

## Application of High-Performance Capillary Electrophoresis to Quantification of Products of the Reverse Transcriptase-Polymerase Chain Reaction

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### Abstract

To apply the high-performance capillary electrophoresis (HPCE) to the quantification of RT-PCR products, the conditions for the routine measurement were established as follows: the reaction mixture after RT-PCR (1  $\mu$ l) was diluted 20-fold with deionized water and introduced into the capillary tube by electrokinetic injection at -10 kV for 40 sec. Electrophoresis was performed at -13 kV for 21 min. DNA was quantified spectrophotometrically at 260 nm during electrophoresis. This method was applied to the quantification of gliostatin mRNA in A 431 cells and compared to the amount of corresponding protein. The amount of gliostatin mRNA was well correlated with that of the protein ( $r=0.733$ ), when RT-PCR products derived from  $\beta$ -actin were used as internal standards. The data indicate that HPCE is useful to quantitate mRNA levels in cells.

**Key words:** PCR, capillary electrophoresis, gliostatin

### [ I ] Introduction

Quantification of specific cellular mRNAs, as well as their protein products, is of great importance in if we are to understand the intracellular responses to the stimuli that induce the differentiation of cells. A combination of reverse transcription with a subsequent polymerase chain reaction (RT-PCR) allows the detection of minute numbers of transcript. For quantitative determinations, cellular mRNAs have been visualized after RT-PCR with radiolabeled or fluorescence-labeled nucleotide primers and agarose or polyacrylamide gel electrophoresis<sup>1,2</sup>. These methods, while sufficiently sensitive for the semiquantification of products of PCR, are frequently troublesome because of harmful radioactivity or fading of fluorescence under UV light. In addition, the radioactivity of <sup>32</sup>P-labeled decays with a half life of 14.3 days, and fluorescence intensity can only be measured in arbitrary units and is dependent on the intensity of UV light used for excitation. Thus, it is difficult to compare the results from experiments performed at different times.

Compared with the conventional gel

electrophoresis, high-performance capillary electrophoresis (HPCE) allows better quantification of nucleotides by the direct and spectrophotometric quantification of DNA at 260 nm during electrophoresis. HPCE also allows the separation of DNA fragments of very similar molecular size.

In this note, we describe an application of HPCE to the quantification of PCR products. The method provides high resolution and a high degree of sensitivity.

### [ II ] Materials and Methods

**Materials.** The A431 cell line, derived from epidermoid carcinoma, was obtained from the Japanese Cancer Research Resources Bank (JCRB) and cells were cultured in Dulbecco's modified medium (DMEM; GibcoBRL) that contained 10 % fetal calf serum. Gliostatin in the cell lysates was quantified by the enzyme-immunoassay (EIA) as described previously<sup>3</sup>.

**Primers.** Sequences of genes for  $\beta$ -actin and gliostatin (platelet-derived endothelial cell growth factor) were obtained from GenBank and used to

design the primer pairs. Specific primer pairs used to amplify the  $\beta$ -actin mRNA were

5'-TACATGGCTGGGGTGGGTTGAA-3' and

5'-AAGAGAGGCATCCTCACCT-3', and those used to amplify the gliostatin mRNA were

5'-GTGGAGGGGCTGTCCGCTCTGGTGGTGGAC-3'

and

5'-CAGCATCCGCTCGAAGCGCCAAGGGCCGA-3'.

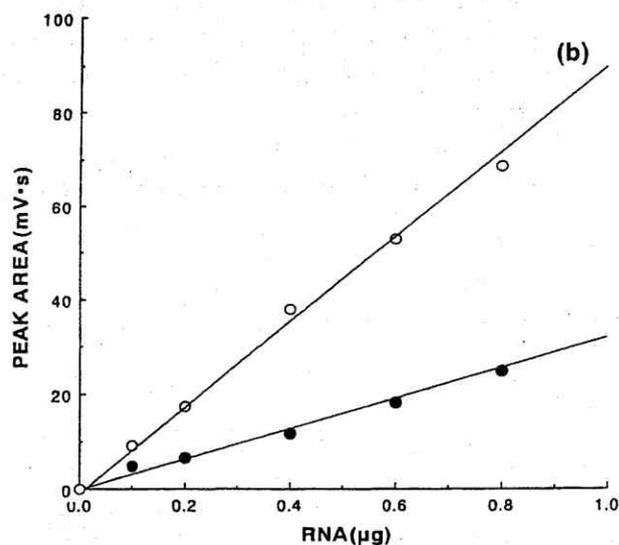
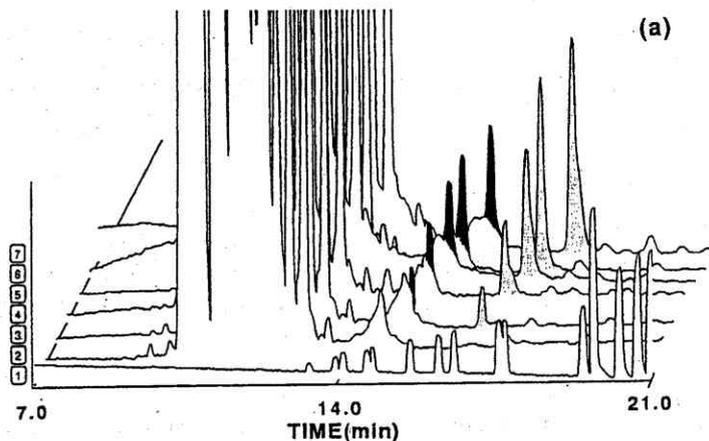
**Preparation of RNA.** Total cellular RNA was obtained as described by Chomczynski and Sacchi<sup>10</sup>. The amount of RNA recovered was quantified spectrophotometrically at 260 nm.

**Reverse transcription reaction.** First-strand cDNAs were synthesized from total RNA by MMLV reverse transcriptase with oligo d(T)<sub>18</sub> priming. Each 20  $\mu$ l of reaction mixture for synthesis of cDNA synthesis reaction mixture contained 1  $\mu$ g of total RNA, 1x PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 1 mM each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 20 units of RNAsin, 160 units of MMLV and 100 pmol of oligo d(T)<sub>18</sub> primer. Reaction mixtures were preincubated for 10 min at 21  $^{\circ}$ C prior to synthesis of cDNA. The reverse transcription reactions were carried out for 30 min at 42  $^{\circ}$ C and reaction mixtures were heated at 95  $^{\circ}$ C for 5 min to terminate the reverse transcription reaction.

**PCR** The "Primer dropping method", as described by Wong *et al.*<sup>9</sup> was used for quantification of gliostatin mRNA, with  $\beta$ -actin mRNA as an internal standard. PCR was carried out in a GeneAmp PCR System 960 (Perkin-Elmer, Applied Biosystems) with 1  $\mu$ l of each primer for gliostatin, 200 mM each dNTP and 2.5 units of Taq DNA polymerase in 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% gelatin, 10% dimethyl sulfoxide in a reaction volume of 100  $\mu$ l. Samples were overlaid with light mineral oil and heated to 95  $^{\circ}$ C for 3 min. Subsequent cycles were conducted in three steps: 1 min at 94  $^{\circ}$ C; 2 min at 50  $^{\circ}$ C; and 3 min at 72  $^{\circ}$ C. Primers for  $\beta$ -actin cDNA were added at the seventh cycle. After 30 cycles, the samples were incubated at 72  $^{\circ}$ C for an additional 7 min. The samples could then be stored at 4  $^{\circ}$ C.

#### High-performance capillary electrophoresis (HPCE)

One  $\mu$ l of the reaction mixture after PCR was diluted to 20  $\mu$ l with deionized water and applied to the model 270A-HT system (Perkin-Elmer, Applied Biosystems) equipped with an HS cell capillary (60



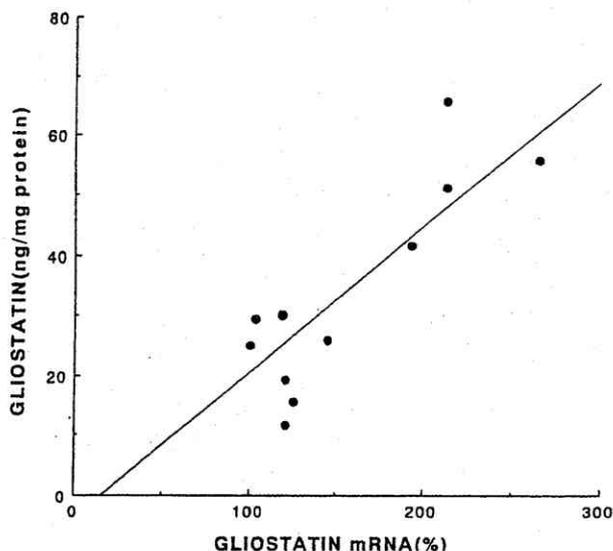
**Fig. 1** (a) HPCE of DNA marker DNAs and products of RT-PCR.

RT-PCR was performed with varying amounts of total RNA from A431 cells (0, 0.1, 0.2, 0.4, 0.6, and 0.8  $\mu$ g) and 1  $\mu$ l of the mixture after RT-PCR was analyzed by HPCE (Electrophoretograms 2-7). Light gray peaks are represent results with primers for of gliostatin, and dark gray peaks those for of  $\beta$ -actin. Electrophoretogram 1, 1 kb ladder markers. The first 11 peaks in electrophoretogram 1 correspond to 75 bp, 134 bp, 154 bp, 201 bp, 220 bp, 298 bp, 349 bp, 396 bp, 506 bp, 517 bp, and 1,018 bp in the order of migration time. The areas of the peaks are proportional to the sizes of the DNA fragments.

(b) Correlation between the quantity of RNA and the peak area after HPCE.

Peak areas in electrophoretograms 2 through 7 in Fig. 1 (a) are plotted against the quantity of RNA used for RT-PCRs.

○, Gliostatin; ●,  $\beta$ -actin.



**Fig. 2** Correlation between cellular levels of gliostatin mRNA and of gliostatin. Gliostatin mRNA was quantitated by HPCE after RT-PCR, relative to the amount of  $\beta$ -actin mRNA as an internal standard, and the concentration of gliostatin was determined by an enzyme-linked immunoassay. The amount of mRNA in unstimulated cells was taken as 100%. The correlation coefficient ( $r$ ) was calculated to be 0.733 ( $n=11$ )

cm, 75  $\mu$ m in diameter) according to the protocol from the manufacturer with minor modifications. Sample was loaded at -10 kV for 40 sec and electrophoresis was performed at 32  $^{\circ}$ C for 21 min, otherwise specified. The data were analyzed with 600E 1.02 software (Perkin-Elmer, Applied Biosystems).

### [III] Results and Discussion

Several factors affect the quantification of peak areas during electrophoresis with continuous monitoring. It is particularly important that the measured peak area be a function of the mobility of the sample and the quantity of the sample, because the lower mobility yields the broader peaks. In this study by HPCE, therefore, the sample was automatically and electrokinetically introduced into the capillary tube to compensate for the apparent dependence of peak area on mobility. Electrokinetic injection allows more accurate quantification than does hydrodynamic injection.

A preliminary study using various loading voltages (-10 kV to -30 kV) and times (10 sec to 80 sec), showed that peak areas were proportional to both the loading voltage and the time taken for

injection. The sensitivity was, however, almost unaffected by the applied voltage and time taken for injection since areas of peaks due only to noise were also proportional to the voltage and the time. It is known that if the ionic strength of the sample, another factor that affects electrophoresis, is lower than that of the electrophoresis buffer, stacking of the sample occurs. To get sharp peaks and improve detectability, the mixture after RT-PCR was diluted 20-fold with deionized water and introduced into the capillary tube by electrokinetic injection at -10 kV for 40 sec. Standard electrophoresis was performed at -13 kV for 21 min.

When a 1-kb DNA ladder (Gibco BRL) was injected electrokinetically and subjected to electrophoresis, 11 peaks of fragments from 75 bp to 1018 bp were well separated from each other and the peak areas were proportional to the sizes of DNA fragments (Fig. 1a, electrophoretogram 1). Under these conditions, DNA fragments of more than about 1 kb were not well separated. Although attempts to utilize HPLC instead of HPCE have been reported<sup>9</sup>, HPCE is different from HPLC in that high resolution strictly depending on molecular sizes of DNA.

RT-PCR products derived from  $\beta$ -actin and gliostatin mRNAs from varying amounts of total RNA prepared from A 431 cells, were also subjected to HPCE for quantification under these conditions. Figure 1a shows the electrophoretogram of these products of RT-PCR. The peak of the cDNA for  $\beta$ -actin (218 bp) was well separated from that for gliostatin (372 bp). Both peak areas corresponded to the amount of RNA used as template for RT-PCR (Fig. 1 b).

To validate the precision of this method, we examined the correlation between the measured amount of mRNA (arbitrary units) and the amount of gliostatin protein (ng/mg protein estimated by a separate EIA) in A431 cells. The amount of gliostatin mRNA was well correlated with that of the corresponding protein ( $r=0.733$ ,  $n=11$ ; Fig. 2).

In summary, HPCE is useful for quantification of the products of RT-PCR without the use of radioactive isotopes and fluorescent chemicals. This method, furthermore, requires only a few microliters of the reaction mixture after PCR and allows the economic use of small amounts of sample RNA and

the reagents for RT-PCR.

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