Highly Sensitive Method to Detect mRNAs in Individual Cells by Direct RT-PCR Using Tth DNA Polymerase

BioTechniques 22:312-318 (February 1997)

ABSTRACT

A new method for detecting the expression of low-abundance mRNA molecules has been developed that combines the sensitivity of PCR, the high efficiency and specificity of reverse transcription (RT) using Tth DNA polymerase at high temperature, and the enhancement of sensitivity and specificity of nested PCR. This method is highly sensitive, reproducible and allows the detection of mRNAs in individual cells by direct RT-PCR.

INTRODUCTION

Reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive technique available to detect low-abundance mRNA transcripts. Although several protocols using this technique have been reported to detect mRNA in single cells (3,5,6,8), they all require an RNA isolation step, which is time-consuming and is accompanied by an unavoidable loss of material, thereby making quantitation difficult. Furthermore, RNA extraction from single cells often requires expensive equipment not readily available in most laboratories. Recently, different reports explored the possibility of amplifying mRNA from single cells by direct RT-PCR to avoid some of these problems (1,2,4); however, a precise quantitative analysis of the limits of these methods was not provided. Moreover, these techniques were either time-consuming or needed radioactive or special material. Additionally, multistep manipulation of the reverse transcription product (1) was necessary or the Tth enzyme property enabling reverse transcription at high temperatures was not used (2,4). We report here a highly sensitive method to detect mRNAs in individual cells by direct RT-PCR using Tth DNA polymerase. This method circumvents the problems

ed or permeable cells were examined (9). The weak staining that remained in the cytoplasm after actinomycin D treatment most likely represents nonincorporated BrUTP. The nature of the RNase-sensitive labeling seen in the incorporated BrUTP. The nature of the treatment most likely represents nonin -

d to resort to microinjection or per -

ting to identify actual transcription sites. These processes may be necessary in or-

REFERENCES


The present study was supported financially by Dr. med. Letten F. Saugstad’s Fund, The Norwegian Cancer Society and the Norwegian Research Council, Oslo, Norway. Skillful technical assistance by Beth Johannessen, Bergen, and valuable discussions with Derick G. Wansink, Nijmegen, are greatly acknowledged. Address correspondence to Gunnar Haukenes, Centre for Research in Virology, Bergen High Technology Center, N-5020 Bergen, Norway. Internet: cannon.haukenes@rnf.uib.no

Received 12 March 1996; accepted 5 August 1996.

University of Bergen
Bergen, Norway
created by manipulating mRNA from a single cell. With this method we have detected actin and cytokine mRNAs from single cells without using radioactivity at any step. In addition, we have amplified one or two molecules of a standard RNA (st-RNA). Our method is a nested PCR amplification that combines the use of the thermostable enzyme Tth for both the reverse transcription and the first DNA amplification, followed by a second PCR step.

**MATERIALS AND METHODS**

**Materials**

Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem-Novabiochem (La Jolla, CA, USA). Tth DNA Polymerase and the AmpliScribe™ T7 Transcription Kit were from Epicentre Technologies (Madison, WI, USA), and Taq DNA Polymerase was from Promega (Madison, WI, USA).

**Cell Line**

The cell line used in the experiments described here is mouse thymoma EL4 (TIB 181; ATCC, Rockville, MD, USA), which expresses interleukin 2 (IL-2) mRNA transcripts upon PMA activation.

**Primers**

The primers used in this study are summarized in Tables 1 and 2. They all span at least one intron.

**Single-Cell PCR Assay**

After activation in bulk culture, the cells were washed twice in phosphate-buffered saline (PBS) and serially diluted to 100 cells/mL. Ten microliters of this dilution (corresponding to 1 cell) were added to individual wells of a Terasaki plate (Nunc, Naperville, IL, USA). The plate was centrifuged (2000 rpm for 2 min), and single cells were collected using an inverted microscope and added to 0.2-mL thin-walled PCR tubes (USA Scientific Plastics, Ocala, FL, USA). After centrifugation at 10,000 rpm for 15 s, the supernatants were removed by aspiration, and 10 µL of dd-H2O containing 200 ng of tRNA were added to the tube. The tRNA was added to act as a substrate for RNases present in the cell and thus limit the potential degradation of the transcript of interest. All the RTs and PCRs were performed with a GeneAmp® PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA). The tubes were heated at 95°C for 1–2 min and cooled down to 65°C; then 10 µL of 2× RT mixture containing 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 1.75 mM MnCl2, 500 µM dNTPs, 1.6 mM of antisense primer(s) and 3.5 U of Tth DNA polymerase were added to the crude cell lysate. Adding RNasin® (Promega) or acetylated bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO, USA) did not improve the results. The mixture was then incubated at

| Table 1. PCR Oligonucleotides Used for the First and Second PCR Amplification |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| **Gene (Mouse)** | **Fragment Size (Bases)** | **Primer Sequence** | **Annealing Temperature (°C)** | **Enzyme** |
| **First Amplification** | | | | |
| β-actin 5’ | 348 | 886-5’TGGAGATCTGTGGCATCCATGAAAC-910 | 60 | Tth |
| β-actin 3’ | | 1234-5’TAAACCGCAGCTGTAACAGTCCG-1210 | | |
| IL-2 - 5’ | 321 | 49-5’TGTACAGCAGCTGCATCCTGTCA-73 | 60 | Tth |
| IL-2 - 3’ | | 370-5’TCAATTCTGTGGCTTGCACTTGCA-346 | | |
| **Second Amplification** | | | | |
| β-actin 5’ | 192 | 961-5’ATGCCACACAGCTGCTGTGGTG-985 | 60 | Taq |
| β-actin 3’ | | 1153-5’CTGATCCACATCTGCTGGAAGGTG-1130 | 55 | Taq |
| IL-2 - 5’ | 227 | 83-5’GACACTTGTGCTCCTTGCTCA-102 | | |
| IL-2 - 3’ | | 310-5’TCAATTCTGTGGCTTGCACTTGCA-291 | | |

| All the primers used spanned an intron. |

| Table 2. PCR Oligonucleotides Used to Quantitate the Limits of the Method |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| **Plasmid Primers** | **Fragment Size (Bases)** | **Primer Sequence** | **Annealing Temperature (°C)** | **Enzyme** |
| **First Amplification** | | | | |
| Antisense 439 | 5’-ATGACTGTGGCTGCTGCTGGGAGC-3’ | 60 | Tth |
| Sense | 5’-TAAAACCGCAGCTGAACAGTCCG-3’ | | |
| **Second Amplification** | | | | |
| Antisense 288 | 5’-TGGAGTCAACAGAGAAGGTGGCAGTAAG-3’ | 60 | Taq |
| Sense | 5’-TCTGACCACAGTGGAAGATGTCCAC-3’ | | |
60°C for 2 min, followed by 10 min at 70°C using a ramp slope program of 4 min. The reaction was then cooled down and kept at 4°C until addition of the PCR mixture. After the RT reaction, 80 µL of the PCR mixture were added. For the first amplification of the β-actin or IL-2 gene, the PCR mixture contained 10 mM Tris-HCl, pH 8.8, 75 mM KCl, 3.5 mM MgCl₂, 0.2 µM sense primer and 50 µM dNTPs. For the first amplification of the IL-2 gene, we added 5% dimethyl sulfoxide (DMSO). For second-strand DNA synthesis, the mixture was denatured at 94°C for 90 s, followed by an annealing at 60°C for 60 s and extension at 72°C for 45 s. The addition of EGTA to chelate Mn²⁺ did not improve the results. The amplification reaction involved 30 cycles of denaturation at 94°C for 20 s, followed by annealing at 60°C for 40 s and extension at 72°C for 40 s. For the last cycle, the extension time was 7 min. Then, 0.5 µL of the first reaction was amplified for an additional 32 cycles by using nested primers under identical conditions, except for IL-2 (annealing temperature 55°C). The PCR mixture contained 10 mM Tris-HCl, pH 9.2, 75 mM KCl, 3.5 mM MgCl₂, 0.3 µM of each sense and antisense primer, 200 µM dNTPs and 1.25 U Taq DNA polymerase. Twenty microliters of the PCR products were analyzed on 1% agarose/2% NuSieve® (FMC BioProducts, Rockland, ME, USA) in the presence of 0.5 µg/mL ethidium bromide.

RESULTS AND DISCUSSION

RT-PCR from the mRNA of a Single Cell

To test this method, we activated EL4 cells in the presence of PMA for 3–12 h and tested for the expression of the IL-2 gene and β-actin as a control. Figure 1 shows a typical result of such an amplification. One-fifth of the respective PCR products was analyzed on agarose gel in the presence of ethidium bromide. The specificity of the reaction was demonstrated by the presence of the single band at the expected size of the PCR product and by sequencing. Controls performed without the RNA-releasing step or without the RT step were both negative for the specific PCR product. Moreover, the same experiments using cells that did not express IL-2 were always negative (data not shown).

Sensitivity of the Amplification

To evaluate the sensitivity of this technique, we performed a calibration using an in vitro-transcribed st-RNA. The st-RNA was synthesized by using T7 DNA polymerase and a plasmid template (7) that contained, in tandem array, multiple primer sequences specific for different cytokine genes and the β-actin gene. By using the combination of primers shown in Table 2, we found that 1–2 copies of input st-RNA can be detected by the method we describe (Figure 2). The results showed that under these conditions the reverse transcriptase activity of Tth is probably greater than 50%. Furthermore, a faint band, corresponding to the amplification of 1–2 copies of input cRNA, was
visible after only 21–23 cycles in the second PCR [corresponding to only 42 or 43 cycles of amplification ($2^{30/200} + 2^{21/2}$)] (Figure 2). This result is in agreement with the fact that in some experiments we could detect a faint band corresponding to β-actin even after the first PCR amplification. Given the levels of dilution, significant sampling errors could arise. Nevertheless, we find that the possibility of amplifying less than 5 molecules of st-RNA is reproducible using this technique. A possible improvement to the technique would be the elimination of contaminating genomic DNA. For this purpose, we tried the technique described by Ziegler et al. (8). Unfortunately, in our hands, a loss of sensitivity of the RT-PCR was observed simultaneously with the elimination of the genomic DNA. Furthermore, treatment of the sample with the DNase I involved the addition of numerous steps to the process, inducing a significant increase in the risk of contamination, and in the loss of material. Finally, the ability to amplify only one species of RNA per cell may be a limitation of the method. However, by using exactly the same protocol we describe here, it is possible to use at least two different antisense primers during the RT, followed by dividing the RT product into two aliquots before PCR amplification, thereby enabling the analysis of the expression of two different genes per cell. The primers and protocol described here can be used for this purpose.

CONCLUSION

The reproducibility, reliability and flexibility of this technique may be useful in solving numerous problems in diverse areas such as oncology (number and cell type expressing a particular mRNA), immunology (Th1/Th2 phenomenon) and virology (number and cell type infected by replicating retroviruses). Furthermore, its simplicity and accuracy suggest that automation of this method is possible.

REFERENCES


This work was supported by grants from the National Institutes of Health (RO1 AI 32122). G. Chiocchia was a recipient of a fellowship from the Charles H. Revson Foundation and also benefits from support by the Philippe Foundation, Inc. Address correspondence to Gilles Chiocchia, INSERM Unité 283, Hôpital COCHIN, Pavillon Hardy A, 27 rue du Faubourg Saint Jacques, Paris 75674, France.

Received 20 February 1996; accepted 7 August 1996.

Gilles Chiocchia and Kendall A. Smith
INSERM Unité 283, Hôpital COCHIN
Paris, France
1The New York Hospital—Cornell Medical Center
New York, NY, USA