PRODUCT APPLICATION FOCUS

Multiparametric duplex real-time nucleic acid sequence-based amplification assay for mRNA profiling

Thibault Verjat, Elisabeth Cerrato, Marcel Jacobs, Philippe Leissner, and Bruno Mougin

BioTechniques 37:476-481 (September 2004)

INTRODUCTION

Gene expression monitoring is becoming increasingly popular in the biomedical research community. This approach requires a reliable amplification method that allows even small amounts of input RNA to be quantified. Nucleic acid sequence-based amplification (NASBA) has been described as an efficient method of amplifying RNA at 41°C using T7 RNA polymerase, RNase H, avian myeloblastosis virus (AMV) reverse transcriptase, and two specific primers (1–3). NASBA has been successfully approved for the detection of various mRNAs (4) and viral and bacterial RNA in clinical samples (5–9). An elegant method of detecting NASBA products is the use of fluorescent molecular beacons (10), which enable homogeneous real-time detection of RNA products (11).

To determine the quantity of target nucleic acids in the sample, they are quantified using external calibration curves. We have developed real-time NASBA-based assays using duplex analysis of the gene of interest and a housekeeping gene. This duplex approach could be employed to monitor several target genes in parallel, thereby providing a convenient tool for multiparametric mRNA profiling.

In the present study, we describe the development of two duplex real-time NASBA assays to investigate, in breast tumors, level of mRNA coding for the estrogen receptor α (ESR1) and the progesterone receptor (PGR) with the cyclophilin B (PPIB) housekeeping gene, whose expression has been shown to be stable in breast cancer cell lines and tumors (unpublished observations).

MATERIALS AND METHODS

Cell Line Culture and Total RNA Extraction

Two tumor cell lines were used in the study, breast carcinoma MCF-7 and BT-549 cells. Both cell lines were purchased from ATCC (Manassas, VA USA). The MCF-7 cell line was grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen Canada, Burlington, ONT, Canada), while the BT-549 cells were grown in RPMI 1640 (Invitrogen Canada). Both media were supplemented with 10% fetal bovine serum (Invitrogen Canada), 2 mM L-glutamine (Sigma-Aldrich, Lyon, France), 1% nonessential amino acids (Invitrogen Canada), and 10 μg/mL streptomycin (Invitrogen Canada) at 37°C in a 5% CO₂ atmosphere. Total RNA was purified from cell lines using TRIzol® reagent as recommended by the manufacturer (Invitrogen Canada). RNA quality and quantity were determined using RNA 6000 Nano LabChips® (Agilent Technologies, Waldbronn, Germany). RNA samples were stored at -70°C until use.

bioMérieux, Marcy l’Etoile, France
Standard RNA

NASBA quantification was based on a standard curve with a known input of RNA transcribed from plasmids. These plasmids were generated by cloning a specific PCR product for each target gene. For ESR1 (GenBank® accession no. X03635), forward primer (5'-TACAGGCAAAATTCA-GATAATCGAC-3') and reverse primer (5'-GGAACCGAGATGATGTAGCCA-3') were located at positions 808 and 1666, respectively, generating a 858-bp fragment. For PGR (GenBank accession no. NM_000926), forward primer (5'-TGCAAGTCTTAATCAACTAGG-3') and reverse primer (5'-TCACTTTTTATGAAAGAGAAGGG-3') were located at positions 2319 and 2977, respectively, generating a 658-bp fragment. For PPIB (GenBank accession no. M60857), forward primer (5'-ACATGATCAACTGGGCGAAGA-3') and reverse primer (5'-GATAATCGAC-3') were located at positions 11 and 650, respectively, generating a 639-bp fragment. Each PCR fragment was cloned into the pGEM-T plasmid (Promega, Madison, WI, USA) and verified by sequencing. In vitro transcription was generated from these plasmids using T7 RNA polymerase (MEGAscript™ Kit; Ambion, Austin, TX, USA) and treated with DNase to remove the plasmid. RNA products were purified using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Integrity and quantity of RNA were evaluated with the RNA 6000 Nano LabChips by the nature of the RNA 18S- and 28S-peaks and the concentration in ng/mL, respectively. The calculation of the RNA copy number can then be performed based on the concentration and the length of the NASBA product. The resulting RNAs were used to set up the ESR1-, PGR-, and the PPIB-standard curves.

Duplex Real-Time NASBA

Real-time detection of NASBA products was performed with the NucliSens EasyQ® Analyzer (bioMérieux bv, Boxtel, The Netherlands) using molecular beacons, which are DNA oligonucleotides labeled with the fluorophore FAM (6-carboxy-fluorescein) or ROX (6-carboxy-X-rhodamine) at the 5' end and a quencher (Dabsyl) at the 3' end. Probes were synthesized by Eurogentec (Seraing, Belgium). Molecular beacons hybridized to the products during amplification and emitted a fluorescence signal that was measured every 45 s, thereby allowing real-time detection.

Duplex NASBA was performed using the NucliSens Basic Kit (bioMérieux bv). Briefly, 5 µL (5 ng) RNA were added to 10 µL NASBA buffer [final concentration in 20 µL 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]. For ESR1/PPIB duplex NASBA, 0.2 µM ESR1 primers, 0.2 µM PPIB primers, and 0.1 µM of each cognate molecular beacon were added, while for the PGR/PPIB duplex NASBA, 0.1 µM PGR primers, 0.2 µM PPIB primers, and 0.1 µM of each cognate molecular beacon were added. The mixture was then pre-incubated at 65°C for 2 min, followed by 2 min at 41°C. Five microliters of enzyme mixture (0.08 U RNase H, 32 U T7 RNA polymerase, 6.4 U reverse transcriptase) were then added to start the RNA amplification and incubated at 41°C for 90 min. Primer sets used as well as molecular beacon sequences are shown in Table 1.

RNA Quantification in the NASBA Assay

The time necessary to generate a fluorescent signal that rises above the threshold of detection and becomes positive is termed time-to-positivity (TTP). Using NucliSens EasyQ Analysis software (bioMérieux bv), the signal positivity threshold was determined, as described previously (12,13), by setting the fluorescence emitted in the first five measurement points as a background. Several duplex calibration curves were drawn using either 10-fold serial dilutions of RNA transcripts (10^8–10^2 copies) of the gene of interest and the housekeeping gene or using 10^8–10^2 copies of RNA transcripts of the target gene mixed with a constant quantity (10^3, 10^3 or 10^4 copies of the housekeeping gene. Thus, for each experiment, the number of mRNA copies per sample input could be extrapolated from those standard curves. Quantification of

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR1 P1(T7)</td>
<td>5'-aattctaatcctacatattggagaaggCTCCACCATGCCTCTACACA-3'</td>
<td>1609–1629</td>
</tr>
<tr>
<td>ESR1 P2</td>
<td>5'-ACATGATCAACTGGGCGAAGA-3'</td>
<td>1427–1447</td>
</tr>
<tr>
<td>PGR P1(T7)</td>
<td>5'-aattctaatcctacatattggagaaggTCCCTGCAATATCTTGGGTA-3'</td>
<td>2925–2945</td>
</tr>
<tr>
<td>PGR P2</td>
<td>5'-AGTTGTGTCGACCTACAGC-3'</td>
<td>2761–2780</td>
</tr>
<tr>
<td>PPIB P1(T7)</td>
<td>5'-aattctaatcctacatattggagaaggCAGGCTGTCTTGAAGTGTGTA-3'</td>
<td>449–470</td>
</tr>
<tr>
<td>PPIB P2</td>
<td>5'-AGGAGAAAGATTGTGCT-3'</td>
<td>231–250</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular Beacon</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR1</td>
<td>5'-FAM-cgatcggATCCTGAGATTTGTTGCTCGcgatcgg-Dabsyl-3'</td>
<td>1515–1534</td>
</tr>
<tr>
<td>PGR</td>
<td>5'-FAM-cgatcggCGGACCATGAGTGTTGATGTcgatcgg-Dabsyl-3'</td>
<td>2870–2889</td>
</tr>
<tr>
<td>PPIB</td>
<td>5'-ROX-cgatcggGTCCAGGGCCGAGACCTACagatcgg-Dabsyl-3'</td>
<td>291–310</td>
</tr>
</tbody>
</table>

The 3’ antisense primers are elongated with a T7 promoter recognition sequence indicated in lowercase characters. The stem sequences of the beacons are indicated in lowercase italic characters. The positions are in reference to the GenBank accession nos. X03635 for ESR1, NM_000926 for PGR, and M60857 for PPIB, respectively. ESR1, estrogen receptor α; the progesterone receptor (PGR) in breast tumors by means of duplex reactions using cyclophillin B (PPIB).
the target gene expression was expressed as the mRNA copy number per 5 ng of total RNA.

RESULTS AND DISCUSSION

Specificity and Sensitivity of Duplex ESR1/PPIB and PGR/PPIB Real-Time NASBA with RNA Transcripts

The specificity of the NASBA assay was strengthened by triple hybridization of the target RNA with two specific primers and a specific detection probe. The specificity of the assay was tested by mixing the ESR1 molecular beacon with $10^6$ PGR RNA molecules and vice versa. In both cases, no signal was observed (data not shown). In addition (see Figure 3), no fluorescence signal was observed when NASBA was performed with total RNA extracted from BT-549 cells known to be estrogen receptor $\alpha$-negative and progesterone receptor-negative when assessed by the ligand binding assay (data not shown), confirming that molecular beacons do not interact with nonspecific targets.

To evaluate the efficacy of duplex NASBA, amplification curves of a 10-fold serial dilution of in vitro-synthesized RNA were plotted. The input of RNA transcripts was $10^2$–$10^8$ molecules for both genes in the same tube. In each such “equimolar” duplex NASBA reaction, molecular beacons specific to ESR1 and PPIB and to PGR and PPIB mRNA were added. As shown in Figure 1, the TTP was linear over a range of at least six orders of magnitude of input RNA molecules.

In the ESR1/PPIB duplex NASBA, the linear quantification of ESR1 was determined to be between $10^2$ and $10^8$ copies of RNA per reaction (Figure 1A) and between $10^3$ and $10^8$ copies per reaction for PPIB (Figure 1B). The limit of detection (LOD) determined with the amplification plots (data not shown) as well as the limit of quantification (LOQ) at which accurate TTP values can be calculated could be determined. The LOD and the LOQ for ESR1/PPIB duplex could be determined at $10^2$ copies and $10^3$ copies, respectively. In the PGR/PPIB duplex NASBA, the linear quantification of both markers was determined to be between $10^2$ and $10^8$ copies of RNA per reaction (Figures 1C and 1D). The LOD was observed at $10^2$ copies for PGR and PPIB, while the LOQ was reached at $10^2$ and $10^3$ copies for PGR and PPIB, respectively. These results illustrate that two different genes can be amplified and detected simultaneously in real-time with NASBA.

Dynamic Range of the Duplex NASBA

In order to assess a putative influence of the quantity of PPIB on the dynamic range of ESR1 and PGR amplification in duplex NASBA experiments, we mixed $10^2$–$10^8$ transcripts of ESR1 or PGR with an equimolar amount of PPIB gene. A serial dilution of target RNA transcripts ($10^2$–$10^8$ copies) was also co-amplified with a constant concentration ($10^3$ or $10^5$ or $10^7$ copies) of the housekeeping gene. As shown in Figure 2, when a low copy number ($10^2$ or $10^5$) of the ESR1 gene was mixed with a high copy number ($10^5$ or $10^7$) of PPIB, the amplification efficacy of the target gene decreased. Similar...

Figure 1. ESR1/PPIB and PGR/PPIB duplex NASBA standard curves with in vitro synthesized RNA. Relationship of time-to-positivity (TTP) to (A) ESR1, (B and D) PPIB, and (C) PGR RNA copy number. The input of RNA copies present in the reaction is indicated on the logarithmic x-axis while the TTP value (in min) is indicated on the ordinate. The TTP values are the mean (± SEM) of eight independent experiments. NASBA, nucleic acid sequence-based amplification; ESR1, estrogen receptor $\alpha$; the progesterone receptor (PGR) in breast tumors by means of duplex reactions using cyclophilin B (PPIB).
results were observed with PGR in a PGR/PPIB duplex NASBA reaction (data not shown). These results demonstrate that, in duplex NASBA, the amplification efficacy of a given gene can be influenced by high numbers of a second gene present in the reaction. This finding, which has also been described for reverse transcription PCR (RT-PCR) (14,15), suggests that the housekeeping gene should have a gene expression level comparable to that of the target gene. Thus, care should be taken to select the best normalized gene for each target in a given tissue. Another way to minimize the variation generated by multiplex amplification would be to optimize the primer concentration to achieve efficient amplification for each gene. An internal calibrator RNA has also proven useful for quantifying gene expression, but this system requires time-consuming optimization of primer concentrations (16,17). However, while accurate quantification is necessary for various applications, such as infectious diseases, our objective here is to propose relative quantification of hormone receptor RNA as a marker in correlation with the clinical features of the tumor. Thus, rather than calculating an absolute number of ESR1 and PGR copies, the main issue will be to determine a gene expression cut-off strongly associated with the presence of receptors in the cytosol, beyond which hormonotherapy could be proposed to the patient.

**Measurement of ESR1 and PGR mRNA Levels by Duplex Real-Time NASBA with Total RNA from Cell Lines**

In order to evaluate the performance of ESR1/PPIB and PGR/PPIB duplex NASBA from small quantities of total RNA, 5 ng extracted from two breast cancer cell lines were used as starting material. MCF-7 cells were shown to be estrogen receptor α-positive/progesterone receptor-positive and BT549 cells to be estrogen receptor α-negative/progesterone receptor-negative by immunohistochemistry (ATCC). This data was confirmed by a ligand binding assay that measures the content of functional cytosolic receptors (data not shown).

ESR1, PGR, and PPIB genes were amplified and quantitatively detected using an equimolar duplex standard curve. Both ESR1 and PGR messengers were found in MCF-7 cells. In contrast, messengers seemed to be weakly expressed (below the LOD) or not at all in the BT-549 cells (Figure 3). The internal control PPIB was found to be positive and gene expression level similar in both cell lines. Quantitative RT-PCR analysis of ESR1 expression was performed and confirmed the NASBA results (data not shown). Therefore, relative quantification of ESR1 and PGR gene expression can be performed by determining the ratio between the number of mRNA molecules of the gene of interest and the housekeeping gene (Table 2). These results demonstrate that duplex real-time NASBA is a suitable technology for simultaneously monitoring expression of ESR1 and PGR genes in a very small amount of total RNA.

In conclusion, we are able to amplify and to detect ESR1 and PGR mRNA by decreasing the starting amount of total RNA to 5 ng, thereby confirming the high amplification power of duplex NASBA. This efficiency in nucleic acid amplification makes the NASBA technique a suitable tool for
analysis of small clinical biopsies. The decreasing size of surgical tumor samples should progressively restrict the use of ligand binding assays and biochemical techniques. Along with immunohistochemistry, molecular biology-based methods can provide the clinicians with relevant information from very small clinical samples, such as biopsies and fine needle aspiration. Finally, using the eight-tube strip format developed for the NucliSens EasyQ platform, rapid, one-shot, easy-to-use, multiparametric, real-time NASBA assays could also advantageously be set up for any mRNA profiling applications.

**ACKNOWLEDGMENTS**

*We thank Dr. Pierre-Jean Lamy at the CRLC Val d‘Aurelle in Montpellier for performing the ligand binding analysis on cell lines and B. Dei-man for her helpful input.*

**COMPETING INTERESTS STATEMENT**

*The authors declare no conflicts of interest.*

**REFERENCES**


Address correspondence to Philippe Leissner, Human Genetics Department, bioMérieux, 69280 Marcy l’Etoile, France. e-mail: philippe.leissner@eu.biomerieux.com