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Genome Res. 1992 1: 283-285
Access the most recent version at doi:10.1101/gr.1.4.283

Analysis of Gene Expression: Use of Oligonucleotide Primers for Glyceraldehyde-3-Phosphate Dehydrogenase

G.S. Dveksler, A.A. Basile, and C.W. Dieffenbach

Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

Analysis of gene expression using RNAPCR is a valuable technique for determining the presence or absence of a particular mRNA. The use of a well-characterized internal control in the PCR amplification procedure provides a standard on which sample-to-sample comparisons can be made.[1] By using this internal control, meaningful information regarding gene expression can be obtained easily by RNAPCR. For this purpose, we have developed a set of PCR primers that amplify the cDNA for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) from at least four species (Fig, 1); and this primer set has been used extensively as an internal control in many cell systems.[2–10] RNAPCR can be made quantitative if coamplification of an artificially made RNA containing the sequence of the primers corresponding to the message of interest is achieved and different cycles and amount of the internal control message are used.[11,12] Although this method is the most accurate, it is very time consuming.

When relative levels of expression of a certain mRNA are desired, coamplification of GAPDH is an alternative approach. The GAPDH message is a good choice for an internal control because its level in tissue culture cells is independent of the degree of confluency of the culture (Fig. 2) and is constant upon a variety of different treatments. GAPDH has proven to be a better internal control than actin and is widely accepted for this purpose on Northern blots, RNase protection, and in nuclear run-on experiments.[13,14] The only system in which GAPDH will not serve as an internal control is adipocyte differentiation where GAPDH is transcriptionally regulated.[15]

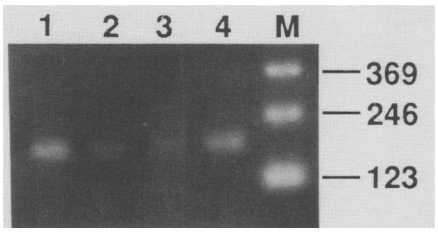

FIGURE 1 Demonstration of utility of GAPDH primers in different species. Reactions for mRNA phenotyping using 1 μg of total cellular RNA from human fibroblast (lane *1*) mouse L929 (lane *2*) rat fibroblast (lane *3*), and chicken epithelium (lane *4*) were performed as follows: 400 ng of antisense GAPDH primer and RNase-free water plus each RNA preparation were mixed and heated to 70°C for 10 min then chilled on ice. Following brief centrifugation, Moloney murine leukemia virus reverse transcriptase (MMLVRT) buffer (1x buffer is 50 mM Tris.Cl, pH 8.3, 50 mM KCl, 10 mM DTT) and 0.8 mM nucleotides was added to each tube. Then 250 units of MMLVRT was added to the reactions (final volume 25 μl) on ice, then incubated at 37°C for 30 min. The MMLVRT reaction was terminated by boiling for 10 min. The PCR reaction was set by addition of a master mix containing 400 ng of sense and antisense GAPDN primers, additional nucleotides (200 μM or 0.2 mM), *Taq* polymerase buffer (1x buffer is 50 mM KCl, 20 mM Tris•Cl, pH 8.45, 1.5 mM $MgCl_2$, 0.01% gelatin) and 2 units of *Taq* polymerase. The 100-μl reactions were overlaid with 150 μl of mineral oil and run for 20 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min. The photograph shows the stained gel using 10% of the total PCR reaction.

Due to the presence of GAPDH pseudogenes in the mammalian genome,[16] special precautions have to be taken to be assured that the GAPDH product is the result of reverse transcription of the GAPDH message fol-

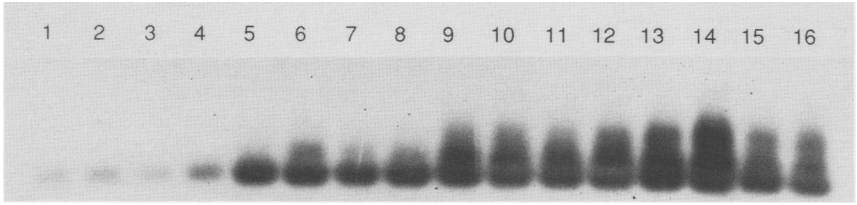

FIGURE 2 Amplification of GAPDH from RNA prepared from the mouse cell line JLSV9 at different stages of cell growth. Total RNA was prepared form the cells that were in log phase (lanes *1*, *5*, *9*, *13*), semiconfluent (lanes *2*, *6*, *10*, *14*), confluent (lanes *3*, *7*, *11*, *15*), and over confluent (lanes *4*, *8*, *12*, *16*). The RNAPCR was performed as indicated in Fig. 1 for different cycles. (Lanes *1-4*) Result of hybridization of the Southern blot with the ^{32}P end-labeled GAPDH probe of the products obtained after 15 cycles of amplification; (lanes *5–8*) 20 cycles; (lanes *9–12*) 25 cycles; (lanes *13–16*) product of 30 cycles of amplification.

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TABLE 1 Primer Sequences and Positions in Published GAPDH cDNAs

Primer	Sequence	Position
Sense primer	5′-CCATGGAGAAGGCTGGGG	388–405[16] 334–351[18]
Antisense primer	5′-CAAAGTTGTCATGGATGACC	582–563[16] 528–509[18]
Mammalian probe	5′-CTAAGCAGTTGGTGGTGCA	549–531[16]
Chicken probe	5′-CCTGAAAATTGTCAGCAATGC	452–472[18]

lowed by amplification. We routinely perform two control reactions to validate the results of our experiments (data not shown). The first control reaction omits reverse transcriptase in the cDNA reaction and the difference in volume is compensated by the addition of H_2O. Any GAPDH product obtained in this reaction will then be explained by the presence of contaminating DNA in the RNA preparation. For the second control reaction, H_2O is added instead of template RNA. The no-template control is very important to demonstrate the absence of contamination with GAPDH PCR product from previous reactions or plasmids containing the GAPDH cDNA in the laboratory. Table 1 lists the sequences of the primers, including a chicken-specific probe. Maier et al. has recently published a human-specific GAPDH RNAPCR primer set.[17]

GAPDH coamplification can be performed two ways (Fig. 3). First we have directly coamplified GAPDH along with specific cytokines and growth factors (ref. 2, data not shown). This protocol works well when the mRNA of interest is expressed at levels similar to the GAPDH control. A second approach employed to obtain the results presented in Figure 4 is to divide the reaction into two parts following the reverse transcription step.[4–6,8–10] This approach is necessary when the mRNA of interest and GAPDH mRNA are present at widely different levels. When the cDNA reaction is divided for further amplification, the specific antisense primers or random primers can be employed to prime the cDNA synthesis by reverse transcriptase and each cDNA product can be amplified for different numbers of cycles with its specific set of primers. In this way, each product assayed can be maintained within the linear range of amplification so that semiquantitative comparisons can be made. The linear range is achieved for the GAPDH amplification usually between 15 and 20 cycles, as shown in Figure 2, while the amplification-detection for the message of interest may require additional cycles.[4,8] It is important to notice that additional bands appear upon hybridization of the PCR product when GAPDH is overamplified. The linear range for a particular mRNA can be determined empirically upon amplification by first varying the cycle number, followed by densitometry scanning of the hybridization signal obtained upon probing of the PCR product with a ^{32}P-labeled oligonucleotide probe that hybridizes to a sequence between the PCR primers.

This brief report cites the utility of coamplification of the GAPDH mRNA as an internal control for RNAPCR. Amplification of GAPDH cDNA is a logical choice because the level of expression of this message is constant within each cell and tissue and is ubiquitous.

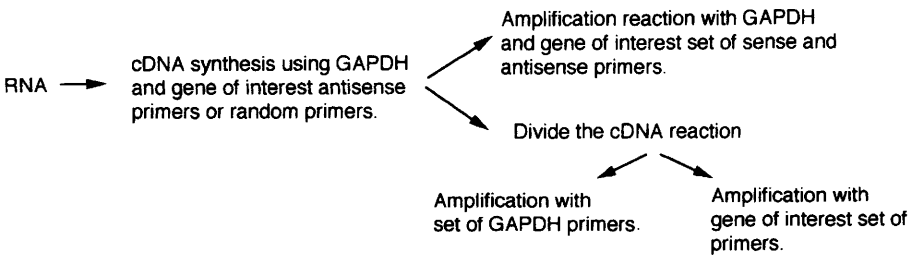

FIGURE 3 Alternative coamplification strategies for the use of GAPDH as an internal control.

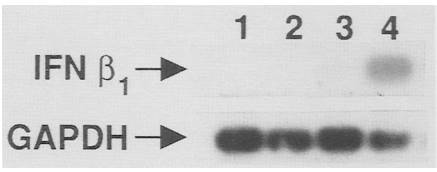

FIGURE 4 Use of GAPDH as an internal control for the amplification of human interferon-β (IFN-β). Because GAPDH was amplified successfully in all reactions to approximately the same extent, the lack of an IFN-β signal arising from mRNA isolated from untreated and TNF treated HeLa cells reflects the absence of IFN-β induction by TNF. Additionally, the differences in IFN-β signal in HEp-2 cells (lanes *3* and *4*) accurately reflect the relative difference arising from TNF treatment, as previously reported.[19]

ACKNOWLEDGMENT

The opinions expressed here are those of the authors and do not necessarily reflect the views of the Department of Defense.

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Received December 9, 1991; accepted in revised form February 25, 1992.