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Research Report
Multiplex Fluorescent RT-PCR to Quantify Leukemic Fusion Transcripts

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ABSTRACT
The detection of chimeric transcripts derived from aberrant chromosomal fusion events provides an exceptionally valuable tool for the diagnosis of leukemia. We have developed a simple, inexpensive, reproducible, and automated method to quantify RT-PCR products. Our approach utilizes fluorescent PCR for the co-amplification of the specific fusion transcript with an internal control (HPRT). We have also combined the advantages of real-time quantitative PCR, namely continuous fluorescent detection of PCR products with the low cost of an endpoint assay by examining in a novel manner the amount of fluorescent PCR product generated during the exponential phase of amplification. This has been achieved by using the automated loading and quantification capacity of a laser-induced fluorescence capillary electrophoresis system, the ABI Prism® 310A, so that we can effectively monitor amplification during the exponential phase cheaply, reproducibly, and in a sensitive manner. We have carefully verified our new technique using five leukemia cell lines, each expressing a different fusion transcript. Specificity and reproducibility (CV within 10%) have been examined and demonstrate the excellent precision of our technology. The high sensitivity levels of at least 10⁴ to 10⁶ obtained for the serial dilutions of the five cell lines validate the choice of our fluorescent PCR as a comparable method to other more complicated and expensive methods. Our results have allowed us to quantify PCR products and the amount of chimeric mRNA originating from the translocation breakpoint. We demonstrate that our novel fluorescent method is useful to detect and quantify residual leukemic cells in patients undergoing therapy.

INTRODUCTION
The detection of leukemic specific fusion transcripts by RT-PCR has revolutionized the field of molecular diagnosis. Since the initial discovery of the fusion between the BCR and ABL genes in t(9;22) (3), many other medically relevant chromosome abnormalities have been identified that result in the juxtaposition and fusion of gene sequences. The fused genes involved in these chromosome aberrations that lead to leukemia play a major role in the development and function of cells from the myeloid and lymphoid lineages. Many studies have shown that the identification of chromosomal aberrations are useful for risk group classification such as t(9;22) and 11q23 aberrations that are associated with a poor prognosis (12,15). Conversely, t(12;21) in pediatric acute lymphoblastic leukemia (ALL) and t(8;21), t(15;17), inv(16) involved in acute myeloid leukemia (AML) are associated with a good prognosis (6,16). Moreover, the detection of remaining leukemic cells (minimal residual disease, MRD) during and after medical treatment has been shown to provide a valuable tool with prognostic relevance (2,4,11).

The most sensitive techniques are based on the analysis of specific fusion genes by conventional RT-PCR using oligonucleotide primers spanning the breakpoint region. After gel electrophoresis, the amount of PCR product is determined by comparison with serial 10-fold step dilutions of leukemic cells or RNA in a semi-quantitative manner. One of the first quantification methods was competitive PCR (2), but this technique is difficult, prone to error, and time-consuming. Advances in quantitative strategies include real-time PCR (7), where the amount of PCR product is quantified following each cycle of the reaction. Real-time PCR is known to be a sensitive method to detect DNA products, ensures detection during the linear range of amplification, and eliminates the need for post-PCR analysis. This technique is now increasingly used for MRD detection but unfortunately requires very expensive instrumentation and reagents.

Our goal was to develop a simple, inexpensive, reproducible, and automated method to quantify RT-PCR products. This new approach incorporates a multiplex fluorescent PCR method (10) in conjunction with direct analysis of the PCR products using a laser-induced fluorescence capillary electrophoresis system, the ABI Prism® 310A (Applied Biosystems, Foster City, CA, USA). Five different leukemia cell lines avail-
able in our laboratory were used to validate our novel technique. The criteria used to evaluate this method included sensitivity, linearity, and reproducibility.

MATERIALS AND METHODS

Cell Lines

The following five cell lines with their corresponding gene fusion were used: K-562 for BCR/ABL (ATCC, Biovalley, France), REH for TEL/AML1, NB-4 for PML/RARA, KASUMI-1 for AML1/ETO, and 697 for E2A/PBX1 (DSMZ, Braunschweig, Germany). The cell lines were maintained in complete lymphocyte medium (Cytocell Ltd., Amplitech, Compiègne, France) until RNA extraction. Mononuclear cells were obtained by Histopaque®-1077 (Sigma, St. Quentin Fallavier, France) density gradient centrifugation and used immediately or stored in liquid nitrogen.

RNA Preparation

Total RNAs of cell lines were prepared by TRIzol® extraction (Invitrogen, Cergy Pontoise, France). Cells (1 × 10^7) were homogenized in 1 mL TRIzol reagent, 0.2 mL chloroform were added, and after 5 min at room temperature, the tubes were centrifuged at 12 000×g for 15 min at 4°C. The soluble RNA in the aqueous phase was then precipitated in an equal volume of isopropanol. After washing in 75% ethanol, RNAs were re-suspended in 30 μL RNase-free water and stored at -80°C. The amount of extracted RNA was determined by measuring absorbance at 260 nm.

Serial dilutions of each cell line were prepared once for all tests presented in this study and stored at -80°C. Each cell line RNA was diluted into normal RNA in 10-fold steps from undiluted to 10^{-6}. Normal RNA used for the dilution experiments was obtained from phlebotomy of patients followed for hemochromatosis and was confirmed as negative for all the specific transcripts explored in this study.

cDNA Synthesis

One microgram of RNA (undiluted or diluted) was preliminarily denatured in a 10-μL partial reaction at 70°C for 10 min and used for the synthesis of cDNA in a 25-μL total reaction containing 0.25 μg random hexamers, 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen), and 20 U RNasin® (Promega, Charbonnières, France). RT reactions were performed at 23°C for 10 min, followed by 42°C for 45 min and 99°C for 3 min. Negative controls without reverse transcriptases were made for each RNA preparation. Aliquots of cDNA were stored at -20°C before use.

Table 1. Sequences of Primers Used in Different Mixtures for Fluorescent RT-PCR

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Cell Lines</th>
<th>Primer Code</th>
<th>Sequence (5’→3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K-562</td>
<td>BCR-b2-C</td>
<td>CAGATGCTGACCAACTCGTGT</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABL-a3-B</td>
<td>GTTTGGGCTTCACACCATCC</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>REH</td>
<td>AM3</td>
<td>AAGGGCTCGCTCATCTTGCTTG</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B12a</td>
<td>CGTGGATTTCAACAGTCCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>697</td>
<td>GCACAACCACCGGCCC</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JN23</td>
<td>CCACGCCTTCGCTAACACGC</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NB-4</td>
<td>B</td>
<td>GTCATAGGAAGTGGAGGTCTTC</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D’a</td>
<td>CTCACAGGGCGCTGACCCCAT</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>KASUMI-1</td>
<td>AML1-A</td>
<td>CTACGCAGCCATGAAGAACC</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ETO-B’a</td>
<td>AGAGGAAGGCCCATTGCTGAA</td>
<td></td>
</tr>
<tr>
<td>all internal mixtures control</td>
<td>HPRT-Fb</td>
<td>TGTAATGACCAGTCAACAGGG</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPRT-R</td>
<td>TGGCTTATATCCAACACTTCG</td>
<td></td>
</tr>
</tbody>
</table>

a5’ HEX-labeled

b5’ NED-labeled
Fluorescent PCR

PCR co-amplifications were performed with 3 μL cDNA in a total volume of 28 μL containing the following final concentrations: 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 μM each dNTP, 0.15 μM each primer, and 0.62 U AmpliTaq Gold™ (Applied Biosystems). Amplification was performed in a thermal cycler (PTC-100™; MJ Research, Labover, Montpellier, France): first cycle at 95°C for 9 min, then 22–35 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 2 min, with a final extension at 72°C for 10 min.

Table 1 shows the primers used in multiplex RT-PCR analysis. The hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene (13) was co-amplified as an internal control. HPRT-F and HPRT-R were incorporated in each mixture to amplify a fragment of 213 bp. To avoid competitive co-amplification, the HPRT primers were added at a lower concentration (0.075 μM) in all mixtures. One primer of each pair was 5’ end-labeled: HEX-labeled (green) for chimeric gene products (Table 1) and NED-labeled (dark) for HPRT-F. After amplification, we added 2 μL each PCR to 17.5 μL deionized formamide and 0.5 μL ROX-labeled (red) Genescan-400® size-standards (GS400HD; Applied Biosystems). After heating for 5 min at 95°C and rapidly chilling on ice, samples were automatically loaded onto the ABI Prism 310A laser-induced fluorescence capillary electrophoresis system, according to the manufacturer’s instructions. Amplified fragment sizes and peak height values were determined after treatment by Genescan software (Applied Biosystems). Co-amplification results were expressed as the ratio between the chimeric PCR product and the HPRT internal control PCR product (gene: HPRT ratio). Some amplification products performed by fluorescent PCR were checked by electrophoresis on a nondenaturing 8% acrylamide gel. Expected sizes for each specific transcript were found following hybridization with a biotinylated end-labeled specific probe (data not shown).

The co-amplification efficiencies were determined for each specific fusion transcript using cDNA obtained from each cell line. Multiplex fluorescent PCRs were performed in different rounds (22, 24, 26, 28, 30, 32, and 35 cycles) with HPRT as the reference gene. In this way, the optimal PCR cycle number could be evaluated to determine the definitive conditions of our method.

In the final protocol, the five different cell line serial dilutions were analyzed in duplicate. A standard curve was established for each cell line by plotting the gene:HPRT ratio versus the known RNA dilution on a logarithm scale. The relative quantity of fusion gene transcript in unknown samples could be calculated automatically by reference to this curve.

RESULTS AND DISCUSSION

Recent technological advances in strategies to quantify PCR products include very expensive methods. We present here a simple method to quantify RT-PCR products based on a fluorescent PCR methodology. In this study, we performed a specific, reproducible, and sensitive method to quantify specific fusion transcripts in leukemic patients to follow the MRD. Using our multiplex fluorescent PCR method, we detected specific amplification products with the expected sizes following analysis using Genescan software, according to the design of the selected primers (references in Table 1).

The reproducibility of the 310A apparatus was evaluated by loading 10...
aliquots from one RT-PCR and the CV between peak heights or peak height ratios was found to be less than 5% (13). The intra-assay reproducibility showed only minimal variation of the gene: HPRT ratio. The inter-assay reproducibility was evaluated on two different sets of PCR and showed variations less than 10% using the peak height ratio.

The independent housekeeping gene HPRT has been selected in this study because it is moderately expressed in mammal cells compared with other housekeeping genes (5). The use of the HPRT gene as an internal PCR control in our method was an excellent way to eliminate false-negative results and in conditions that avoided competitive co-amplification. We have evaluated the PCR product yield for each target chimeric transcript and compared them to the HPRT transcripts using cDNA from each cell line. PCR co-amplification efficiencies were determined at different PCR rounds (22–35 cycles) to evaluate optimal conditions (Figure 1). The PCR co-amplification ratio showed lower variation in the later part of the exponential amplification phase. This assumes that any preferential amplification of one cDNA did not occur between the chimeric cDNA and the HPRT cDNA. These results demonstrate that the gene:HPRT ratios were most constant between 30 and 32 amplification cycles. All subsequent analyses were thus performed in this range of PCR co-amplification efficiencies.

It is worth noting that expression levels of HPRT in the five cell lines are not very different. This is demonstrated in Figure 2, where we have brought together the peak height values of HPRT PCR products obtained in the experiments presented in Figure 1. Only with REH and 697 cell lines the expression of HPRT is slightly lower than the values of the others.

One of the advantages of our method is that we could quantify together the two co-amplified products from the same loading. In this way, the internal control was analyzed at the same time and the ratio gene:HPRT allowed us to normalize the possible variable steps from RNA extraction to PCR.

The standard curves presented in Figure 3 showed that quantification of each target transcript was linear on a
scale of at least four orders of magnitude with good correlation factors. The study of the sensitivity levels of each cell line showed that we can detect, using a single PCR, all the fusion transcripts with a sensitivity level of at least $10^{-4}$ to $10^{-6}$. It is important to note that these results were obtained without the use of nested PCR, thereby minimizing the associated risk of contamination. These results validate our choice of fluorescent PCR as a sensitive method that compares favorably to others (1,18,19). Our aim to quantify RT-PCR products was possible, as we could construct a linear standard curve from the gene:HPRT ratio versus the known RNA dilution. Therefore, our method allowed us to quantify the unknown level of chimeric RNA and to detect and quantify the remaining leukemic cells for MRD detection.

In summary, we have developed an RT-PCR method using fluorescent 5′ end-labeled primers with a good specificity that does not require gel loading or time-consuming steps such as Southern blotting and hybridization. In addition, excellent reproducibility of the fluorescent PCR method has been demonstrated in this study. Comparison with real-time PCR invites several comments. Real-time PCR requires a unique hybridization probe for each RT-PCR target so that, although a high degree of specificity is obtained, it is prohibitively expensive for many laboratories. Furthermore, real-time PCR also requires expensive instrumentation. A recent study by Schmittgen et al. (17) compared real-time PCR with endpoint PCR methods. We have combined the advantages of real-time PCR, namely continuous fluorescent detection of PCR products, with the low cost of an endpoint assay by examining the amount of fluorescent PCR product generated during the exponential phase of amplification. This has been achieved by utilizing the automated loading and quantification capacity of the ABI Prism 310A laser-induced fluorescence capillary electrophoresis system so that we can effectively monitor amplification during the exponential phase cheaply, reproducibly, and in a sensitive manner. The linearity of the standard curves drawn by plotting the fusion transcript:HPRT ratio versus the known RNA dilution demonstrates the PCR efficiency.

In conclusion, our fluorescent quantitative RT-PCR method is a successful alternative strategy for quantifying MRD during clinical studies.

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REFERENCES


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