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# Similar sequence-free amplification of human glyceraldehyde-3-phosphate dehydrogenase for real time RT-PCR applications

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**Abstract**

One of the major applications of real time polymerase chain reaction (PCR) is relative quantification, where the expression of a target gene is determined as a ratio to a stably expressed reference gene, the so-called housekeeping gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPD) is a glycolytic enzyme, which is active in all mammalian tissues and is frequently used as housekeeping gene in expression studies. The functional locus maps to human chromosome 12p13, but several GAPD-related sequences, including processed pseudogenes, GenBank homologous sequences and computationally predicted sequences are present along the human genome. Due to the high level of GAPD-related sequences it is very important to avoid genomic DNA amplification when GAPD is used as endogenous control in mRNA quantification. We have outlined a GAPD couple of primers that avoid any genomic DNA amplification for real time reverse transcription PCR applications by SYBR-Green Dye. These new designed primers are an useful and chip alternative to probe technologies, and can carry out specific and reproducible data in mRNA expression studies.


*Keywords:* Glyceraldehyde-3-phosphate dehydrogenase; Pseudogene; Gene expression; Real time PCR; Housekeeping gene

## 1. Introduction

Reverse transcription (RT)-polymerase chain reaction (PCR) is a high sensitive method for detection of mRNA (often obtained from small tissue samples). Various methods are available to investigate low tissue specific expression: compared to classical competitive RT-PCR or RT-PCR with dot blot analysis, real time RT-PCR assay is increasingly used for its high sensitivity and wide quantification range. It has also simplified the problems connected with reproducibility, because it uses an internal control gene for normalization of the mRNA quantification [1]. However, the high sensitivity of this tool requires a particular attention on data normalization and processing [2]. It is important that the chosen control gene is stably expressed in both processed and unprocessed samples. In general more than one gene has to be tested before to find a satisfactory reference for a specific work, and it could be useful to use more than one reference to perform a good normalization with stably expressed control genes [3]. For many genes, genomic DNA (gDNA) sequences exist, named 'processed pseudogenes' [4]. They are sequences present in the genome that are identical, or nearly identical, to the mRNA sequence, and typically contain a poly(A)3′ end [5]. Processed pseudogenes lack promoters and introns, which renders the design of a couple of primers able to distinguish between mRNA and gDNA pseudogenes, extraordinarily challenging [6].

GAPD is one of the more used housekeeping genes, and there is evidence of a stable expression for GAPD mRNA [7]. However, some criticisms were moved because GAPD displays variations in the levels of mRNA transcription [8] and its expression may be differentially regulated under some pathological and experimental conditions [9,10]. Numerous GAPD pseudogenes and GAPD-like sequences have been described within the human genome [5,6].

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A GAPD couple of primers has been reported with an amplicon preventing amplification of X-linked pseudogene (GenBank accession no. X01111) [11]. Subsequently, a set of primers for a pseudogene-free amplification of GAPD has been published, but the analysis of the similar sequences was only restricted to some GAPD pseudogenes [12].

In our study, we tested the GAPD mRNA and its similar sequences in order to design a couple of primers, able to amplify only cDNA and avoiding gDNA, that can be used as internal control for the quantification of gene expression in real time RT-PCR applications.

## 2. Material and methods

### 2.1. GAPD primer design

GAPD mRNA sequence (GeneBank accession no. NM_002046) was analyzed with nucleotide–nucleotide BLAST against the non-redundant database available at the NCBI website. Attention has been focused on 72 sequences of the search results, including 11 designated pseudogenes, 57 GenBank similar sequences and four computationally predicted sequences, which were analyzed with Genome Browser (http://genome.ucsc.edu/) and Map Viewer tools (http://www.ncbi.nlm.nih.gov/mapview/). The analysis has been carried out into divergent regions to make a primer set able to amplify mRNA sequences and avoiding any genomic similar sequence. In particular the 5′-UTR of GAPD mRNA, when present, had a region (from 40 to 68 bp) highly divergent from GAPD-related sequences. A couple of primers, named A-69, has been selected into this 5′-UTR with an amplicon of 69 bp and the following sequences: forward primer 5′-CTC-TCT-GCT-CCT-CCT-GTT-CGA-C-3′ and reverse primer 5′-TGA-GCG-ATG-TGG-CTC-GGC-T-3′. A blast for short, nearly exact matches available at the NCBI website to test the specificity of the primers, has been performed. The couple of primers did not reveal matches with sequences different from GAPD mRNA. Table 1 shows the sequence and positions of binding of each of the A-69 primers to genomic sequences showing that the homology is not complete. The forward primer has a total match on two genomic sequence of chromosome 22q13 (pseudogene GeneBank accession no. AK128367) and on chromosome 15q22 (GeneBank accession no. AC090543), but no similarity was found for reverse primer in those regions. Reverse primer was designed without similarity to other regions outside the GAPD mRNA. In order to avoid the GAPD gDNA (GeneBank accession no. J04038) amplification, the reverse primer, has been designed into intron–exon boundary. In addition, another couple of primers, named B-137 (forward primer 5′-GAA-GGC-TGG-GGC-TCA-TTT-3′ and reverse primer 5′-CAG-GAG-GCA-TTG-CTG-ATG-AT-3′), has been designed; both primers spann intron–exon boundary, so that they avoid GAPD gDNA, but not pseudogene and similar GAPD mRNA regions. The PCR primers for GAPD were synthesized commercially (MWG Biotech AG, Florence, Italy) and were designed using the online PrimerQuest software of IDT Corporation (www.idtdna.com). All analyzed sequences were obtained from NCBI (National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov).

### 2.2. Genomic DNA and total RNA extraction

gDNA was extracted from human whole blood (10 mL) by use of a salting-out procedure (Miller et al. 1988 [13]; modified).

Total RNA extraction from 30 mg of human adrenal-cortex tissue was performed with the SV Total RNA Isolation System (Promega Corporation, Madison, WI) according to the manufacturer protocol. Each sample was treated with DNAse contained into the kit.

### 2.3. Reverse transcription

One microgram of total RNA was used dissolved in 20 μL of final volume reaction mixture. Briefly, oligo dT (PE Biosystems, Warrington, UK) and RNA target, incubated at 70 °C for 15 min and at 4 °C for 5 min, were added to the reaction mixture, which contained the following components: 0.5 mM dNTPs (Deoxynucleotide-Ser, SIGMA, Milan, Italy), 3 mM $MgCl_2$, 1U/μL ribonuclease inhibitor, ImProm-II Reaction Buffer and ImProm-II Reverse Transcriptase (Promega). The mixture was incubated at 25 °C for 15 min, at 42 °C for 60 min, and at 70 °C for 15 min to inactivate reverse transcriptase.

### 2.4. Real time PCR

Real time PCR was performed using a ICycler iQ detection system (Bio-Rad Laboratories, Milan, Italy). Reactions were performed in a 25 μL of final volume reaction mixture, containing 800 nM of specific primers, 12.5 μL iQ SYBR Green Supermix (Bio-Rad), and 2 μL of reverse transcription reaction solution or 0.1 μg of gDNA. The protocol used was: denaturation program (95 °C for 3 min), 45 cycles of two steps amplification (95 °C for 15 s and 60 °C for 30 s), and melting curve (60–90 °C with a heating rate of 0.5 °C/10 s).

### 2.5. Analysis of PCR products

The specificity of amplification was tested with a 3.5% agarose gel (SeaKem LE agarose; FMC BioProducts, Rockland, MD), and with real time PCR melting analysis by iCycler iQ software 3.0 (Bio-Rad).

Table 1
Homologies between the couple of primers A-69 and whole human sequences after BLAST search

| Accession Number | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|
| **NM_002046.2** | **ctctctgctcctcctgttcgac** | **agccgagccacatcgctca** |
| AC114496.2 | ctctctgctcctcctattcgac | agccg-----catccctaa |
| AL359553.14 | | agccacatccctga |
| AC016995.7 | | agccacatc-ctga |
| AC073358.14 | | agccacatccctga |
| BX648936.1 | ctctctgctcctcccgttcgac | agctg-----catccccaa |
| NG_003027.1 | tctctgctcctcccgttcgac | agccg-----catccctga |
| AL133475.14 | ctctctgctcctcctgtttgac | aacc-----acatccctga |
| AL035604.16 | | gccacatccctga |
| AL627443.6 | ctctttcctcctccgattcagc | agcc-----acatccctga |
| AC096553.1 | ctctctgctcctccagtttgac | attgg-----catccctga |
| AC015998.8 | tctctgctcctcctgtttgac | |
| AP003120.4 | tctgctcctcccatttgac | agcct-----catccctga |
| AC025264.16 | ctctctgctcctcccatttgac | agctg-----catccctga |
| AL596092.8 | ctgcttctcctgttcgac | agccg-----catccctga |
| AL391122.9 | tctctgcttctcctgtttgac | agctgtg-----tccctga |
| AC090543.12 | tctctgctcctcctgttcgac | agctg-----catccctga |
| AC084794.3 | ctctctgctcctcccattcgac | |
| AC020947.6 | ctctctgctcctcccatttgac | |
| AC011551.3 | ctctctgctcctcccgttcaac | agccacgt-acgtccctga |
| AK128367.1 | ctctctgctcctcctgttcgac | aattgcg-----tccctga |
| X01111.1 | ctctctgctcctcctgttctac | agccaaaccacatccctga |
| AL139811.30 | ctctctgctcctcttgttcgac | agccg-----catccatga |
| NG_002924.1 | ctctctgctcctcccgtttgac | -----agttacatccctga |
| XM_351282.1 | | agccatatccctga |
| XM_294070.2 | | agccacatccctga |

## 3. Results

The couple of primer B-137 gave a 137 bp product using both gDNA and cDNA templates (Fig. 1a). Real time PCR showed similar Ct and melting points for cDNA and gDNA (Fig. 1b, c). The couple of primers A-69 gave a product of the expected size (69 bp) with cDNA as template (Fig. 2a). Real time PCR showed specific Ct (Fig. 2b). Melting analysis was specific because it showed a single melt peak without primer dimer or elongation products. Melting temperature of GAPD products was 86 °C (Fig. 2c). No results were obtained when the template was gDNA: after 35 cycles there were some non-specific Ct values (Fig. 2b) and no specific melting peaks (Fig. 2c). In gel, non-specific bands, having nothing in common with the specific products of 69 bp were observed (Fig. 2a).

In order to test robustness of A-69 RT-PCR conditions in different patterns of mRNA expression, an experiment with

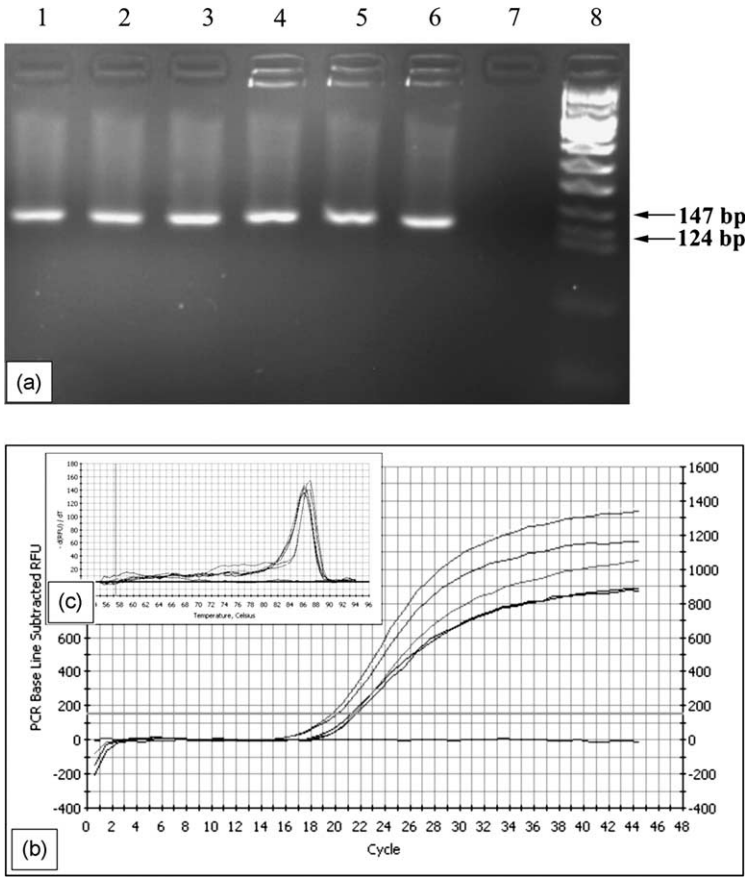


Fig. 1. (a) Pseudogene amplification using primers B-137. Lanes 1–3 show PCR products from human adrenal cortex cDNA, and lanes 4–6 PCR products from human whole blood gDNA. Lanes 7 and 8 show negative control and the ladder (Roche Diagnostics GmbH, Mannheim, Germany), respectively. (b) Real time PCR plots performed with cDNA and gDNA. (c) Melting point plots of the 137 bp PCR products.

some different tissues and cell lines was carried out: cDNA from periurethral and peripheral prostate gland, adrenal gland, HUVEC and HEK293 cell lines have showed specific melting peaks. Negative control reactions, on the other hand, gave no melting peaks or non-specific melting peaks after 35 cycles.

Primers was tested in real time PCR by performing a 10-fold serial dilution (from 0.01 to 100 ng) and a standard curve was obtained with a 0.99 correlation coefficient and a 96.7% efficiency.

## 4. Discussion

Housekeeping genes are a large group of genes that code for proteins whose activities are essential for the maintenance of cell function. Due to the similar and essential role of these genes for cell viability, it was generally assumed that they are expressed at similar levels in different cell types. In particular, the gene chosen as control must be stably expressed in treated and untreated samples in order to enable the comparison of signals and to normalize variations and pipetting errors from sample to sample [2]. However, various studies have recently shown that the expression level of housekeeping genes is actively regulated and may vary among different cells types. Hence, for relative quantification, one must choose a housekeeping gene that fits into the investigated system. β-Actin, HPRT1, 18S rRNA, cyclophillin, β-2 microglobulin and GAPD are usually used as reference genes, but unfortunately some of them (e.g. β-Actin and GAPD), have pseudogenes [14,15]. Processed pseudogenes are created by endogenous RT activity. They are very similar to their closest corresponding human gene, being almost complete in coding regions. Their chromosomal distribution appears random and dispersed [16]. Therefore, the use of these housekeeping genes as internal standard in RT-PCR, requires a careful selection of primers to distinguish between mRNA and gDNA. Contamination of RNA sample with gDNA might interfere in the experimental protocol and prejudice the estimation of the efficiency of the RT step. RNA samples must be treated with RNAse-free DNAse before PCR analysis to prevent contamination. Some commercial RNA isolation kits allow DNAse treatment on column, minimizing losses of RNA material. Furthermore, this procedure may be unsuitable for the presence of residual RNAse into DNAse that may degrade the sample or affect his storage. Hence, some investigators prefer to use DNAse only when the primers or

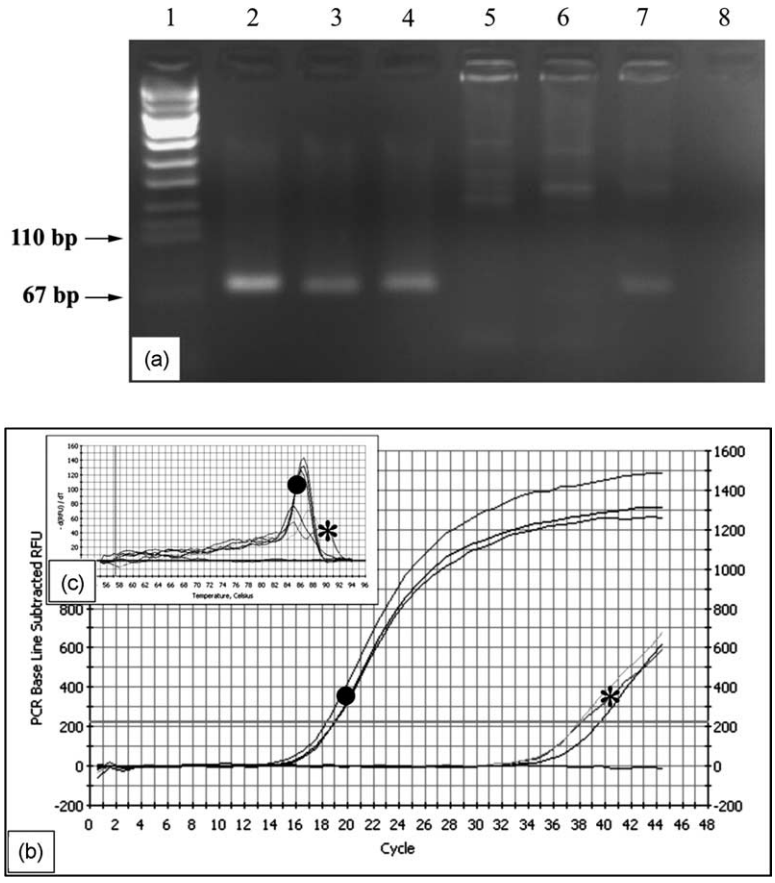


Fig. 2. (a) Pseudogene-free amplification using primers A-69. Lanes 2–4 show the PCR products from human adrenal cortex cDNA, and lanes 5–7 the PCR products from human whole blood gDNA. Lanes 1 and 8 show the ladder and negative control, respectively. The couple of primers A-69 shows a specific amplification of cDNA. (b) Real time plots performed with cDNA (●) and gDNA (*). A late Ct (after 35 cycles) for gDNA is show. (c): Specific melting point plots of cDNA products (●) and non-specific melting point plots (*) of gDNA products.

probes cannot prevent genomic amplification [17]. Moreover, in the case of laser-microdissected and paraffin-embedded derived tissues, where cDNA synthesis has to be performed using very low mRNA amounts [18,19], the opportunity to avoid DNAse-treatment step can preserve from the loss of precious material.

Here, we have studied human GAPD sequence and investigated a wide range of similar and highly homologous and computationally predicted RefSeq sequences. We designed a couple of primers that avoid GAPD pseudogene amplification. As mentioned in the introduction, some investigators have previously tried to find primers useful to avoid pseudogene amplifications [11,12]. Anyway, the analysis of GAPD similar sequences was partial and the designed primers were useful only for conventional RT-PCR, but not optimized for real time RT-PCR. For this specific application, the amplicon for the PCR product should be as small as reasonably possible, 70–150 bp for hybridization probes and less than 300 bp for SYBR-Green assay [2]. At present, the use of human GAPD as reference gene in real time RT-PCR avoiding genomic amplification can be performed with probe technologies. Applications with commercially available GAPD primers to SYBR-Green Dye are possible with good DNAse-treatment. However, the complete removal of contaminating DNA from RNA cannot be guaranteed. The herein described set of primers (A-69) allows the selective amplification of human GAPD cDNA, avoiding any similar sequence. In addition, they ensure reliable and accurate expression studies, whenever GAPD is used as internal reference.

## References

[1] Klein D. Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 2002;8:257–60.
[2] Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 2002;30:503–12.
[3] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3 [RESEARCH0034].
[4] Hurteau GJ, Spivack SD. mRNA-specific reverse transcription-polymerase chain reaction from human tissue extracts. Anal Biochem 2002;307:304–15.
[5] Mighell AJ, Smith NR, Robinson PA, Markham AF. Vertebrate pseudogenes. FEBS Lett 2000;468:109–14.

[6] Benham FJ, Povey S. Members of the human glyceraldehyde-3-phosphate dehydrogenase-related gene family map to dispersed chromosomal locations. Genomics 1989;5:209–14.
[7] Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, et al. Housekeeping genes as internal standards: use and limits. J Biotechnol 1999;75:291–5.
[8] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169–93.
[9] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 2000;46:69–81.
[10] Schmid H, Cohen CD, Henger A, Irrgang S, Schlondorff D, Kretzler M. Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. Kidney Int 2003;64: 356–60.
[11] Lehmann MH, Weber J, Gastmann O, Sigusch HH. Pseudogene-free amplification of human GAPD cDNA. Biotechniques 2002;33: 766–70.
[12] Harper LV, Hilton AC, Jones AF. RT-PCR for the pseudogene-free amplification of the glyceraldehyde-3-phosphate dehydrogenase gene (gapd). Mol Cell Probes 2003;17:261–5.
[13] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res 1988; 16:215.
[14] Dirnhofer S, Berger C, Untergasser G, Geley S, Berger P. Human beta-actin retropseudogenes interfere with RT-PCR. Trends Genet 1995;11:380–1.
[15] Garcia-Meunier P, Etienne-Julan M, Fort P, Piechaczyk M, Bonhomme F. Concerted evolution in the GAPD family of retro-transposed pseudogenes. Mamm Genome 1993;4:695–703.
[16] Zhang Z, Harrison PM, Liu Y, Gerstein M. Millions of years of evolution preserved: a comprehensive catalog of the processed pseudogenes in the human genome. Genome Res 2003;13:2541–58.
[17] Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29:23–39.
[18] De Preter K, Vandesompele J, Heimann P, Kockx MM, Van Gele M, Hoebeeck J, et al. Application of laser capture microdissection in genetic analysis of neuroblastoma and neuroblastoma precursor cells. Cancer Lett 2003;197:53–61.
[19] Lehmann U, Bock O, Glockner S, Kreipe H. Quantitative molecular analysis of laser-microdissected paraffin-embedded human tissues. Pathobiology 2000;68:202–8.