraction and real-time PCR. *J. Clin. Microbiol.* 40, 2392–2397.
- Guatelli, J., Whitfield, K., Kwok, D., Barringer, K., Richman, D., Gingeras, T., 1990. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retrovirus replication. *Proc. Natl. Acad. Sci. U.S.A.* 87, 1874–1878.
- Heim, A., Grumbach, I.M., Zeuke, S., Top, B., 1998. Highly sensitive detection of gene expression of an intronless gene: amplification of mRNA, but not genomic DNA by nucleic acid sequence based amplification (NASBA). *Nucleic Acids Res.* 26, 2250–2251.
- Hochberger, S., Althof, D., Gallegos de Schrott, R., Nachbaur, N., Rock, H., Leying, H., 2006. Fully automated quantitation of hepatitis B virus (HBV) DNA in human plasma by the COBAS AmpliPrep/COBAS TaqMan system. *J. Clin. Virol.* 35, 373–380.
- Ono, Y., 1983. The complete nucleotide sequence of the cloned hepatitis B virus DNA; subtype adr and adw. *Nucleic Acids Res.* 11, 1747–1757.
- Ryffel, C., Kayser, F.H., Berger-Bachi, B., 1992. Correlation between regulation of *mecA* transcription and expression of methicillin resistance in staphylococci. *Antimicrob. Agents Chemother.* 36, 25–31.
- Simpkins, S.A., Chan, A.B., Hays, J., Popping, B., Cook, N., 2000. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. *Lett. Appl. Microbiol.* 30, 75–79.
- Sooknanan, R., Van Gemen, B., Malek, L., 1995. *Molecular Methods for Virus Detection*. Academic Press, London, UK, pp. 261–285.
- Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R.F., Rossau, R., 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J. Gen. Virol.* 81, 67–74.
- Thibault, V., Pichoud, C., Mullen, C., Rhoads, J., Smith, J.B., Bitbol, A., Thamm, S., Zoulim, F., 2007. Characteristics of a new sensitive PCR assay for quantification of viral DNA isolated from patients with hepatitis B virus infections. *J. Clin. Microbiol.*, doi:10.1128/JCM.01180-07.
- Van Beuningen, R., Marras, S., Kramer, F., Oosterlaken, T., Weusten, J.J.A.M., Borst, G., Van de Wiel, P., 2001. Development of a high throughput detection system for HIV-1 using real-time NASBA based on molecular beacons. In: *Proteomics Technologies*, Proceedings of Society of Photo-Optical Instrumentation Engineers (SPIE), Washington, DC 4264, pp. 66–72.
- Weusten, J.J.A.M., Carpay, W.M., Oosterlaken, T.A.M., Van Zuijlen, M.C.A., Van de Wiel, P.A., 2002. Principles of quantitation of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. *Nucleic Acids Res.* 30 (6), e26.