

Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample

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ABSTRACT

We describe a semiquantitative RT-PCR protocol optimized in our laboratory to extract RNA from as little as 10,000 cells and to measure the expression levels of several target mRNAs from each sample. This procedure was optimized on the human erythroleukemia cell line TF-1 but was successfully used on primary cells and on different cell lines. We describe the detailed procedure for the analysis of Bcl-2 levels. Aldolase A was used as an internal control to normalize for sample to sample variations in total RNA amounts and for reaction efficiency. As for all quantitative techniques, great care must be taken in all optimization steps: the necessary controls to ensure a rough quantitative (semi-quantitative) analysis are described here, together with an example from a study on the effects of TGF- β 1 in TF-1 cells.

INTRODUCTION

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts (1,2). In most cases, when RNA analysis is required, a qualitative study is not sufficient to deliver a satisfactory answer. A common question is the quantification of specific RNA transcripts and the detection of any variation in their expression levels under different experimental conditions. We have often faced the problem of detecting poorly expressed transcripts, as well as that of handling small amounts of unique samples, such as primary hematopoietic cells (3,4) or tumor biopsies from human patients (5-7). When studies are performed in human primary models or in established and transformed cell lines in conditions requiring expensive reagents, analysis has to be performed with a sensitive but reliable technique because only a limited number of experiments can be run on each sample. A number of protocols and improved PCR techniques are now available, which will be discussed in the present manuscript,

but these are not all easily accessible to a standard laboratory and have pitfalls together with the advantages for which they were created. Although reproducibility is always an essential requirement, extreme accuracy may not be: in most studies the focus is not to measure minor changes or the exact number of molecules, but an increase or decrease by at least 1.2-fold in expression levels. Hence, despite the greater accuracy of recently developed techniques, semi-quantitative methods are still widely used and appropriate for many purposes. Here we describe the standard procedure, optimized in our laboratory, to assess Bcl-2 levels with Aldolase A as an internal control, and all the necessary controls to ensure a quantitative analysis. Examples of the analyses of multiple markers and of the data obtained are shown.

MATERIALS AND METHODS

RNA extraction

RNA extractions were carried out with the RNeasy mini kit (Quiagen, Hilden, Germany), according to the manufacturer's instructions. We normally used 2×10^5 cells, but cell numbers ranging from 1×10^4 to 2×10^6 were successfully utilized. The following protocols are optimized on the human erythroleukemia TF-1 cell line (8), and were tested with Phytohemagglutinin-activated lymphocytes, but different cell lines, primary hematopoietic cells and tumor biopsies were also used successfully. Samples were vortexed for 1 min to shear genomic DNA before loading onto the RNeasy mini columns, and then eluted in a minimum volume of 30 μ l and a maximum volume of $2 \times 50 \mu$ l RNase-free water. RNA obtained with this procedure was essentially free of genomic DNA. When using different extraction procedures, or working with tissue samples, a DNase I treatment, followed by phenol extraction and ethanol precipitation, was applied to remove traces of contaminating DNA (9).

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Reverse Transcription

RNA obtained from 20,000 cells was reverse transcribed in the presence of 5 mM MgCl₂, 1X PCR Buffer II, 1 mM dNTPs, 25 u MuLV Reverse Transcriptase, 1 u RNAGuard Ribonuclease inhibitor (Amersham Pharmacia Biotech, Uppsala, Sweden), 2.5 μM Random hexamers in a final reaction volume of 20 μl. All reagents were from PE Applied Biosystems except when otherwise specified. Reactions were carried out at 42°C for 30 minutes in a Gene Amp PCR system 9600 (PE Applied Biosystems), followed by a 10 minute step at 99°C to denature the enzyme, and then by cooling to 4°C.

PCR

a) Standard reaction for Bcl-2

Two μl of cDNA products were amplified with 1 unit of Ampli Taq Gold (PE Applied Biosystems) in the buffer provided by the manufacturer which contains no MgCl₂, and in the presence of the specific primers for Bcl-2, together with the Aldolase-A primers (6), used as an internal control as described below. The amount of dNTPs carried over from the reverse transcription reaction is fully sufficient for further amplification. Reactions were carried out in the Gene Amp PCR system 9600. A first cycle of 10 minutes at 95°C, 45 seconds at 65°C and 1 minute at 72°C was followed by 45 seconds at 95°C, 45 seconds at 65°C and 1 minute at 72 °C for 30 cycles (see below). The conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification, and that the two sets of primers used in each reaction did not compete with each other (see below for a description of the necessary controls and determination of the specific parameters). Each set of reactions always included a no-sample negative control. We usually performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination. We have tested Taq polymerases from different sources, but have standardized our protocol with AmpliTaq Gold (PE Applied Biosystems).

b) Primer selection and determination of T_m

Our primers were synthesized by Amersham Pharmacia Biotech. The sequence was determined using the software Primer 3 (developed by Steve Rozen, Helen J. Skaletsky, 1996, 1997) available on-line at <http://www-genome.wi.mit.edu>. Primers were always chosen according to the following parameters: length between 18 and 25 bases, optimal 20-22 bases; T_m comprised between 57 and 65°C, optimal T_m 60-62°C; length of amplification product between 200 and 500 bp, so as not to overlap with the 176 bp Aldolase A amplification product. Occasionally, the primers were selected manually according to the following criteria: C+G content, >60 %; repetitive sequences, absent; repetitive bases, stretches of >3 identical bases (such as poly Ts) were normally avoided (although 4 was shown to work); sequence, perfectly homologous to the RNA of interest and so that the 3' end base was preferably G or C. To determine specificity, all sequences were compared with the Genbank using the program Blast available at the National Center for Biotechnology Information

website (www.ncbi.nlm.nih.gov). When both primer sequences showed homology to the same gene, different from that of interest, they were discarded. Moreover, when possible, it is advisable to select the PCR primers in such a way that the 5' and the 3' primers span different exons, so that the amplification product obtained from the cDNA would be of different length from that obtained from any contaminant genomic DNA comprising intronic sequences.

The following primers were used: Bcl-2 5'-CGACTT CGCCGAGATGTCCAGCCAG-3' (T_m 63.5°C), and 5'-ACTTGTGGCCCAGATAGGCACCCAG-3' (62°C); Aldolase A A25, 5'-CGCAGAAGGGGTCCTGGTGA-3' (59°C) and H20, 5'-CAGCTCCTTCTTCTGCTCCGGGGT-3' (62.2°C). The T_{ms} indicated in brackets were calculated according to the formula reported by Sambrook et al. (9), see below for comments. Bcl-2 yielded an amplification product of 388 bp and Aldolase A of 176 bp. See (10) and (11).

c) Primer concentration.

Each primer was used in a concentration range of 0.05 to 0.3 μM. Lower concentrations may be limiting, in which case the amplification would not be quantitative, whereas higher concentrations may leave a large amount of unused primers which could give rise to non-specific amplification products. In our system, a concentration of 0.06-0.15 μM was usually optimal. We optimized Bcl-2 with 0.15 μM of each of the two Bcl-2-specific primers and 0.06 μM of each Aldolase A-specific primer. The higher concentration used for Bcl-2 is due to an attempt to favor amplification of Bcl-2, which is expressed at lower levels than Aldolase A.

d) Determination of optimal MgCl₂ concentration

Determination of the optimal concentration for each primer set was performed using different MgCl₂ concentrations in the standard PCR reaction conditions (see below) and by adjusting the water volume consequently. We normally tested the following MgCl₂ concentrations: 1, 1.5, 2, 3 and 5 mM. The products were run on an agarose gel (see below) to choose the condition that gave the highest yield and specificity. Bcl-2 amplification gave the best results in 1 mM MgCl₂ (Fig 1).

e) Determination of cycling parameters

The annealing temperature was calculated as described above. The standard program comprised a cycle of 10 minutes at 95°C, 45 seconds at 65°C and 1 minute at 72°C, followed by 45 seconds at 95 °C, 45 seconds at 65 °C and 1 minute at 72°C for a number of cycles to be determined (30 for Bcl-2). It is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel (see below) and can be quantified, but also so that amplification is in the exponential range and has not reached a plateau yet. The optimal number of cycles has to be in the same range for the specific RNA of interest (i.e. Bcl-2) and the control, in this case Aldolase A, so that both can be measured on the same gel. When the annealing temperature of the two primer sets was different, annealing temperatures ranging between the optimal temperature of each primer set were tested.

f) Control for competition between primer sets

To determine whether the selected conditions are suitable for semiquantitative RT-PCR with both primer sets (Bcl-2 and Aldolase A) at the same time, we performed a competition control, by amplifying the same sample at the same time in the presence of the specific primers for Bcl-2, the internal control Aldolase A, and both sets together. The samples were then run on the same agarose gel for quantitation. When competition was detected, different reaction conditions were tested (see Results and Discussion).

Gel Electrophoresis

The PCR products were loaded onto Ethidium Bromide-stained, 1 to 2 % (depending on the size of the amplification products) agarose gels (1 % for Bcl-2) in TBE (9). A 1 kbp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run on every gel to confirm expected molecular weight of the amplification product.

Acquisition of gel images and quantitative analysis

Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a Cohu High Performance CCD camera (Cohu Inc. San Diego, CA) and quantification of the bands was performed by Phoretix 1 D (Phoretix International Ltd., Newcastle upon Tyne, UK). Band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be determined and Aldolase-A was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization to Aldolase A.

Further control: hybridization of PCR products with specific primers

When the PCR yield is very low and hence barely detectable or not visible at all on agarose gel, or when non-specific amplification products cannot be eliminated, it may be necessary to blot the agarose gel containing the PCR amplification products and hybridize it with a labeled specific probe. This can either be the cDNA (obtained by cloning or by PCR) or a specific oligonucleotide. This step is normally not required but we had to perform it with a few markers which were otherwise difficult to detect or quantify (3-6).

RESULTS AND DISCUSSION

When choosing a quantitative or semi-quantitative protocol to determine RNA expression levels by RT-PCR, many parameters must be taken into consideration (12). These include: 1) feasibility in the laboratory; 2) necessity to study different markers in the same sample; 3) availability of sample; 4) accuracy required for the specific application (i.e. need to measure the specific number of RNA molecules or rather rough variations in RNA levels). Relatively accurate methods have been developed, such as competitive PCR (MIMIC) or real-

time PCR based on the use of fluorogenic probes. The MIMIC technique requires an internal competitor that has to be specifically designed and then constructed for each specific RNA to be studied (13,14). An amplification curve with the internal competitor is then built for each sample. Quantification is rather accurate, but this technique requires a relatively large amount of cDNA and a large number of amplification reactions per sample and moreover, requires intensive initial work when different RNAs have to be analyzed and hence different mimic competitors need to be constructed. The PCR techniques that were initially devised (competitive, non-competitive, etc.) are all based on an "end point" measurement, that occurs when the PCR reaction is completed. Real-time PCR is a very promising quantitative method that is based on the concept of monitoring the PCR reaction in the thermal cycler as it progresses. A number of real-time methods have been devised (15,16,17), which require a dedicated instrument. Although this technique undoubtedly has a number of advantages, especially in terms of accuracy, it is not easily available to the standard laboratory and may be cumbersome to set up when used as only one of many techniques running in the laboratory. Another interesting, recently devised method, is the single-tube PCR, which permits reverse transcription and PCR in the same tube using the tTh enzyme (18). This is a very convenient method when a single RNA marker has to be analyzed in a large number of samples. On the other hand, assaying several RNAs on the same sample, we preferred to work on the same cDNA product in order to reduce the variability due to the reverse transcription reaction. For a rather comprehensive discussion of advantages and pitfalls, see a recent review by W. M. Freeman *et al.* (12). In our laboratory we have successfully utilized a semi-quantitative PCR method employing Aldolase A as an internal control in a number of systems and different types of samples (3-7). This method is based on the use of an internal control, which is a housekeeping gene (19,11) and hence on the assumption that the experimental conditions do not alter the expression levels of the selected control RNA. Since determination of RNA concentration is often inaccurate, and consumes a considerable amount of sample, we chose to work with a fixed number of cells (2,000 cells per PCR reaction). In our conditions Aldolase A content did not vary significantly (20) within the same type of sample (i.e. the same cell line or the same tissue type). We describe here the controls that were performed to establish the conditions to amplify Bcl-2 RNA in the human cell line TF-1, and that need to be established whenever studying a new marker or a new system in order for the data to be at least roughly quantitative.

Reaction conditions in the Reverse Transcription step are mostly dependent on the enzyme and the primers of choice. Whereas other protocols require use of specific primers, we prefer to reverse transcribe the total RNA population with random hexamers so that different PCR analyses could be performed on the same cDNA sample. Alternatively, a poly T primer could be used to amplify RNAs from their 3' tail, but in our hands this approach gave a lower yield than that with random hexamers.

A number of parameters can be varied in the PCR step and are

extremely important for the reaction to be quantitative. A variety of thermostable polymerases are now available on the

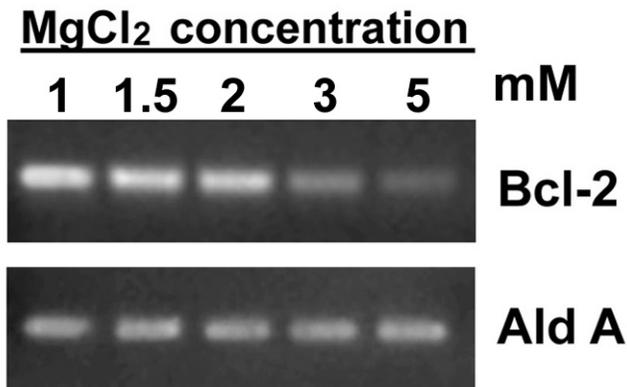


Fig. 1: Effect of different MgCl₂ concentrations in the PCR reaction. Analysis was performed on the human erythroleukemia cell line TF-1. Bcl-2 amplification was optimal at 1 mM MgCl₂ and decreased gradually at higher concentrations. The intensity of the Aldolase A amplification product did not change significantly. Ald A, Aldolase A.

market. In the past “hot-start” was often employed to avoid immediate start of transcription before denaturation and proper annealing of the primers. In recent years an easy alternative has become available from different manufacturers, by the addition of “blocking” antibodies to the polymerase, which keep the polymerase inactive till it is heated to 92-95°C for at least 10-12 minutes. The enzyme is hence not active during the time required to set up the reactions. Moreover, use of this enzyme can sometimes increase specificity.

Selection of the internal control RNA is also critical. Other control RNAs were tested (such as GAPDH, or actin), but most of the best known housekeeping genes are expressed at very high levels, hence their amplification profile reaches a plateau much earlier than the relatively low abundant RNAs we studied. Moreover, suitable conditions for amplification of Aldolase A were very flexible, ranging from a MgCl₂ concentration of 1 to 5 mM (Fig. 1), 27 to 32 cycles (Fig. 2), and an annealing temperature of 55 to 65°C, which made this gene the ideal control, easily adaptable to combination with most other primer sets.

MgCl₂ is an essential factor for the Taq polymerase to function, in a range of 1-5 mM, but efficiency of amplification with specific primers is strictly sequence-dependent. As shown in Fig.1, Bcl-2 worked best at 1-2 mM MgCl₂, whereas the yield of Aldolase A did not change at the concentrations tested (Fig. 1). Any primer set behaves differently, and a narrower concentration range (for example 1, 1.5, 2, 2.5, 3 mM) can be

tested, if necessary. As a matter of fact we have come across primer sequences that gave optimal amplification at 1.5 mM but no amplification at all at 1 nor at 2 mM MgCl₂ (21). The calculated T_ms differ according to the formula chosen for its calculation and number of different formulas have actually been described in the literature. We normally refer to the T_m calculated by the program Primer 3 (see Materials and Methods) or to the T_m calculated by Amersham Pharmacia Biotech and provided with each oligonucleotide data sheet. The actual annealing temperature (T_{ann}) utilized in the PCR can be up to 2-5°C higher than the calculated T_m. Alternatively, we referred to the formula described by

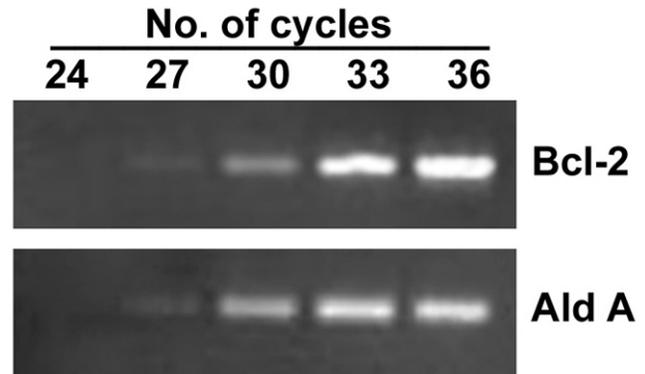


Fig. 2: Determination of the exponential range of amplification for Bcl-2 and Aldolase A. The analysis was performed in 1 mM MgCl₂ at 65°C. The intensity of the Bcl-2 amplification product increased up to 36 cycles, whereas Aldolase A(Ald A) reached a plateau at 33 cycles.

Sambrook *et al.* (9): $T_m = 58 + 0.4 * (\% G + C) - 500/l$, in which *l* is the oligo length. In this case, the optimal T_{ann} was normally about 3-5°C higher than the calculated T_m. Only in a few particular cases we found it necessary to test different temperatures (slightly above and below the calculated T_{ann}).

Another parameter to be analyzed thoroughly is the number of amplification cycles to perform. It is not sufficient to visualize the amplification product on a gel. It is well known that amplification is initially exponential but reaches a plateau when the activity of the enzyme declines and when any of the reagents become limiting in the reaction. At plateau, RNAs initially present at high levels may give products of equal intensity to low abundant RNAs. In our experiments we tested a number of cycles ranging from 24 to 36 (Fig 2). Fig. 2 shows that whereas Bcl-2 intensity increased up to 36 cycles, no increase could be seen in Aldolase A at more than 33 cycles, i.e. it had already reached a plateau. Another problem that we have often encountered is non-predictable cross-hybridization that occurs when two or more primer sets are enclosed in the reaction.

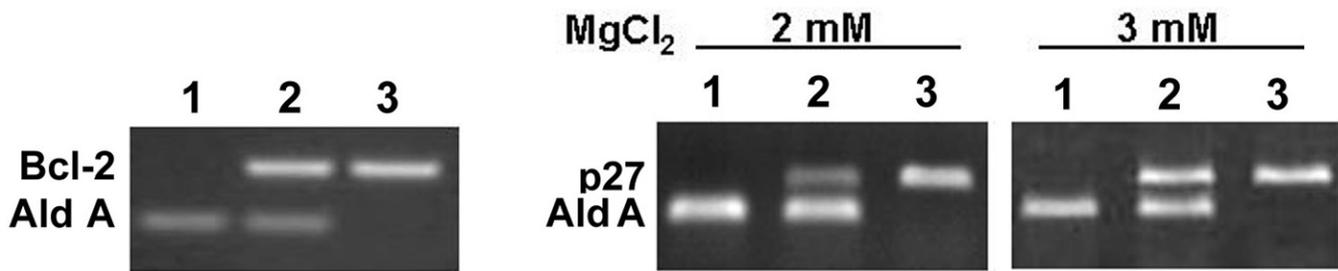


Fig. 3: Control for competition between different primer sets. Reactions were performed in the same conditions for lanes 1, 2 and 3. Lane 1 contained the Aldolase A primer set only, lane 3 the Specific primers for the target RNA, and lane 2 both primer sets. In the selected conditions (1 mM MgCl₂, 65°C, 30 cycles) Bcl-2 and Aldolase A did not compete (left panel). The middle panel shows an example in which the specific primers for the target RNA (p27) competed with the Aldolase A primers (left, 2 mM MgCl₂). The right panel shows the same samples as in the middle panel: substitution of 2 mM with 3 mM MgCl₂, eliminated competition, i.e. the intensity of the p27 band did not change when the Aldolase A primers were included in the reaction (lane 2). Ald A, Aldolase A.

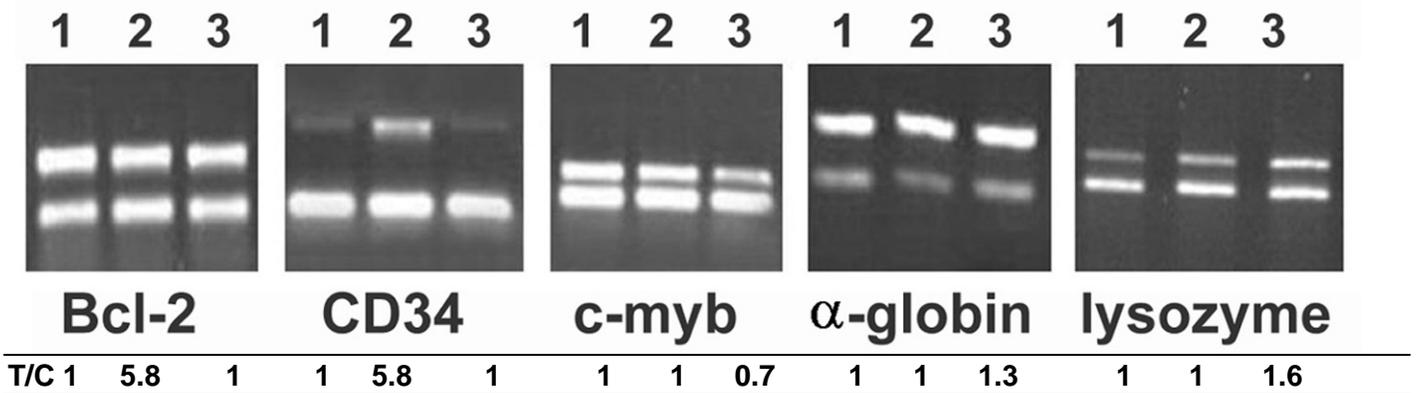


Fig. 4: Example of analyses performed on different target RNAs in the same set of samples: effect of TGF-β1 on TF-1 cells. Lane 1 is control TF-1 cells, lane 2 treatment with TGF-β1, lane 3 treatment with anti-TGF-β1. In all panels, the upper band is the target and the lower band is the amplification product of Aldolase A. T/C indicates the relative level of the target amplification product (T) over the Aldolase A internal control (C) after normalization to the control sample, i.e. it expresses the change in folds with respect to the untreated control (lane 1).

Quantitation has to be performed on the individual amplification products obtained with single primer sets, and only if the amplification products obtained with each separate primer set had the same intensity as that obtained with both primer sets together, were the conditions considered as optimal (Fig. 3, left panel). Otherwise, when competition showed as a decrease in intensity of the target RNA in the presence of Aldolase A (Fig. 3, right panel), different reaction conditions were tested (in terms either of MgCl₂ concentration or annealing temperature, or both, see above). We show in Fig. 3 how Bcl-2 and Aldolase A did not compete when combined at 30 cycles in 1 mM MgCl₂. On the other hand, we show as an example that p27 primers did compete with Aldolase A, and that the amplification product for p27 was greatly reduced in the presence of the Aldolase A primers when the reaction was performed in 2 mM MgCl₂. When the MgCl₂ concentration was increased to 3 mM competition was completely eliminated (Fig. 3, right panel). We have seen so far that a number of parameters are very critical in performing a duplex PCR reaction in condition such that the amplification is roughly

quantitative. As a consequence, the probability that more than two primer sets can be optimized in identical reaction conditions is very low. Therefore, it may be very cumbersome to establish optimal reaction conditions for multiple sets of primers at the same time (multiplex PCR). It may be easier and safer to perform separate reactions for each target RNA. When reaction conditions are optimized, quantification becomes reliable (22,23). When performing reactions on different sets of samples in the same experimental conditions, variability after normalization to Aldolase A was never greater than 15% in our hands. In our case, when working with the smallest number of cells (10,000) we could only perform at most 5 PCR reactions. When working with larger samples, the number of target RNAs that can be analyzed is almost unlimited. We show in Fig. 4 an example of RT-PCR analysis performed on the erythroleukemia cell line TF-1 in different experimental conditions. We have previously shown that TGF-β1 has anti-differentiating properties in hematopoietic progenitors (4). We measured the RNA levels of some common markers of differentiation of hematopoietic cells (Bcl-2, CD34, c-myb, α-

globin, and lysozyme) following exposure to TGF- β 1 or anti-TGF- β 1. TF-1 cells were exposed to 5 ng/ml TGF- β 1 or to 20 μ g/ml anti-TGF- β 1 blocking antibodies for 48 hours (24). In this example Bcl-2 levels did not change significantly and CD34 increased by 5.8-fold in the presence of TGF- β 1, indicating a shift of the cells to a more primordial state. c-myc decreased to 0.7-fold, α -globin increased by 1.3-fold and lysozyme by 1.6-fold when TGF- β 1 was depleted with blocking antibodies. These values are the mean of three independent experiments, in which the deviation was always comprised between 12 and 15 %. When the calculated value (T/C, in the figure) was different from the corresponding control by less than 20%, it was considered as equal to 1.

Our experiments confirmed that semiquantitative RT-PCR provides reliable information as long as the proper controls are all performed correctly. The protocols we have described have been reproduced on different types of samples and different target RNAs and some of the analyses were also confirmed by Western blotting or by flow cytometry and produced analogous information (4,5,6,24).

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