Quantification of mRNA by RT-competitive-PCR and high performance liquid chromatography*

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Abstract The use of RT-competitive-PCR with high performance liquid chromatography (HPLC) detection to quantify the absolute number of mRNA copies in mammalian cells is reported. As an example, the glutathione transferase (GST)-α mRNA in human hepatoma Hep G2 cells has been estimated. A PCR-generated internal standard was used as a competitor, co-amplified with the GST-α target sequence. The RT-competitive-PCR method was improved by designing target and competitor molecules which differed in only 30 base pairs. This allowed the two sequences to be co-amplified with the same efficiency. This improvement also facilitated a wider ratio to be used than previous methods (target:competitor ratio between 0.2 and 5). Products were baseline separated by HPLC using an ion-exchange column readily quantified at 260 nm. To validate the improved methodology, the effect of a known GST-α inducer, the anticancer drug oltipraz, was shown to induce GST-α mRNA up to 3-fold in Hep G2 cells. The RT-competitive PCR-HPLC method provides a reliable and sensitive way to quantify the amount of specific mRNA with 0.1 ng of total RNA.

Keywords: **RT-PCR, glutathione transferase, oltipraz, Hep G2 cell, HPLC.**

Gene expression can be studied by Northern blotting, ribonuclease protection assays, quantitative reverse transcriptase-PCR (QRT-PCR)[1,2] and real-time RT-PCR (TaqMan assay)[3]. Because of the cost of TaqMan equipment, it is not yet widely available. RT-PCR enables the amplification of specific mRNA molecules and has been shown to be about 1000 times more sensitive than the traditional RNA blotting techniques[4,5]. Although RT-PCR has many advantages over RNA blotting methods, it is difficult to obtain quantitative information due to the exponential nature of the PCR amplification, where small variations in amplification efficiency will dramatically affect the yield of products. However, this problem can be overcome by competitive RT-PCR[5~7]. In competitive PCR, a DNA fragment containing the same primer template sequences as the target competes for primer binding and amplification. The basic assumption is that target and competitive fragments are co-amplified with equal efficiencies within a defined number of cycles[8,9]. One of the advantages of using competitive PCR is that quantitative information from both exponential and nonexponential (plateau) phases of the amplification can be obtained[4,10], and it is the most reliable method for nucleic acid quantification when the amount of starting material is limited[7]. In a sample containing a fixed amount of target, spiked with a known amount of competitor, the amount of product generated reflects the initial number of target molecules; but this is only within certain folds of ratios of target to competitor[11]. In this paper, we describe the combination of RT-competitive PCR and HPLC to measure the gene expression of GST-α in human hepatoma Hep G2 cells. A PCR-generated, homogeneous internal standard was used as a GST competitor, which was only 30 bp longer than the GST target fragment. This would improve the quantification of products since the molecules were amplified with the same efficiency, within a wider range of ratios. The GST-α target and competitor PCR products were separated by HPLC on an ion-exchange, non-porous column and quantified at 260 nm. The induction of GST-α was shown by treating Hep G2 cells with oltipraz (4-methyl-5-(2-pyrazinyl)-1, 2-dithiole-3-thione), a synthetic derivative of dithiolethiones found in cruciferous vegetables originally developed as an antischistosomal agent[12] and it has been used as anticancer drug in Qidong County, China[13]. It is a proven inducer of GST protein and mRNA in mammalian cells as shown by Western and Northern blotting[14~16].

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1 Materials and methods

1.1 Chemicals and regents

Oltipraz was a gift from Dr. M. Manson, MRC Toxicology Unit (Leicester, UK). A Bio/RNA-XCell kit for isolation of RNA, a Nucleotrap kit for nucleic acids from gels and the RT-PCR kit (Calypso^{TM}) were all from Bio/Gene Ltd. (Bedfordshire, UK). AmpliTaq DNA polymerase primers and RNase inhibitor were purchased from Perkin Elmer. A Shodex IEC DNApak column was purchased from Phenomenex (Cheshire, UK).

1.2 Cell culture

The human Hep G2 hepatoma cell line was from the European Collection of Animal Cell Cultures (UK). Hep G2 cells were routinely grown in Eagle's minimal essential medium with L-glutamine, non-essential amino acids and 10% fetal calf serum (FCS) at 37℃, 5% CO_2 in air. The medium was changed twice a week, and confluent cells in 10 cm diameter dishes were sub-cultured weekly at a split ratio of 1:36 into a 3.5 cm diameter dish by treatment with 0.25% trypsin-EDTA. Normal Hep G2 cells were grown in 10% FCS medium for the first day and then changed to 5% FCS medium on the second day. On the fifth day the medium was changed to 1% FCS medium containing insulin (10 μg/mL) and transferrin (5 μg/mL). After 24 h, serum free medium was used and the cells were treated with oltipraz at a final concentration of 1 ~ 10 μmol/L. The control cells were treated with dimethyl sulfoxide at a final concentration of less than 0.5% (v/v).

1.3 Isolation of RNA and RT-competitive-PCR

Total RNA from Hep G2 cells was isolated using a Bio/RNA-XCell total RNA isolation kit according to the manufacture's instruction. Primers for measurement of gene expression and synthesis of competitor sequences were designed from the published sequences of human GST-α (GST-A1 and A2)[17]. The forward primer (nt 501~nt 519) is 5'-AGA CTA CCT TGT TGG CAA C-3' and the reverse primer (nt 602~nt 620) is 5'-TTC AGG GCC TTC AGC AGA G-3'.

A primer pair of 5'-ATA GAG ATG GAC CTG CGT CAC TAT TTC TAG AGC CGG GCT GAC ATT-3' and 5'-CTA GAA ATA GTG ACG CAG GTC CAT CTC TAT CAG CTT GTT GCC AAC-3' was used for the amplification of GST insert seqence.

The design of a PCR-generated competitor was based on a published protocol[18] with modification. The GST-α competitor was synthesised by five separate PCR reactions by using two sets of primers: GST-forward and reverse, GST-insert 1 and 2 which contained a 30 bp complementary sequence at the 5' ends for inserting a 30 bp sequence into the GST-α target. A schematic representation is shown in Fig. 1.

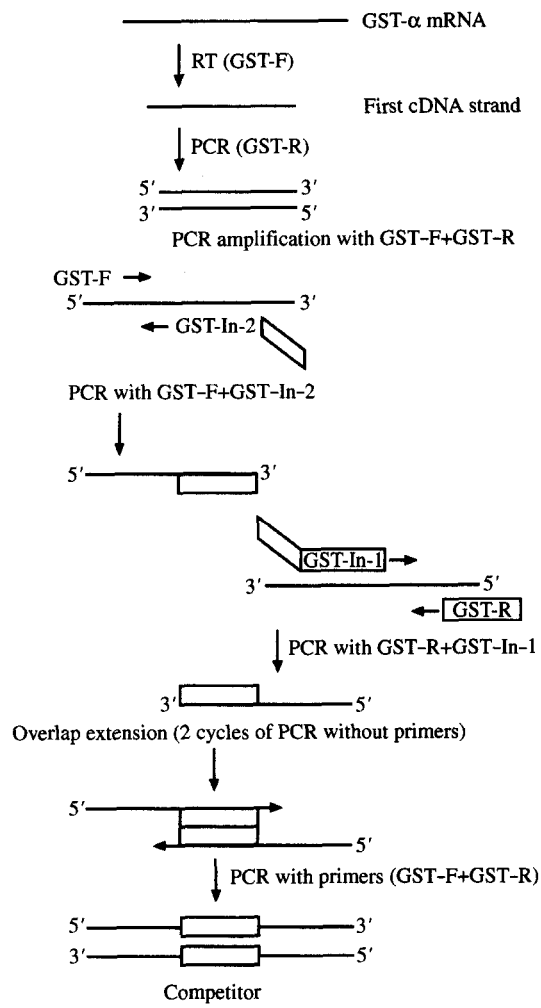

Fig. 1. Synthesis of a cDNA competitor. The scheme is designed to illustrate the insertion of a 30 bp sequence for overlap extension. Reaction 1: reverse transcription (RT) and PCR to amplify the GST-α target sequence. Reaction 2: PCR with GST-forward and Insert 2 primers. Reaction 3: PCR with GST-reverse and Insert 1 primers. Reaction 4: overlap PCR extension of products from reactions 2 and 3 (2 cycles without addition of primers). Reaction 5: PCR with GST-forward and reverse primers to generate competitor (150 bp) which is 30 bp longer than that of the GST-α target sequence (120 bp).

RT-competitive-PCR was carried out using an

RT-PCR system (Bio/Gene). Total RNA (0.1 ~ 20 ng) together with addition of competitor (0.1 ~ 100 fg) was used in RT-PCR buffer containing 0.2 mmol/L dNTPs, 0.4 μmol/L primers, 0.5 μL Accurase™ and AMV reverse transcriptase in a total volume of 50 μL. Reverse transcription was performed at 50 ℃ for 30 min, followed by an initial denaturation step at 94 ℃ for 2 min. Amplification was performed at 94 ℃ for 30 s, 50 ℃ for 30 s and 68 ℃ for 30 s for 25 ~ 32 cycles, with a final 5 min extension step at 68 ℃. A Perkin Elmer GeneAmp PCR System 2400 was used for all experiments. Negative controls were run containing no RNA template and also one sample containing every component, but only placed into the PCR thermal cycler after the reverse transcription step. The reaction products were diluted with water (1∶1, v/v), and centrifuged at 13000 g for 2 min prior to HPLC analysis. The GST-α and competitor DNA fragments were purified on a 4% agarose gel and the bands were cut out and purified by using a Nucleotrap kit (Bio/Gene). The concentration of target and competitor were determined by a spectrophotometer at 260 nm (1 OD = 50 μg/mL). The GST-α target fragment was sequenced, which was 100% identical to the published sequence[17].

1.4 PCR

The GST-α target and competitor molecules were amplified using PCR. Each reaction contained 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.3 mmol/L dNTPs, 0.4 μmol/L primers and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer). The thermal cycler conditions included an initial denaturation step at 94 ℃ for 2 min, followed by amplification at 94 ℃ for 30 s, 50 ℃ for 30 s and 72 ℃ for 30 s, for a certain number of cycles as indicated.

1.5 HPLC conditions for quantification of PCR products

The HPLC system included an autoinjector, two pumps, a UV-detector, controlled by a Gilson 715 HPLC controller. The separation of GST-α target and competitor was performed on a Shodex IEC DNApak column (6 × 50 mm) (Phenomenex, UK) with a linear gradient program for binary mobile phase as follows: buffer A contained 0.1 mol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0) and buffer B was buffer A + 1 mol/L NaCl (pH 8.0). The mobile phase was a linear gradient from 50% to 75% buffer B in 16 min, then to 50% B in 1 min and then re-equilibration for 5 min with 50% B. The flow rate was 1.0 mL/min with detection at 260 nm. The column oven temperature was set at 30 ℃. The injection volume was 20 μL.

2 Results and discussion

Most often, PCR products are separated by gel electrophoresis and quantified by image scanning or by detection of an incorporated radiolabel. These methods are either not particularly quantitative or using hazardous reagents, and also not readily automated. HPLC with a non-porous column and UV detection has been shown to be a powerful technique for separation and quantification of PCR products[19~21]. Our interest was to develop a reliable method to detect and quantify enzyme induction in cultured mammalian cells with HPLC detection to improve the conditions for RT-competitive-PCR. Fig. 2 shows the separation of PCR marker standards on the HPLC. DNA fragments up to 500 bp can be separated under the conditions described in Sec. 1. The conditions were optimised for sizes between 50 and 200 bp products and the minimum size difference required for baseline separation of target and competitor was 30 bp. Linear standard curves of target (120 bp) and competitor (150 bp) were obtained by plotting the amounts of the target or competitor (up to 160 ng) against the absorbance t 260 nm (Fig. 3).

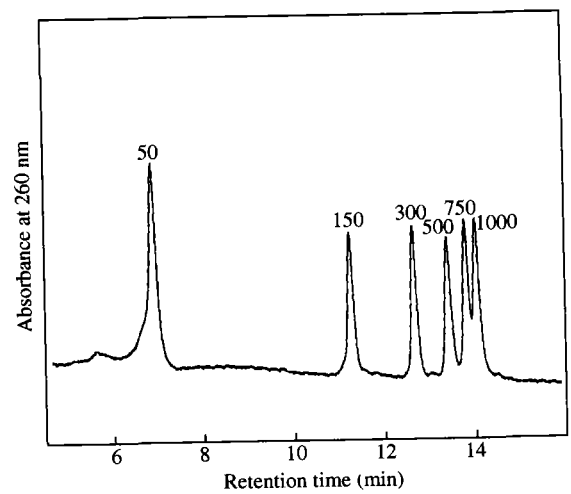

Fig.2. HPLC separation of PCR markers (50 to 1000 bp). Each peak contained 40 ng double stranded DNA.

Quantitative PCR requires the synthesis of a competitor which can be amplified with the same efficiency as the target. An ideal competitor should re-

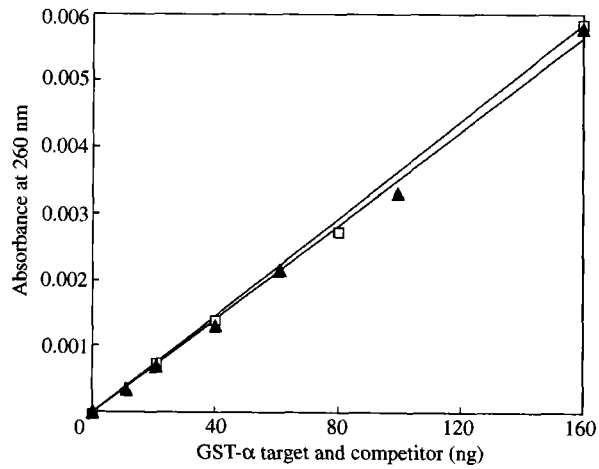

Fig. 3. Standard curves of GST-α target and competitor. Various amounts of GST target or competitor (10 ~ 160 ng) were injected onto an ion-exchange column. The standard curves of target (□) and competitor (▲) were plotted by using amount (ng) against peak height at 260 nm; R^2 values were 0.9985 and 0.9973 respectively.

semble the target sequences as close as possible both in length and sequence[7,9]. The designed 150 bp competitor has a similar G + C content (52.6%) to the 120 bp target (51.6%), and only 25% longer in length. The competitor and target were baseline separated directly on the HPLC. The use of a PCR-generated competitor has some advantages over the method of incorporating an *Eco*RI site into a competitor[18]. Incorporation of an *Eco*R1 site requires determination of the efficiency of digestion by restriction enzyme, and it also requires an extra step of digestion before analysis with an associated risk of product loss[18,22].

A key issue is whether the competitor is amplified with the same efficiency as the target. To demonstrate this, we determined what the suitable ratio of target to competitor molecules is and how cycle numbers affects the accuracy of quantitative RT-PCR. With known amounts of target and competitor molecules mixed in different ratios, the final quantification of the PCR reaction was determined. The known number of starting target molecules was termed "target molecules added" and the number of molecules calculated using PCR was referred to as "target molecules measured". The total number of molecules was 2.45×10^5 and the ratio of target/competitor molecules was from 0.2 to 5 at the beginning of the experiment. All of the target molecules measured were within 90% of the target molecules added (the ratios of molecules added/molecules measured are all within 0.95 ~ 1.05, and the chromatograms are shown in Fig. 4. This confirms that the competitor molecules are amplified with the same efficiency as the target molecules in the PCR amplification, and in RT-PCR. Therefore the 150 bp competitor is a suitable internal control for quantification of GST-α gene expression by RT-competitive-PCR analysis. This extends the method described previously[11]. The amount of PCR products of target and competitor increased with the cycle number is shown in Fig. 5. After 21 cycles, both products could be detected by HPLC and the ratio of these two products remained constant until the formation of a heteroduplex at cycle 27. Because the nucleotide sequences of target and competitor are identical except for the insert, there will be heteroduplex DNA formation due to the cross annealing between the target and the competitor molecules when the reactants such as primers become a limit in the later stage of the PCR amplification[18,23,24]. Heteroduplex formation can thus be

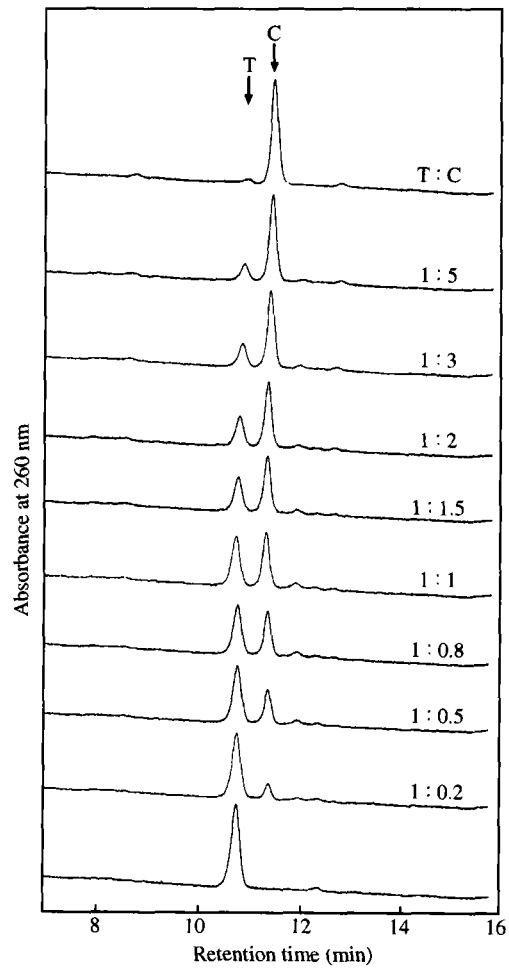

Fig. 4. Chromatograms of different ratios of target and competitor (26 cycles). The first chromatogram contains only competitor (C) and the final contains only target (T). The other ratios are indicated on the chromatograms.

controlled by limiting the cycle numbers to 26 as shown in Fig. 5. The formation of heteroduplex increased with the cycle number, and after 30 cycles there was relatively little increase in the amounts of target and competitor products, but the heteroduplex increased substantially. The amount of the heteroduplex was maximum at a ratio of target/competitor = 1 (data not shown). However, the quantification range can be extended into the non-exponential phase of the amplification[4,25,26], so it is possible to work in this range provided that the heteroduplex can be resolved from peaks of target and competitor. It has been reported that the amount of target molecules and competitor molecules can be calculated according to the molecular weight ratio, and added back to the target and competitor peaks, but this may decrease the accuracy of the results[23].

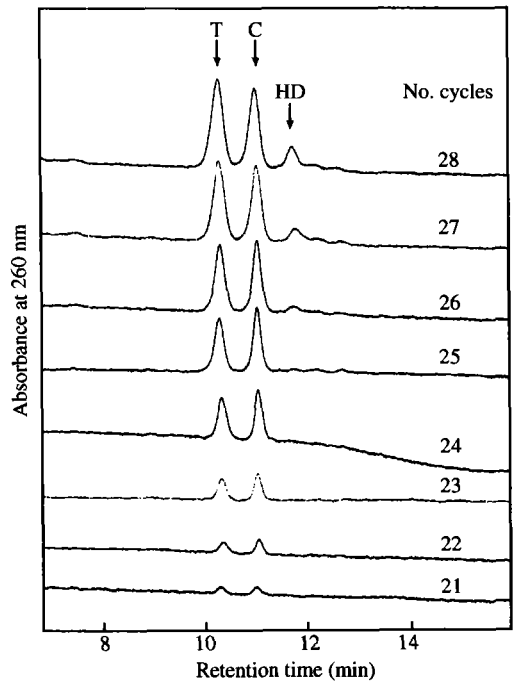

Fig. 5. Dependence of cycle number of PCR on competitor and target peaks. The ideal range of detection is between the detection limit (21 cycles) and before heteroduplex (HD) formation (26 cycles).

The combination of RT-competitive PCR and HPLC in analysis of the induction of GST-α was performed by setting up replicate reactions containing equal amounts of unknown GST-α target with different known amounts of GST-α competitor spiked into each sample. After RT-PCR, the products were separated by HPLC and quantified at 260 nm. The unknown amount of GST-α target was determined by extrapolating the data based on the competitor molecules using the formula: $\lg(Y_C/Y_T) = \lg X_C - \lg X_T + n\lg[(1+E_C)/(1+E_T)]$[9], where Y_C is the amount of competitor after PCR amplification; Y_T the amount of target after PCR amplification; X_C the amount of competitor added; X_T the amount of target; E_C and E_T are the efficiencies of competitor and target respectively. When the efficiencies are equal, the formula is simplified into $\lg(Y_C/Y_T) = \lg X_C - \lg X_T$; $\lg X_T = \lg X_C - \lg(Y_C/Y_T)$; i. e. $X_T = X_C \cdot Y_T/Y_C$.

Using these conditions, the expression of mRNA encoding GST-α was induced by oltipraz in human hepatoma Hep G2 cells as expected. The two chromatograms show the amounts of GST-α target in control and in oltipraz-treated (2 μmol/L) Hep G2 cells (Fig. 6), showing a 2.5-fold induction. The induction was dose-dependent as shown in Fig. 7. The absolute copy number of GST-α in control cells was 250 ± 60/cell; oltipraz (10 μmol/L) elevated this to 800 ± 175/cell. The cDNA from the GST mRNA is single stranded, whereas the competitor molecules added are double stranded, so the copy numbers were multiplied by two. The starting total RNA in this assay is 0.1 ~ 20 ng, which is insufficient for quantification on Northern blots. An automated HPLC can run 30 samples overnight and the column can be used for up to 3000 injections, without using radioactive and fluorescent markers. One potential disadvantage of the use of a DNA competitor in RT-PCR is that there is no control of the RT-step[4]. However, the DNA competitor shows less inter-assay standard variation[27].

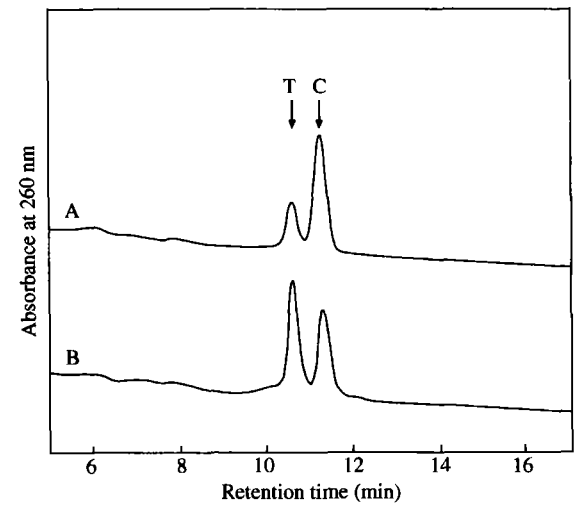

Fig. 6. Comparison of GST-α target molecules in control and oltipraz-treated Hep G2 cells. Total RNA from control (a) and oltipraz-treated (b) Hep G2 cells was spiked with 15fg competitor before RT-PCR.

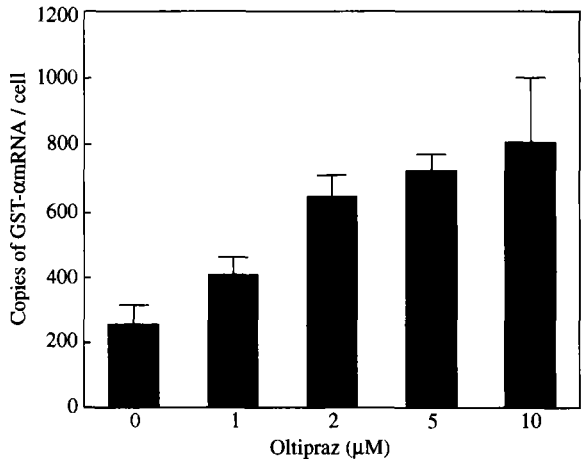

Fig. 7. Dose-dependent effect of oltipraz on induction of GST-α in human Hep G2 cells. Hep G2 cells were treated with oltipraz (0～10 μmol/L) for 16 h and total RNA was isolated by Bio/RNA Xcell solution. GST-α mRNA was assayed by RT-PCR and HPLC as described in Section 1.

In summary, we have presented the data showing an RT-competitive-PCR and HPLC assay useful for the quantification of gene expression which extends and improves the previous methods. Theoretically, this method should have a broad spectrum of application in measurements of many biological markers. The combination of RT-competitive-PCR and HPLC analysis for quantification of gene expression is a sensitive and a powerful system for determination of mRNA in very small amounts of clinical samples.

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