
Address correspondence to Anthony M. Smith, Department of Medical Microbiology, MRC Pneumococcal Diseases Research Unit, School of Pathology, South African Institute for Medical Research and University of the Witwatersrand, P.O. Box 1038, Johannesburg, South Africa. Internet: 174ant@chiron.wits.ac.za

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Anthony M. Smith and Keith P. Klugman
South African Institute for Medical Research and University of the Witwatersrand
Johannesburg, South Africa

Quantitative RT-PCR for Human Fas (CD95) Expression

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Fas, a 45–48-kDa protein belonging to the tumor necrosis factor receptor family, has been shown to play a central role in the regulation of apoptosis and programmed cell death in lymphoid cells, hepatocytes, epithelial cells and possibly other cell types by interactions with its ligand, FasL (1,8). It has also been implicated in the pathogenesis of several viral infections, including human immunodeficiency virus (HIV)-associated apoptosis of CD4+ and CD8+ T lymphocytes (7,9). Fas expression has been shown to be up-regulated in association with these apoptotic processes (4,5,7,8). However, a method to quantitate Fas mRNA expression has not been reported yet. Here we present a polymerase chain reaction (PCR)-based method for such quantitation.

Reverse transcription (RT)-PCR is used with increasing frequency to examine levels of gene transcripts due to its high sensitivity and time efficiency. However, quantitation of mRNA levels has been problematic because of the exponential nature of the reaction, where small variations can lead to dramatic changes in final results. One approach to this problem has been competitive RT-PCR, where one set of primers is used to amplify both a target gene cDNA and another homologous or non-homologous DNA fragment of a different size, which competes for the same set of primers and thus acts as an internal standard (2,10). We have successfully used competitive RT-PCR with a nonhomologous internal standard to quantitate Fas gene expression in normal and apoptotic human endothelial cells (EC).

The steps of our protocol are summarized in Table 1. A nonhomologous internal standard DNA fragment for Fas was constructed using a PCR mimic construction kit (CLONTECH Laboratories, Palo Alto, CA, USA). We synthesized two composite primers of 41 nucleotides each, in which 21 nucleotides from the 5′ end are part of a Fas primer sequence, used by our group previously in nonquantitative reactions (5,6), followed by 20 nucleotides complementary to the heterologous DNA fragment (CLONTECH). These composite primers were used in the first PCR amplification according to the manufacturer’s protocol to generate a 425-nucleotide “Fas mimic”, which has both the Fas primer sequences on its two ends but is 130 nucleotides short of the Fas fragment normally obtained by using those primers with cDNA. A dilution of the first PCR product was then amplified using the original Fas-specific (21-mer) primers to ensure that all fragments have the complete Fas-specific primer sequences. This fragment was then purified with a CHROMA SPIN™ Column (CLONTECH) and quantitated using absorbance spectroscopy at 260 nm. The Fas mimic was then diluted to 100 amol per µL stock solution (25 pg/µL) and was used in competitive PCR experiments, described below. Fas primers capable of amplifying a segment from nucleotides 271 to 820 of Fas cDNA are: Fas1 - 5′ CAAGTGACTGACATCAACTCC, Table 1. Protocol

1. Construction of a nonhomologous internal standard fragment, referred to as “Fas mimic”.
2. Isolation of total RNA from normal and apoptotic cells and synthesis of first-strand cDNA.
3. First PCR amplification with constant amounts of cDNA and serial 1:10 dilutions of Fas mimic.
4. Second PCR amplification with constant amounts of cDNA and serial 1:3 dilutions of Fas mimic in a narrower range, based on the results of the first amplification.
5. Gel and data analysis of products of the second PCR for quantitation of Fas.

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Benchmarks

Fas2 - 5′ CCTGGGTTTTCCTTTCTG′ TGC. The composite primers for making Fas mimic are: Fasm1 - 5′ and Fasm2 - 5′ CCTTGGTTTTCCTTTCTG′ GTGCATTGATCTGGACCATG′ C.

To check the efficiency of Fas 1 and Fas 2 primers for amplifying Fas cDNA and Fas mimic, we have used a plasmid pBLF58-1, which contains full-length human Fas cDNA (3), obtained from Dr. S. Nagata of Osaka Bioscience Institute, Osaka, Japan. As shown in Figure 1, Fas 1 and 2 primers were able to amplify the Fas cDNA from the plasmid and the Fas mimic efficiently (lanes 1 and 3). When equimolar quantities (200 amol) of the plasmid and mimic were used together in a PCR with Fas 1 and 2 primers, equal PCR signals were obtained (lane 2).

To isolate RNA and to synthesize first-strand cDNA, we used as a test system primary human microvascular EC of renal origin (HMVEC-G; Cell Systems, Kirkland, WA, USA) treated with normal human plasma or plasma from a patient with a thrombotic microangiopathy, thrombotic thrombocytopenic purpura (TTP). This disease has been associated with plasma-induced EC apoptosis and induction of Fas (6). After an overnight incubation at 37°C, total cellular RNAs were isolated from 1–2 × 10⁶ EC per sample by the TriZOL™ reagent (Life Technolo-

![Figure 1. Efficiency of Fas 1 and 2 primers in PCR amplification of Fas cDNA and Fas mimic DNA.](image)

![Figure 2. Competitive PCR analysis of Fas expression in renal endothelial cells.](image)
gies, Gaithersburg, MD, USA) (4). RNAs were treated with RNase-free DNase (Life Technologies), and the concentration of RNA was determined by absorbance spectroscopy at 260 nm.

A constant amount (10 µg) of RNA from normal and apoptotic EC was reverse-transcribed into cDNA using 400 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies) in a 100-µL volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM each dGTP, dATP, dTTP, dCTP, random hexamer (1 µM) and RNase inhibitor (40 U) for 60 min at 37°C followed by a 5-min incubation at 95°C to destroy the enzyme and then chilling the tubes on ice.

For the first PCR amplification, 10-µL aliquots of cDNA were amplified with Taq DNA Polymerase (PE Applied Biosystems, Foster City, CA, USA) in standard PCR (7) along with serial 1:10 dilutions of Fas mimic in 4 concentrations ranging from 500 fg to 0.5 fg. Briefly, the cDNAs were denatured for 2 min at 97°C before 35 runs in a Model 9810 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA), with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min in each cycle. A final extension at 72°C for 5 min was included. The products were separated by electrophoresis in a 1.4% agarose gel and stained with ethidium bromide (EtBr). The range within which the Fas target and Fas mimic bands come to nearly equal intensity was noted.

In a second PCR amplification, a serial 1:3 dilution of the Fas mimic was used in a more narrow range with a constant volume of cDNA (10 µL), which in this case was between 5 and 0.5 fg for EC with normal plasma and 50–5 fg for EC with TTP plasma. The PCR was carried out the same way as described above. The products were separated on an agarose gel, stained with EtBr and photographed.

To analyze the gel and data, a photograph of the gel was then scanned by a Scanman® Easytouch Scanner (Logitech, Fremont, CA, USA) to input the figure to the gel analysis software SigmaGel (Jandel Scientific, San Rafael, CA, USA). The software was used to calculate the intensities of individual bands in each lane. The values were plotted as a Fas target:Fas mimic ratio vs. input concentration of Fas mimic in femtograms. Linear regression analysis was used to plot a straight line. The inverse of the value where the Fas target:mimic ratio of 1 intersects the X axis gives the concentration of cDNA present in the given sample. Assuming that the efficiency of reverse transcription is equivalent for all reactions run in parallel on a given day, an assumption checked using duplicate samples, the number of Fas mRNA molecules can be calculated as fg/µg input RNA. Figure 2A shows a plot for renal microvascular EC cultured with normal plasma. Normal plasma does not induce EC apoptosis (6). These cells have a concentration of Fas (3 fg), which is almost...
four times lower than the same cells when induced to undergo apoptosis by treatment with TTP plasma (11.5 fg, Figure 2B).

Our method can be used to similarly quantitate Fas gene expression in various apoptotic cell death systems as an aid to understanding the role of Fas in a particular system. We would be willing to provide our construct to investigators interested in studying additional systems of Fas-related apoptosis.

REFERENCES


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Debashis Mitra and Jeffrey Laurence
Cornell University Medical College
New York, NY, USA

Nonradioactive PCR-SSCP Assay to Detect apoB Arg3500 to Gln Mutation Using a Minigel Format

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Elevated levels of low density lipoprotein (LDL) are positively correlated with increased risk of coronary heart disease. Approximately 70% of LDL is cleared by the liver. The high affinity interaction between LDL and the LDL receptor occurs by apolipoprotein B-100 (apoB-100). It has been shown that a point mutation in apoB-100, a CGG to CAG change in codon 3500 resulting in the substitution of glutamine for arginine (2), can decrease the normal binding affinity to the LDL receptor leading to increased plasma levels of LDL (2). Investigators in several countries are routinely screening for this mutation. Detection of this mutation has generally been accomplished by allele-specific oligonucleotide hybridization of genomic DNA amplified by the polymerase chain reaction (PCR) technique (2,5). Another technique uses primers designed to introduce a mismatch base into the normal but not mutant allele by PCR. The wild-type, but not the mutant, is cleaved by restriction endonucleases (1). Both methods are laborious, and the former also requires 32P-labeled probes.

PCR single-strand conformation polymorphism (PCR-SSCP) analysis is a powerful method for identifying sequence changes in amplified DNA (3). Variations in the sequence as small as one base pair can alter the secondary structure of single-stranded DNA; these variations are detected by shifts in electrophoretic mobility. We have adapted the PCR-SSCP technique for nonra-