

DNA nucleic acid sequence-based amplification-based genotyping for polymorphism analysis

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Nucleic acid sequence-based amplification (NASBA) is a sensitive isothermal transcription-based amplification method known to be a suitable tool for RNA research. We demonstrate that NASBA technology can be applied to single nucleotide polymorphism (SNP) analysis using human genomic DNA as a template. Combination of DNA NASBA with multiplex hybridization of specific molecular beacons makes it possible to unambiguously discriminate the presence of the SNP of interest. This protocol is easy-to-use, robust, and makes it possible to rapidly detect single nucleotide substitutions in clinical or cell line DNA sequences using a large range of DNA input. Such a real-time genotyping DNA NASBA assay can find broad application in clinical diagnostics.

INTRODUCTION

Single nucleotide polymorphisms (SNP) represent the largest source of diversity in the human genome. Some of these variations have been directly linked to human diseases, though nearly all are neutral. Usually, the mutation is detected after or during PCR amplification of the region of interest using specific labeled probes such as molecular beacons (1–6). Indeed, molecular beacons are uniquely suited for SNP analysis because they recognize their targets with significantly higher specificity than conventional oligonucleotide probes (7–10).

Nucleic acid sequence-based amplification (NASBA) is a sensitive and efficient isothermal transcription-based amplification method specifically designed for the detection of RNA targets (11). NASBA has proved successful in the detection of various messenger RNAs (mRNAs) and in the detection of viral, bacterial, and fungal RNA in clinical samples and food (12–19). Furthermore, this technology has also been described as capable of amplifying DNA with or without a denaturation step in the process (11,20,21). Here we describe a DNA NASBA-based protocol for analyzing SNP from DNA as

the sole template. DNA NASBA results in the production of large amounts of single-stranded antisense RNA that can be detected through the hybridization of specific fluorescent molecular beacons. Using Factor V Leiden (G1691A) mutation as a model (22), we propose a real-time DNA NASBA-based assay that allows simple, clear, and fast discrimination of SNPs in genomic DNA extracted from cell lines and clinical samples.

MATERIALS AND METHODS

Plasmid DNA

Based on template DNA genotyped for Factor V Leiden, 374-bp fragments containing either the G or A nucleotide at position 1691 were generated by PCR using forward (5'-AGTGCTTAA-CAAGACCATACTA-3') and reverse (5'-AACAGACCTGGAATTTGAA-ACTAA-3') primers located at position 36568–36589 and 36918–36941, respectively (GenBank® accession no. NT_004668). PCR amplification was performed in a Model 9600 thermal cycler (Perkin-Elmer, Wellesley, MA, USA) (2 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C;

followed by 7 min at 72°C). The two PCR fragments were cloned into the PCR-Trap vector (GeneHunter) and verified by sequencing. Plasmids carrying the G1691G or A1691A genotype were amplified and purified using the Plasmid Maxi Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer.

Cell Line Culture and Genomic DNA Extraction

Three lymphoblastoid cell lines of known Factor V (G1691A) genotype were used in the study: (i) GM14899 cells carrying the A1691A genotype; (ii) GM16000C cells carrying the G1691G genotype; and (iii) GM16028B cells carrying the heterozygous G1691A genotype. All cell lines were purchased from Coriell Cell Repositories (Camden, NJ, USA). Cell lines were grown in RPMI 1640 supplemented with 15% fetal bovine serum, 2 mM L-glutamine, and 0.2 µg/µL penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere. Genomic DNA was extracted by treating the cellular pellet with 0.1 µg/µL proteinase K for 12 h followed by a phenol/chloroform step and ethanol precipitation. The genomic DNA was then recovered in 500 µL of DNase-free water. DNA quality and quantity were determined on ethidium bromide-stained agarose gel and by UV spectrophotometry. Genomic DNA samples were stored at -20°C until use.

Clinical Samples and Genomic DNA Extraction

Two hundred microliters of whole blood was collected in EDTA tubes from three individuals of known Factor V (G1691A) genotype. Genomic DNA was extracted using the NucleoSpin® Kit (Marcherey-Nagel, Hoerdt, France). Genomic DNA was then recovered in 20 µL of DNase-free water. DNA quality and quantity were determined on ethidium bromide-stained agarose gel and by UV spectrophotometry. Genomic DNA samples were stored at -20°C until use.

DNA NASBA

NASBA is based on the activity of

avian myeloblastosis virus (AMV) reverse transcriptase (RT), *Escherichia coli* RNase H and T7 RNA polymerase with two oligonucleotide primers to amplify the desired fragment more than 10^{12} -fold in 90 min (20). Briefly, based on the NucliSens[®] Basic Kit (bioMérieux bv, Boxtel, The Netherlands), 5 μ L genomic DNA were added to 10 μ L of NASBA buffer [final concentration in a 20- μ L reaction mixture: 40 mM Tris-HCl, pH 8.5, 12 mM MgCl₂, 70 mM KCl, 5 mM dithiothreitol, 15% (v/v) dimethylsulfoxide (DMSO), 1 mM of each dNTP, 2 mM of each NTP, 0.2 μ M of each primers, 0.2 μ M of Factor V (1691-G) molecular beacon, and 0.1 μ M of Factor V (1691-A) molecular beacon] and pre-incubated at 95°C for 2 min before incubation at 41°C for 2 min. Five microliters of enzyme mixture (0.08U RNase H, 32 U T7-RNA polymerase, 6.4 U RT) were added, and the reaction was incubated at 41°C for 90 min. Primer sets used for the amplification and molecular beacon sequences are shown in Table 1.

Real-Time NASBA Detection of the SNP

Real-time detection of NASBA products was performed by NucliSens EasyQ Analyzer (bioMérieux bv) using molecular beacons that are DNA oligonucleotides labeled with FAM (6-carboxy-fluorescein) or ROX (6-carboxy-

X-rhodamine) fluorophores at the 5' end and a quencher (Dabsyl) at the 3' end. Probes were synthesized by Eurogentec (Seraing, Belgium). Molecular beacons hybridize with the amplicon during amplification, and emitted fluorescence is measured every 45 s, allowing real-time detection.

Detection of the polymorphic nucleotide was performed by mixing molecular beacons specific to the G or A nucleotide in the same tube. Based on the raw fluorescent signal data, the open/closed (O/C) signal ratio of the hybridization reaction, corresponding to the ratio between the end point signal value determined at 90 min and the background signal value at 45 s, can be determined for each molecular probe. Therefore, for each genotyping assay, the O/C signal ratio of both G and A probes was automatically calculated by the software of the NucliSens EasyQ Analyzer, thus clearly discriminating the genotype of the DNA tested.

RESULTS AND DISCUSSION

We have reported here that NASBA, which is known to be a suitable tool for RNA research, can be adapted to amplify DNA for genotyping application if a preliminary denaturation step is added in the process. Indeed, when denaturing purified genomic DNA at 95°C for 2 min, amplification primers can an-

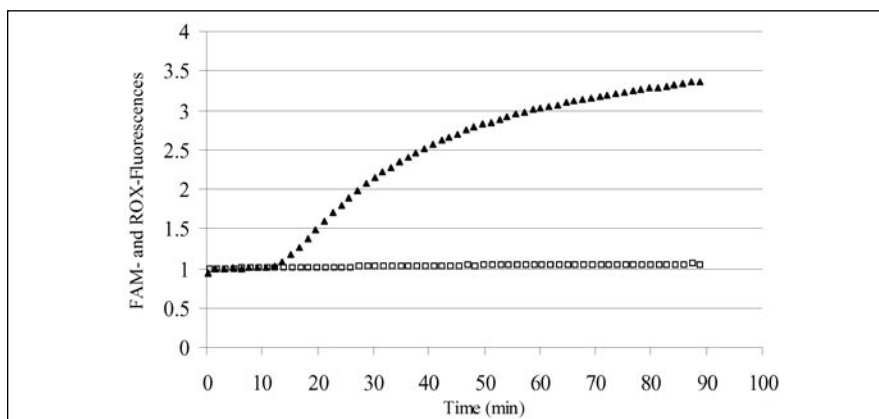


Figure 1. Multiplex detection of the Factor V Leiden allele in a real-time nucleic acid sequence-based amplification (NASBA) reaction using 50 ng plasmid DNA carrying the Factor V (A1691A) genotype. Two differently colored molecular beacons were present in the reaction. Each kinetics curve is depicted using the symbol of the corresponding molecular beacon used [ROX-labeled G-specific probe (□) and FAM-labeled A-specific probe (▲)]. No fluorescence was observed using either the FAM- or the ROX-labeled molecular beacon in a control reaction containing only water (data not shown). Amplification plots are representative of triplicate experiments.

neal to the single strand target and initiate the NASBA process. When combining this amplification process with a hybridization and detection step using specific fluorescent molecular beacons, SNP can be detected in real-time with the NucliSens EasyQ Analyzer.

We first tested the amplification efficacy and hybridization specificity of

molecular beacons on plasmid DNA samples. As shown in Figure 1, using 50 ng of Factor V (A1691A) plasmid, we observed a positive signal with the A probe while no background fluorescent signal was obtained with the G probe, showing that NASBA technology is well-suited to amplifying pure DNA with no RNA contamination.

Similar results were obtained with the Factor V (G1691G) plas-

mid (data not shown). We then tested 50 ng of genomic DNA extracted from three lymphoblastoid cell lines carrying either homozygous G or heterozygous or homozygous A genotype for the Factor V gene at position +1691. We observed that the DNA carrying a homozygous (G1691G) genotype generated a positive signal with the G molecular beacon and only a weak fluorescent signal with the A probe (Figure 2A). Heterozygous (G1691A) DNA generated a positive signal with both the G and the

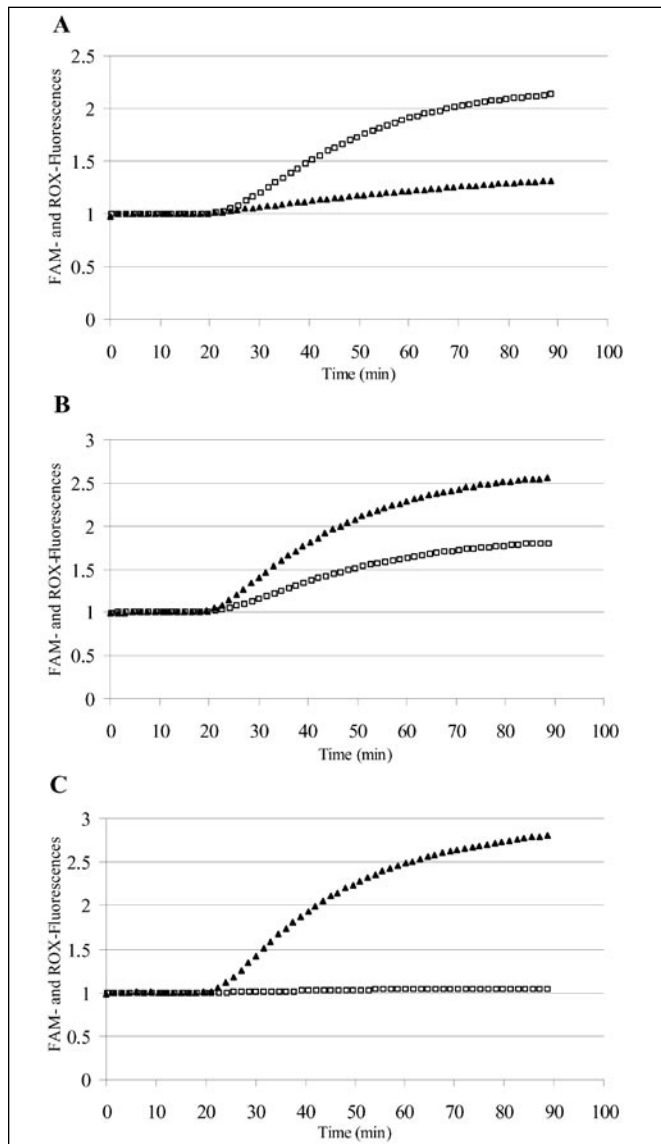


Figure 2. Multiplex detection of the Factor V Leiden allele in a real-time nucleic acid sequence-based amplification (NASBA) reaction using 50 ng genomic DNA extracted from cell lines carrying (A) the Factor V (G1691G) genotype, (B) the Factor V (G1691A) genotype, and (C) the Factor V (A1691A) genotype. Two differently colored molecular beacons were present in each reaction. Each kinetics curve is depicted using the symbol of the corresponding molecular beacon used [ROX-labeled G-specific probe (□) and FAM-labeled A-specific probe (▲)]. No fluorescence was observed using either the FAM- or the ROX-labeled molecular beacon in a control reaction containing only water (data not shown). Amplification plots are representative of triplicate experiments.

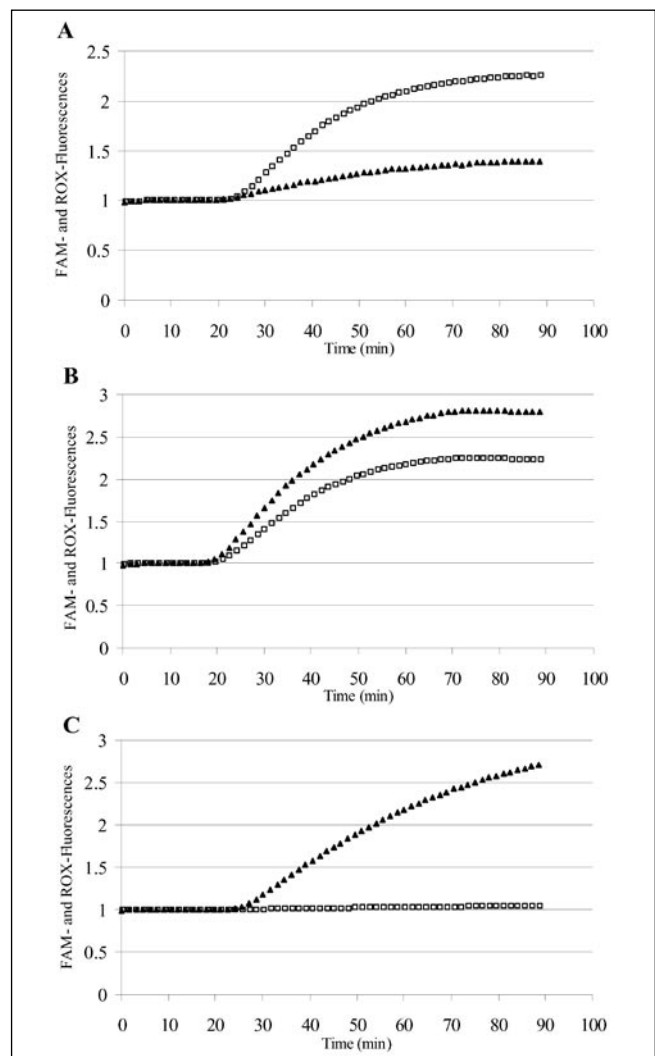


Figure 3. Multiplex detection of the Factor V Leiden allele in real-time nucleic acid sequence-based amplification (NASBA) reaction using 3 µL genomic DNA extracted from blood samples from individuals with (A) the Factor V (G1691G) genotype, (B) the Factor V (G1691A) genotype, and (C) the Factor V (A1691A) genotype. Each kinetics curve is depicted using the symbol of the corresponding molecular beacon used [ROX-labeled G-specific probe (□) and FAM-labeled A-specific probe (▲)]. No fluorescence was observed using either the FAM- or the ROX-labeled molecular beacon in a control reaction containing only water (data not shown). Amplification plots are representative of triplicate experiments.

Table 1. Sequences of the Primers and Molecular Beacons Used for Factor V Leiden Genotyping

Primer	Sequence	Position
P1(T7)	5'-aattctaatacgcactactatagggagaAAATTCTCAGAATTTCTGAAAGG-3'	36704–36726
P2	5'-AGTGCTTAACAAGACCATACTA-3'	36568–36589
Molecular Beacon	Sequence	Position
+1691-G	5'-ROX- <i>cgatcg</i> CTGGACAGGCGAIGA <i>A</i> Acgatcg-Dabsyl-3'	36663–36678
+1691-A	5'-FAM- <i>cgatcg</i> CTGGACAGGCAAIGA <i>A</i> Acgatcg-Dabsyl-3'	36663–36678

The 3' antisense primer is elongated with a T7-promotor recognition sequence indicated in lowercase characters. The stem sequences of the molecular beacons are indicated in lowercase italic characters. The positions are in reference to the GenBank accession no. NT_004668.

Table 2. Cell Line and Blood DNA Genotyping

Cell Line DNA	DNA Sample (FV G1691G)	DNA Sample (FV G1691A)	DNA Sample (FV A1691A)
O/C ratio of 1691-A MB	1.33	2.57	2.83
O/C ratio of 1691-G MB	3.77	3.04	1.13
Blood DNA	DNA Sample (FV G1691G)	DNA Sample (FV G1691A)	DNA Sample (FV A1691A)
O/C ratio of 1691-A MB	1.33	2.41	2.5
O/C ratio of 1691-G MB	3.51	3.31	1.11

Open/closed (O/C) signal value corresponding to the ratio between the end point positive signal and background signal for each molecular beacon (MB).

A probes (Figure 2B), while homozygous (A1691A) DNA generated a positive signal with the A probe and only a background fluorescent signal with the G probe (Figure 2C). The O/C signal value confirmed the specificity of the hybridization of those probes and was thus helpful for discriminating between the two alleles (Table 2).

Successful genotyping was similarly observed when using 3 µL of genomic DNA extracted from 200 µL of clinical blood samples. Homozygous and heterozygous DNA were clearly identified (Figure 3, A–C). Again, the O/C signal values confirmed the discrimination among the different haplotypes (Table 2). Based on 200 clinical blood samples, we confidently determined that an O/C ratio, for the 1691-A molecular beacon, higher than 1.5 was always associated with the presence of the A nucleotide at position 1691. The presence of a G at position 1691 was also associated with an O/C ratio of the 1691-G molecular beacon higher than 1.5 (data not shown). A range of 50 ng to 1 µg genomic DNA input per assay was successfully tested (data not shown), suggesting that the detection is robust with a large range

of DNA input. This will be particularly useful in laboratories that perform high-throughput genotyping. In addition, we have shown that two standard extraction-purification protocols (i.e., phenol/chloroform and commercial column-based methods) give good genotyping results.

Altogether, our results demonstrate for the first time that NASBA technology using human genomic DNA as a template can be applied to SNP analysis. Combination of DNA NASBA and molecular beacons makes it possible to unambiguously discriminate the presence of the SNP of interest in real-time, using the NucliSens EasyQ Analyzer. Unlike PCR, in which a thermal cyler is required, the isothermal nature of NASBA allows to minimize the instrumentation requirements for such genotyping assays. In addition, detection of the single-stranded RNA product of NASBA by probe-based hybridization does not require a denaturing step as needed with PCR. By determining an O/C ratio threshold for each sequence, SNP detection can be automated with respect to the fluorescent signals generated. This protocol is easy-to-use and rapid, since

it allows mutations to be detected in 90 min. In conclusion, we propose a real-time DNA NASBA protocol that allows simple, clear, and rapid discrimination of single nucleotide substitutions in DNA sequences and that could find broad application in clinical diagnostics.

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COMPETING INTERESTS STATEMENT

The authors declare no conflicts of interest.

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