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17 were positive in PCR.

One apparent pitfall of the technique was the fact that reactions were carried out first on the plate supernatants and then on individual plugs from the plates, suggesting the possibility of a general contamination of the plugs. We could see that it was not the case and that the contamination level was very low compared with the signal obtained with positive plugs. Therefore, this technique makes it possible to screen a phage library in about a week, without the expense of membranes, and at a high stringency using PCR.

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Pseudogene-Free Amplification of Human GAPDH cDNA

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RT-PCR has become a common method in molecular biology laboratories involved in research, diagnostics (9), and forensic medicine (4,7,10). Much effort has been spent to improve this powerful method (12). However, there is still an ongoing discussion about the reliability of the method for quantitative mRNA analysis. One reason is the possibility of amplifying contaminating DNA, which is normally easily distinguished from the cDNA PCR product by its greater size because of the presence of introns. However, if a pseudogene exists that arose because of retrotransposition of mRNA (6), then amplification of cDNA and contaminating DNA results in PCR products of the same sizes. This would lead to false-positive results or at least to an unknown error in the quantitative data. A complex method that avoids pseudogene amplification was introduced (11), but specific primers that fail to amplify related pseudogenes are rare. Housekeeping genes are used to normalize quantitative RT-PCR data. Here accurate determination of cDNA concentration is essential. Primer pairs for human and rat β -actin, which do not amplify their corresponding pseudogenes, were designed (5,8). We demonstrate the problem and then solve it for the human housekeeping gene GAPDH (1) since a corresponding pseudogene (2) exists and the GAPDH mRNA (13) is frequently used to normalize RT-PCR.

First, genomic DNA was isolated from human whole blood with the QIA-amp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). No RNase treatment was performed. The human monocytic cell line U-937 (ACC 5) obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was used as RNA source. Total RNA was isolated with the RNeasy® Mini Kit (Qiagen). No DNase treatment was performed. Subsequently, cDNA was synthesized from total RNA using the Reverse Transcription System

(Promega, Mannheim, Germany). The reaction volume of total 20 μ L contained 1 μ g RNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100, 1 mM each of four dNTPs, 5 mM MgCl₂, 20 U recombinant RNasin® ribonuclease inhibitor, 0.5 μ g Oligo(dT)₁₅ Primer, and 15 U avian myeloblastosis virus reverse transcriptase. The mixture was incubated at 42°C for 60 min and then heated at 95°C for 5 min to inactivate the reverse transcriptase.

Second, 1 μ L cDNA and 100 ng genomic DNA were amplified simultaneously under the same conditions. Initially, nucleic acids were denatured at 95°C for 3 min, amplified with 29 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 45 s, and finally extended at 72°C for 5 min in a Mastercycler® gradient (Eppendorf, Hamburg, Germany). The reaction volume of total 25 μ L contained 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μ M each of four dNTPs, 1.5 mM MgCl₂, and 0.2 U DynAzyme™ II (Biometra, Göttingen, Germany). The human GAPDH sense primer 5'-TGAAGGTCGGAGTCAA-CGGATTTGGT-3' and antisense primer 5'-CATGTGGGCCATGAGGTCC-ACCAC-3' (BD Biosciences Clontech, Heidelberg, Germany) were used at a final concentration of 200 nM. The amplification of cDNA and genomic DNA resulted in a PCR product of the same size (Figure 1). The PCR product size

Figure 1. Amplification of cDNA and genomic DNA. PCRs were performed from cDNA (lane 1) and genomic DNA (lane 2) as described using a commercially available human GAPDH primer pair for RT-PCR. Negative control (lane 3) without nucleic acid. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The gel was photographed by a Polaroid® camera. Phage X174 DNA-HaeIII digest and λ DNA-HindIII digest (Biometra) served as a molecular weight marker (M).

from cDNA amplification met expectations. The primer pair used encompasses six introns of the human GAPDH gene (GenBank® accession no. J04038) and therefore fulfilled the criterion necessary to avoid a PCR product with the same size. Additionally, this primer pair fails to amplify the human GAPDH DNA since 19 nucleotides of the sense primer hybridized to the 3'-end of exon 1 and the other 7 nucleotides to the 5'-end of exon 2. However, this did not exclude the possibility of pseudogene amplification. To define the PCR product obtained by genomic DNA amplification, a global alignment from the human GAPDH mRNA (GenBank accession no. M17851) and the X chromosome-linked human GAPDH pseudogene (GenBank accession no. X01111) was created by the program GAP (3). The analysis of the alignment revealed that (i) there is a high degree of similarity between the human GAPDH gene and its corresponding pseudogene and (ii) each of the corresponding primer binding sites on the GAPDH pseudogene had only one mismatch. Therefore, we suggest that the PCR product obtained from genomic DNA resulted at least in part from amplification of the human

GAPDH pseudogene. Consequently, the suggested human GAPDH pseudogene amplification should be prevented by using primers that bind to regions on the human GAPDH mRNA that are less conserved in the pseudogene. Such regions were identified from nucleotide numbers 1–33 and from 471–485. The program Prime® (Wisconsin Package Version 9.0; Accelrys, San Diego, CA, USA) was applied to the human GAPDH mRNA (GenBank accession no. M17851) to find a suitable primer pair that covers these regions. We found under standard conditions for this program the sense primer 5'-AGCCA-CATCGCTCAGAACAC-3' and the antisense primer 5'-GAGGCATTGCT-GATGATCTTG-3' (patent pending, DE 199 35 767 A1). According to the computer analysis, this primer pair has (i) an optimal annealing temperature of 57°C, (ii) leads to the amplification of nucleotides 12–485 of the human GAPDH mRNA transcript, and (iii) fails to amplify the human GAPDH DNA since one-half of the antisense primer hybridized to the 3'-end of exon 5 and the other half to the 5'-end of exon 6.

The newly designed human GAPDH primer pair was checked for

its capacity to amplify cDNA and genomic DNA at different annealing temperatures. PCR conditions and subsequent steps were performed as described above, except for the annealing temperature of the PCR cycle, which was set in the range from 50.6°C to 69.3°C. Even at low annealing temperatures, amplification of cDNA yielded a single PCR product that corresponded in size to the expectation from the computer-aided human GAPDH primer design. There was no cDNA amplification observed at annealing temperatures higher than 65.5°C. Identically sized products from PCR with genomic DNA were not observed even by applying an annealing temperature that was more than 6°C below the optimal, theoretical annealing temperature of 57°C. Only a very small PCR product of larger size (about 600 bp) than the cDNA PCR product was observed at an annealing temperature of 52.5°C or lower. However, this is 4.5°C below the optimal, theoretical annealing temperature, and it was not surprising that any mismatch priming could have occurred by such less stringent conditions. Moreover, as expected, no amplification of the human GAPDH gene was observed (Figure 2).

Figure 2. Exclusive amplification of the human GAPDH cDNA. (A) PCRs with cDNA (lanes 1) and genomic DNA (lanes 2) were performed with the newly designed human GAPDH primer pair. A Mastercycler gradient was used for simultaneous amplification of nucleic acids at different annealing temperatures in the range as indicated. PCR products were separated and stained, and the gel was photographed as described in Figure 1. Phage X174 DNA-*Hae*III digest and λ DNA-*Hind*III digest served as a molecular weight marker (M). (B) The plot illustrates the volume of the PCR product in relation to the annealing temperature. Here the gel picture was acquired by the GelDoc 2000™ Gel Documentation System (Bio-Rad Laboratories, München, Germany), and the volume of each PCR product was determined using the Quantity One® Quantitation Software (Bio-Rad Laboratories). The volume of the cDNA PCR product obtained with the annealing temperature of 50.6°C was set to 100. All plotted values represent the mean of three independent experiments.

Figure 3. The GAPDH RT-PCR product arises from mRNA. RT-PCR was performed with the newly designed human GAPDH primer pair, and the annealing temperature was set to the optimal value of 57°C (lanes 1–4). The following modifications were made. Before PCR, RNA was treated with a DNase-free RNase A/T1 mixture for 45 min before cDNA synthesis (lane 2), cDNA synthesis was performed without reverse transcriptase (lane 3), and no cDNA was used (lane 4). Phage X174 DNA-*Hae*III digest and λ DNA-*Hind*III digest (M). Nucleic acid fragments were separated and visualized as described in Figure 1. The gel picture was acquired with the GelDoc 2000 Gel Documentation System.

Benchmarks

To prove that the PCR product from cDNA amplification indeed derives from RNA, two additional control experiments were performed. First, RNA was treated with a DNase-free RNase A/T1 mixture (BD Biosciences, Heidelberg, Germany) before cDNA synthesis. Second, RNA was subjected to PCR without reverse transcriptase. No PCR product was obtained under either condition (Figure 3). Therefore, the PCR product from cDNA amplification indeed arises from mRNA. The identity of this PCR product was confirmed both by sequencing (MWG Biotech AG, Ebersberg, Germany) and by digestion with the restriction enzymes *HinfI*, *MvaI*, and *RsaI* (AGS, Heidelberg, Germany). The obtained fragments corresponded to the theoretical expected sizes of 163, 141, 128, and 42 bp for *HinfI*; 220, 175, and 71 bp for *MvaI*, and 302 and 172 bp for *RsaI*. Note that the 42-bp fragment from the *HinfI* digest is barely visible even on the SFR™ agarose gel (Biometra) used and the 8-bp fragment from the *MvaI* digest not at all (Figure 4).

Further, the approximately 600-bp PCR product derived from genomic DNA was sequenced, and a nucleotide BLAST search revealed 99% identity with nucleotides 31763–32362 of the human chromosome 5 clone CTB-77C3

Figure 4. Verification of the GAPDH RT-PCR product identity. RT-PCR was performed as described in Figure 3, lane 1. Afterwards, the PCR product was purified with the QIAquick™ PCR Purification Kit (Qiagen) and digested with the enzymes as indicated at 37°C for 90 min. Purified PCR product without digestion (I). Phage X174 DNA-*HaeIII* digest and λ DNA-*HindIII* digest (M). Nucleic acid fragments were separated on a 2% SFR agarose gel and visualized, and the gel was photographed as described in Figure 1.

(GenBank accession no. AC008704). This region is completely unrelated to GAPDH, but its amplification occurred since the 5'-end of the newly designed GAPDH sense primer is identical to nucleotides 31763–31777 and the 5'-end of the antisense primer match to nucleotides 32350–32362.

Hence, we show that the newly designed human GAPDH primer pair led to highly selective amplification of the human GAPDH cDNA without amplification of the X chromosome-linked GAPDH pseudogene. Therefore, these primers are useful tools for many scientists working with PCR.

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