A new method of competitive reverse transcription polymerase chain reaction with SYBR Gold staining for quantitative analysis of mRNA

There are several methods available to analyze the mRNA concentration quantitatively. Among them, the competitive reverse transcription (RT-)PCR method is very useful. For this method, Cy5-labeled primers were used, and after gel electrophoresis in 7 M urea, the Cy5-labeled single-strand DNA was measured by a fluorescence detector. However, as the equipment to measure the Cy5-labeled fluorescence is expensive, we developed a new method using SYBR Gold staining. After gel electrophoresis in 7 M urea, the single-strand PCR product DNA was stained with SYBR Gold, and photographed with a standard UV-transilluminator and a standard digital camera with a specific filter for SYBR Gold staining. The photographic image was digitized by an imaging software. We measured β-actin and plasma glutathione peroxidase (Gpx3) mRNA concentrations of HepG2 cell cultured at 5 and 20% oxygen tension. The Gpx3 expression was increased by hypoxia. The result was equivalent to the data obtained by the real-time PCR analysis.

Keywords: Competitive reverse transcription-PCR / Competitive RT-PCR / Glutathione peroxidase / Oxygen tension / SYBR Gold

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Abbreviations: Gpx3, plasma glutathione peroxidase; RT-PCR, reverse transcription-PCR

HepG2 cells were obtained from the Riken Bioresource Center, Tsukuba, Ibaraki, Japan. Cells were incubated at two levels of incubator oxygen. Five percent incubator...
oxygen tensions were generated in a multigas incubator BL-40M (Juji Field, Tokyo, Japan). Gas-phase oxygen tension was controlled by continuous injection of an appropriate amount of nitrogen gas. Cell culture at atmospheric oxygen levels (20% oxygen) was incubated in a standard incubator without an additional supply of nitrogen gas. The CO₂ level was maintained at 5% in all cases. Oxygen tension in the multigas incubator was also measured by an oxygen gas detector glass tube, GASTECC GV-100S (Gastec, Ayase, Kanagawa, Japan). Cells were cultured in 4 mL of DMEM (Asahi Techno Glass, Tokyo, Japan) in a 60 mm dish supplemented with 10% fetal calf serum (FCS) (heat-inactivated; Irvine Scientific, Santa Ana, CA, USA)/50 U/mL penicillin (Dainippon Pharmaceutical, Osaka, Japan)/50 U/mL streptomycin (Dainippon Pharmaceutical, Osaka, Japan) at 20% oxygen tension for 24 h. The medium was changed, and four of the dishes were incubated at 5% O₂ – 5% CO₂ – 90% N₂ gas for 48 h. Four dishes of control cells were incubated at standard oxygen tension (5% CO₂ – 95% air). Total RNA was prepared by a modification of the procedure of Chomczynski and Sacchi [6]. After the culture medium was removed, 1 mL of ISOGEN (Nippon Gene, Toyama, Japan) was added to the dish, and subjected to phenol extraction and isopropanol precipitation followed by ethanol precipitation. After the total RNA was treated by DNase I (Wako, Tokyo, Japan), the cDNA was synthesized by M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) using Oligo (dT) 15 primer (Promega, Madison, WI, USA) according to the manufacturer’s protocol.

The mRNA of β-actin was measured by the competitive RT-PCR method (Fig. 1). The method of competitive RT-PCR was developed based on the protocol of our previous paper [2]. The competitor plasmid for β-actin was a gift from Mitsubishi Kagaku Bio-clinical Laboratories, Tokyo, Japan. The primer sequences for competitive RT-PCR were CTTCTACAATGAGCTGCGTG and TCAT-3’ for the forward primer (Promega, Madison, WI, USA) according to the manufacturer’s protocol.

The mRNA of Gpx3 at 5 and 20% oxygen tension was measured by the competitive RT-PCR method (Fig. 2a) and by the real-time PCR method (Fig. 2b). The internal
Figure 2. Effect of 5% oxygen tension on the expression of Gpx3 measured by competitive RT-PCR and real-time PCR. HepG2 cells were cultured at 5% oxygen tension or normal oxygen tension for 48 h. cDNA was synthesized from total RNA, and the concentrations of β-actin and Gpx3 were measured by competitive RT-PCR (a) and real-time PCR (b) as described above. Standard plasmid was the same in all cases. (*p < 0.05)

Competitive RT-PCR and real-time PCR are useful methods for quantitative analysis of mRNA concentration. These methods are more sensitive methods than the Northern blotting method, but the equipment for those PCR-based methods is expensive. We developed a competitive RT-PCR method without expensive equipment. For this purpose, we chose new dye SYBR Gold that can stain a single-strand DNA [3]. The result we obtained was sufficiently satisfactory to allow a comparison with the result obtained by the real-time PCR. This method does not require expensive equipment, and requires only a specific filter for SYBR Gold dye staining. Although we used a commercial software to digitize the band intensity, a free software is also available. We also performed the competitive RT-PCR using a flat bed image analyzer with Cy5-labeled primers. The result correlated very well with this method (data not shown). We analyzed many genes, and compared the results obtained by both methods, and found that both methods correlated very well as shown in Fig. 2. We are now also using a DNA microarray (Applied Biosystems Japan) to analyze the comprehensive gene expression and have found that those PCR-based analyses correlate with the DNA microarray analysis.

The Gpx3 expression was increased by 5% oxygen tension about four times that of the normal condition. The Gpx3 expression in Caki-2 cell in a hypoxic condition was measured with luciferase reporter assay by Bierl et al. [8]. Exposure to 95% N2 and 5% CO2 for 6-h intervals up to 24 h increased expression about three times that of the normal condition. The conditions were not the same in this experiment, but Gpx3 expression is sensitive to oxygen tension because the promoter region of Gpx3 has a hypoxia-inducible factor-1-binding site [8]. We are now also analyzing the effect of oxygen tension on the many gene expressions using the competitive RT-PCR and the real-time PCR, and have found that the oxygen tension in the incubator influences the expression of the many gene expressions (unpublished data). The baseline of pO2 levels in tissues is considered to be about 50 mmHg [9] which is equal to about 6% O2. Most of the cells in the body are exposed to very low oxygen tension, and high oxygen tension which is used for "standard cell culture conditions" with 5% CO2 and air may not be a suitable condition for the cells to investigate the cell function in the body.
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References


