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TECHNICAL NOTE

A Rapid and Sensitive Protocol for Competitive Reverse Transcriptase (cRT) PCR Analysis of Cellular Genes

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The specific analysis of gene transcripts is of increasing importance for studies in molecular pathology. Competitive RT-PCR with mutagenized exogenous competitor templates has evolved as an attractive approach to quantify individual mRNA levels. The generation of exogenous competitor RNAs usually requires mutagenesis and cloning of the mutant fragment into plasmids followed by in vitro transcription. In contrast to primer directed mutagenesis and in vitro transcription, preparation of the mutant fragments is a time consuming procedure. Here we report on a modified semi-quantitative RT-PCR protocol to circumvent the laborious cloning of mutant exogenous competitors. Templates for the in vitro transcription are generated in a single PCR reaction with simultaneous addition of a promoter sequence 5`of the forward primer and deletion of 10-20 nucleotides at the opposite end just ahead of the reverse primer binding site. The product of this PCR step serves as template for in vitro transcription to yield exogenous competitor RNA of equal quality and amount as conventional cloning strategies. Total RNA amounts are corrected for by analyzing the expression of different housekeeping genes in the same manner. One of the primers used in the following competitive RT-PCR reaction is labeled with a fluorescent dye for the analysis of target and exogenous competitor product on an semiautomated sequencer. In the present study, this protocol was employed to analyze the expression of the PTCH, Fas-receptor, NF-1, ß2-microglobulin and GAPD genes in human brain tumors. It will, however, be widely applicable to studies on cellular transcripts in biological specimens.

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Introduction

Quantification of specific mRNAs poses several problems to the investigator. While northern blotting is unrivaled in accuracy and reproducibility, this method requires considerable amounts of intact RNA. The development of reverse transcription PCR (RT-PCR) strategies has been a major advance. RT-PCR allows detection of low abundance mRNAs in minute tissue specimens. However, the high sensitivity and kinetic properties of PCR reactions can seriously interfere with the quantitative analysis of RT-PCR products. Therefore, the addition of exogenous competitor RNA molecules has been introduced by several RT-PCR protocols (10, 3, 2) . Exogenous competitor ribonucleotides share identical primer sequences with the targets and can be added to the reverse transcription in different concentrations. The use of these standards is based on the principle that target and competitor molecules are transcribed and amplified with similar efficiencies at all stages of the reaction.

Several techniques for the generation of exogenous competitor RNA molecules have been described (1, 9, 5). Common to most of these procedures are cloning steps which involve laborious protocols with plasmids and bacteria. In order to facilitate the generation of RT-PCR standards, we developed a simple and efficient strategy to generate mutant exogenous competitor RNAs. Our approach employs a single step of PCR mutagenesis and circumvents the handling of microorganisms. Detection of the fluorescent PCR products on a semiautomated sequencer allows their quantitative analysis. We here demonstrate the feasibility of this approach and present five examples of exogenous competitor RNAs for the patched (PTCH), beta-2-microglobulin (B2M), FAS-receptor (FASR), glycerol-

aldehyde-phosphate-dehydrogenase (GAPD) and the neurofibromatosis type 1 (NF1) genes and preliminary expression data. This procedure is suitable for the analysis of any gene product, for which primer sequence information is available.

Material and Methods

Generation of homologous exogenous competitors. Total RNA from snap frozen human tumor tissues was extracted using the Trizol™ reagent (Life Technologies, GIBCO, Eggenstein, Germany), followed by DNAse I (Boehringer Mannheim, Germany) treatment. DNAse was removed by an additional round of Trizol™ extraction. Total RNA was reverse transcribed using the Superscript™ preamplification system II (GIBCO, Eggenstein, Germany). Template cDNA product was generated by PCR with primers for the B2M, PTCH, NF1, GAPD and FASR genes (for primer sequences see Table 1). Mutagenesis and T7 promoter addition were carried out with a single PCR reaction in a final volume of 100 μl containing 5 pmol each of the T7-addition primer and the mutagenesis primer, 10 ng of the template cDNA product, 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin and 200 mM of each nucleotide. Initial denaturation at 94°C was followed by 40 cycles on a thermal cycler (ABI 9600, Foster City, USA). This PCR product was column-purified (QIAquick Spin PCR Purification kit, Quiagen, Hilden Germany). 0.5 μg mutated template DNA was in vitro transcribed using 10 U T7 RNA polymerase (Stratagene, Heidelberg, Germany) in a volume of 25 μl. DNA template was removed by addition of 100 U RNase free DNase (Boehringer Mannheim, Germany) and 40 U RNasin™ (Pharmacia, Freiburg, Germany) in a total volume of 30 μl. This mixture was purified with RNeasy Quickspin columns (Quiagen, Hilden Germany) and eluted in 30 μl DEPC water. The preparation of exogenous competitor molecules is illustrated in Figure 1.

Reverse transcription and semiquantitative PCR analysis. The concentration of the homologous exogenous competitors was determined by spectrophotometry. In order to estimate the transcription levels of target RNAs, serial dilutions of the homologous exogenous competitors ranging from 10^{-10} mg to 10^{-6} mg and 0.25 μg of total RNA from a pool of tumor tissues were reverse transcribed. Optimal titration was defined as the

B2M-f	5'-TGTCTTTCAGCAAGGACTGG
B2M-r	5'-GATGCTGCTTACATGTCTCG
B2M-T7	5'-GGATCCTAATACGACTCATATAGGGAGGTGTCTTTCAGCAAGGACTGG
B2M-mut	5'-GATGCTGCTTACATGTCTCGGCTGTGACAAAGTCACATGG
FasR-f	5'-TCCTACCTCTGGTTCTTACG
FasR-r	5'-TTCATCCCCATTGACTGTGC
FasR-T7	5'-GGATCCTAATACGACTCACTATAGGGAGGTCCTACCTCTGGTTCTTACG
FasR-mut	5'-TTCATCCCCATTGACTGTGCCTGGAGGACAGGCTTATGG
PTCH-f	5'-ACATGTACAACAGGCAGTGG
PTCH-r	5'-GCAAAGGAGGTTTACCTAGG
PTCH-T7	5'-GGATCCTAATACGACTCACTATAGGGAGGTGCCAAGGCTGTGGGCAAGG
PTCH-mut	5'-GCTTCACCACCTTCTTGATGTGGCAGGTTTTTCTAGACGG
GAPD-f	5'-TGCCAAGGCTGTGGGCAAGG
GAPD-r	5'-GCTTCACCACCTTCTTGATG
GAPD-T7	5'-GGATCCTAATACGACTCACTATAGGGAGGTGCCAAGGCTGTGGGCAAGG
GAPD-mut	5'-GCTTCACCACCTTCTTGATGTGGCAGGTTTTTCTAGACGG
NF1-f	5'-GTATCTTTCCAGCAACAGGG
NF1-r	5'-CATGTACCTGATGCCTAGTC
NF1-T7	5'-GGATCCTAATACGACTCACTATAGGGAGGGTATCTTTCCAGCAACAGGG
NF1-mut	5'-CATGTACCTGATGCCTAGTCTGGTAAGGTTAAGGCTGGAC

f= forward primer; r= reverse primer; T7= T7 promoter addition primer; mut= mutagenesis primer

Table 1. Primer sequences for mutagenesis, T7 promoter addition and PCR amplification

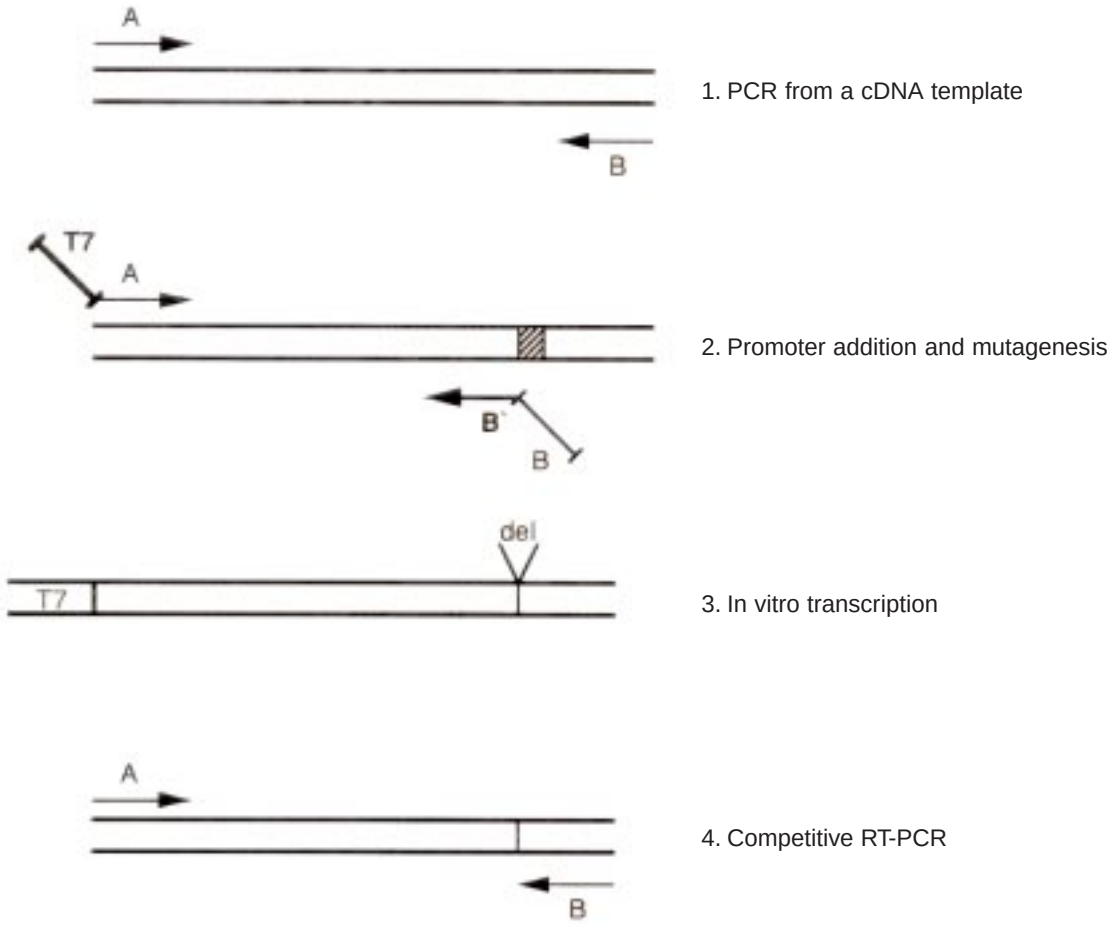

Figure 1. Production of exogenous competitors. cDNA PCR products of the respective genes were subjected to a second PCR step with specific primers for the addition of the T7 promoter 5` of the PCR product and a primer for the deletion of nucleotides ahead of the reverse primer. The T7 promoter attachment primer consists of the original sense primer with the basal T7 promoter sequence GGATCCTAATACGACTCACTATAGGGAGGT in 5` position. The mutagenesis primer consists of 20 bases that anneal ahead of the deletion site with the original reverse primer at the 5` position. In the first cycle of the PCR the complementary sequence A of the T7 promoter addition primer and the fragment B' of the mutagenesis primer anneal to the template. During the following PCR cycles both primers anneal completely to the new template and yield a product containing the T7 promoter at the 5` end of the amplicon and a small deletion ahead of the reverse primer B. Since sense and reverse primer anneal with 48 and 40 nucleotides respectively, the resulting PCR reaction is highly specific and secures precise deletion of the desired bases as determined by direct sequencing. 0.5 μg of this PCR product was used as template for the *in vitro* transcription. In the competitive RT-PCR assay the competitor molecules are amplified with the same primers and efficiency as the target transcript but yield PCR products of smaller size. A precise determination of competitor and target cRT-PCR products can thus be achieved.

point of equal signal intensity of homologous exogenous competitors and target transcript (representative data are shown in Figure 2). Extracts of total RNAs from individual tumors were then added to a mixture of homologous exogenous competitors of concentrations adjusted for the respective predetermined titration points and reverse transcribed. In order to adjust for high fluctuations of target gene expression, two reverse transcriptions with concentrations of homologous exogenous competitors above and below the optimal titration point were performed. These cDNAs were PCR amplified with fluorescent labeled primers. Amplicons were separated and analyzed on a 6% denaturing acrylamide gel on a semiautomated DNA Sequencer (ABI 373A) and the Genescan software (ver. 1.2.1).

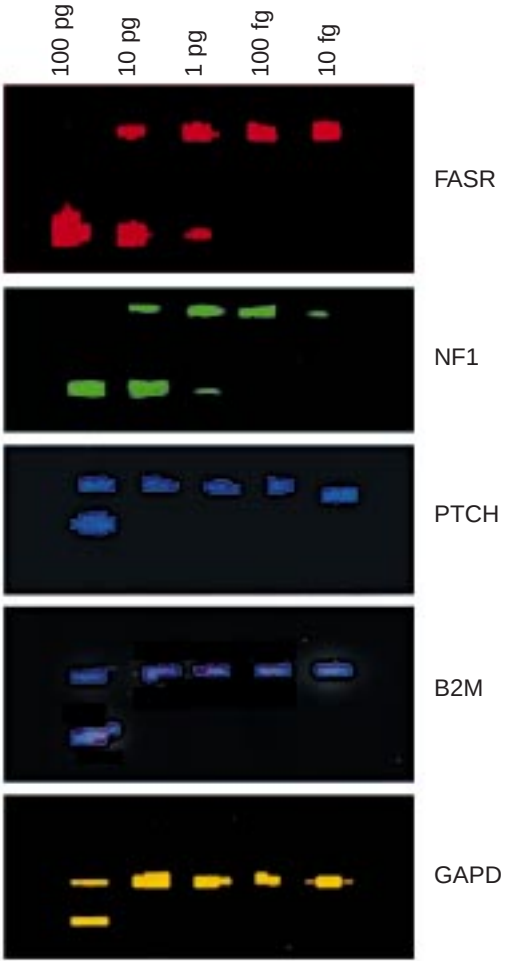

Figure 2. Titration of exogenous competitors and total input RNA.The expression of the genes FASR, GAPD, B2M, NF1 and PTCH in a pool of different tumors of the CNS was determined by titrating tumor total RNA against a serial dilution of exogenous competitors. The amounts of standard molecules were 100pg, 10pg, 1pg, 100fg, 10fg. Each competitor dilution was reverse transcribed with 250 ng of pooled total RNA.

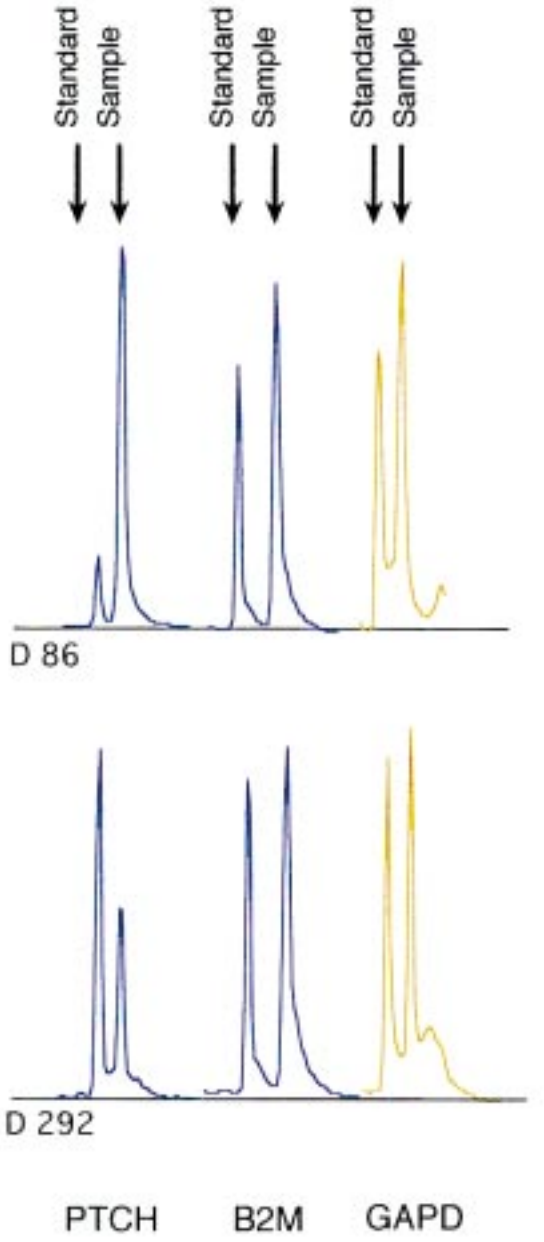

Figure 3. Signal intensities of the PTCH, GAPD and B2M RT-PCR products in two patients with medulloblastoma. Both patients show fairly constant signals for GAPD and B2M and their respective competitors. In contrast, the PTCH signal exceeds the signal for the PTCH competitor in patient D 86 (indicating high PTCH expression). In contrast, the PTCH signal in patient D 292 is lower than the signal for the PTCH competitor indicating low levels of PTCH transcript. The calculated expression levels of PTCH are 3.6 (PTCH/GAPD) and 2.6 (PTCH/B2M) for tumor D 86, and 0.6 (PTCH/GAPD) and 0.3 (PTCH/B2M) for tumor D 292.

Calculation of RNA expression levels. The ratio of target PCR product to homologous exogenous competitor PCR product was used to determine the level of gene expression. Initial differences in the amounts of tumor RNA subjected to reverse transcription were adjusted by dividing the target:target competitor product ratio by the ratio of the housekeeping gene signals to that of its respective homologous exogenous competitor. The expression levels were calculated with the following algorithm:

$$\frac{\text{target} / \text{target}_{\text{competitor}}}{\text{housekeeping} / \text{housekeeping}_{\text{competitor}}}$$

Results

RNA standards. The use of a combined mutagenesis/T7addition primer consistently resulted in high quality templates for in vitro transcription. Successful mutagenesis was verified by direct sequencing. In vitro transcription with T7 RNA polymerase yielded 2.5-5 μg of exogenous competitor RNA molecules sufficient for numerous experiments (100 pg being the maximum requirement for one assay).

Determination of the optimal titration points and RNA expression levels. In order to determine the optimal titration conditions, exogenous competitors were added to 250 ng of total RNA in amounts of 10 fg, 100 fg, 1pg, 10 pg and 100 pg. Those concentrations were chosen for subsequent assays at which the respective amount of standard template and tumor RNA yielded roughly identical signals. Using this approach, the optimal titration point was 100 pg for B2M and GAPD in astrocytic gliomas and medulloblastomas, between 10 and 1 pg for FASR and NF1 in astrocytic gliomas and 100 pg for PTCH in medulloblastomas (Fig. 2).

Expression levels of B2M, GAPD and PTCH in medulloblastoma. In order to evaluate B2M and GAPD as reference genes for brain tumors, we compared the relative expression levels of these genes. The mean ratios of GAPD/B2M readings were 0.8 +/- 0.35 indicating constant levels of transcription for B2M and GAPD in the individual tumors. In contrast to B2M and GAPD, PTCH gene expression was highly variable. Representative signals for PTCH, GAPD and B2M products are shown in Figure 3. In a series of 13 medulloblastoma, mRNA expression levels varied by a factor of 180. Similar variations in PTCH expression were detected with both GAPD and B2M as reference genes (Fig. 4).

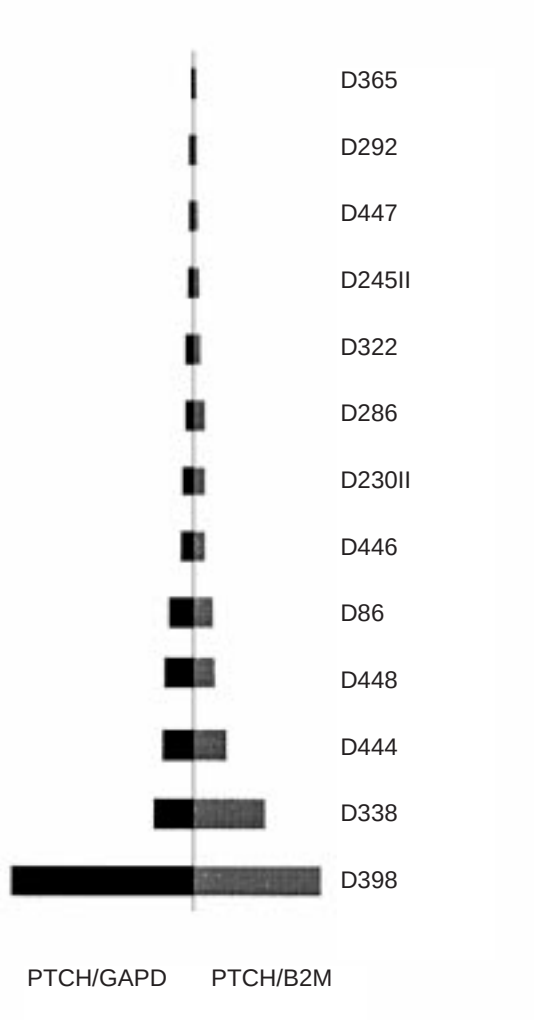

Figure 4. Relative expression levels of the PTCH gene in 13 medulloblastomas. The black and gray columns show the expression values of PTCH adjusted to GAPD and B2M housekeeping gene transcripts respectively. In this assay, tumor D398 expressed high levels of PTCH. Four tumors (D338-D86) showed intermediate and eight tumors (D446-D365) showed lower amounts of PTCH transcripts. This observation is independent of the specific housekeeping gene used to correct for differences in the initial RNA content.

Discussion

Competitive RT-PCR is likely to play an increasing role in the quantitative detection of mRNA levels in tissue samples. This method relies on the simultaneous analysis of target and suitable control transcripts. It combines the advantages of high experimental throughput, high sensitivity and requirement of small amounts of input RNA. These assays require exogenous competitor RNAs. In many protocols, the generation of exoge-

nous competitor RNAs involves a series of laborious steps including multiple rounds of PCR, nucleotide purification, subcloning into vectors and transformation of bacteria. Our modified protocol for single step synthesis of exogenous competitor templates offers several advantages. Mutagenesis and promoter addition can be achieved in a single step, subcloning procedures as well as bacterial work are no longer required. In fact, RNA competitors can be obtained in one day.

To demonstrate the feasibility and potential of this approach we examined a series of 13 medulloblastomas for expression of the PTCH gene. High levels of transcripts were detected in one, low expression in eight and intermediate expression in the remaining four tumors (4). The PTCH RT-PCR products were adjusted for different RNA amounts, by use of reference transcripts from GAPD and B2M genes. Correction for both, GAPD and B2M housekeeping genes yielded very similar results for PTCH mRNA. This experiment strongly supports the use of GAPD and B2M RNAs as appropriate reference transcripts for brain tumor specimens.

Recent studies detected significant differences in GAPD expression in human tumors (8, 7, 6). These findings challenge the use of GAPD as a universal control transcript. In order to avoid pitfalls due to variant expression of the control gene we suggest that more than one housekeeping gene transcript should be included in RT-PCR studies. The most reliable values for quantification are achieved at or close to the titration points. Major deviations to either side of the titration point may result in decreased accuracy. This implies a need for several titration steps. A precise determination of RNA levels can thus be obtained even if the expression of the target gene is highly elevated or reduced.

In summary, the simple procedure for the synthesis of mutagenized RNA standards presented in this report will considerably facilitate semi-quantitative cRT-PCR experiments. It should be particularly useful for analyzing multiple gene transcripts of low abundance in small biological specimens.

Acknowledgements

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