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# Determination of protein phosphorylation and the translocation of green fluorescence protein-extracellular signal-regulated kinase 2 by capillary electrophoresis using laser induced fluorescence detection

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**Abstract**

In this study, we developed a method to monitor the phosphorylation and translocation of the extracellular signal-regulated kinase (ERK2) proteins after PC12 cells have been stimulated by a mitogen. The method involves the use of green fluorescent protein (GFP), capillary electrophoresis and the measurement of laser-induced fluorescence (CE-LIF). We showed the prescence of the non-phosphorylated GFP-ERK2 and phosphorylated GFP-ERK2 in cell lysates by CE-LIF, and then compared the phosphorylations of GFP-ERK2 and GFP-183A. Phosphorylated GFP-ERK2 was detected at 6.7 min and the non-phosphorylated GFP-ERK2 at 5.3–5.5 min. The results were compared with confocal laser scanning microscope imaging and western blot results, and suggest that the developed method can be used to detect other enzymatic modifications.


*Keywords:* Protein phosphorylation; Extracellular signal-regulated kinase; Enzymes; Green fluorescent protein

## 1. Introduction

Capillary electrophoresis has become a powerful analytical tool for the analysis of a wide variety of molecules from small organic molecules, to macromolecules such as DNA and proteins [1]. Recently, CE was used as an alternative to current analytical methods to detect enzyme activity [2,3]. Notably, since the CE-based protein kinase assay was established by Dawon et al., much research has been done on protein kinase assays based on CE [4].

The extracellular signal-regulated kinases (ERKs) are important in signaling pathways, and regulate cellular proliferation and the maintenance of a differentiated phenotype. Two isoforms, ERK1 (p44) and ERK2 (p42), have been well characterized in mammals. ERKs are activated by many different extracellular stimuli such as peptide growth factor and phorbol esters [5–7]. The phosphorylation of two residues (threonine185 and tyrosine183) on ERK2 leads to maximal enzyme activation, moreover, covalently bound phosphates are required to maintain its activity. Following ERK activation, the next key step in the signaling mechanism of the ERK cascade involves the translocation of ERKs to the nucleus. The proper subcellular localization of various components plays an important role in regulating the physiological function of the ERK cascades [8,9]. In PC12 cells, the transient activation of ERKs by epidermal growth factor causes cell proliferation [10], and these proliferating cells differentiate in response to nerve growth factor, which acts partly by inducing the sustained activation of ERKs and which induces the neurite outgrowth [11]. Therefore, it is important that we understand the mechanism underlying the nuclear translocation of ERKs and to characterize their subcellular localizations.

Green fluorescent protein (GFP) has attracted much interest as a reporter for gene expression, in both the biotechnology and cell biology areas, in terms of the monitoring of the

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subcellular transport of various proteins in the secretary and signal transduction pathways of bacteria, plants and mammalian cells, and for detecting protein-protein interactions electrophoretically [12]. The main advantage of offered by GFP is that its excitation spectrum is in the visible range and thus it has an excitation line that is compatible with the output of the frequently used argon ion laser. Wild type GFP, was originally isolated from the jellyfish *Aequorea victoria* [13]. It is an acidic, globular protein with a molecular weight of 30 kDa, a maximum excitation wavelength at 395 nm and a smaller excitation peak at 475 nm [14]. The wild type GFP and its variants can be easily detected in the cellular environment without chemical tagging or otherwise disturbing cells. The fluorescence of enhanced-GFP (EGFP), a variant of GFP, that contains the double-amino acid substitution of phenylalanine 64 to leucien and serine 65 to threonine, is 35 times more intense than that of the wild type GFP [15], and has also been extensively used as a reporter of gene expression. For this reason, EGFP has been used in many studies. Following the increased use of EGFP, a better detection method was required, because classical gel electrophoresis approaches, commonly used for demonstrating the presence of EGFP, have the disadvantage of being time-consuming, difficult to automate and hampered by high material consumptions [16].

Capillary electrophoresis (CE) has proven to be a powerful separation tool for the analysis of biomaterials because it is fast and requires a small sample volume [17,18]. When combined with a detection technique such as the laser-induced fluorescence (LIF), CE can provide very high sensitivities. CE-LIF has been applied to the analysis hormones in biological materials, and for single cell and protein analysis. In particular in the cell biology area, investigations of the use of GFP fusion protein are being increasingly carried out with CE-LIF both qualitatively and quantitatively.

The objective of our study was to develop a CE-LIF method to determine phosphorylation levels and to follow the translocation from the cytoplasm to the nucleus of the green fluorescent protein-extracellular signal regulated protein kinase 2 (GFP-ERK2) protein after its activation by nerve growth factor (NGF) in pheochromocytoma cell line (PC12 cell) extracts.

## 2. Experimental

### 2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM), lipofectAMINE 2000, Optimum minimum essential medium (Opti-MEM), fetal bovine serum (FBS), horse serum, trypsin, penicillin-G and streptomycin solution, and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). Sodium dodecyl sulfate (SDS), 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS), sodium hydroxide, sodium bicarbonate and other chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA).The green fluorescent protein (GFP) gene of pEGFP-C1 vector (Clontech, Palo Alto, CA, USA) was kindly provided by Dr. R. Seger at the Department of Biological Regulation at the Weizmann Institute of Science. Rabbit anti-GFP polyclonal antibody and goat anti-rabbit IgG conjugated horseradish peroxidase (HRP) antibody were purchased from Santa Cruz Biotechnology (Delaware, CA, USA). A pheochromocytoma cell line, PC12 was obtained from the American Type Culture Collection (Rockville, MD, USA). All other chemicals used were analytical grade.

### 2.2. Equipment

A P/ACE 5000 capillary electrophoresis system equipped with a 5 mW air-cooled argon ion laser detector, a P/ACE LIF detector, and an untreated fused silica capillary column were obtained from Beckman Instruments (Fullerton, CA, USA). Data were analyzed using Beckman System Gold software, version 8.1. Confocal images were obtained using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). For gel electrophoresis and western blot analysis, samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V for 90 min in a Mini Protean three units (Bio-Rad, Hercules, CA, USA), and then transferred to a nitrocellulose membrane (Schleidher and Schuell, Keene, NH, USA) using a Bio-Rad Mini-Trans Blot cell in transfer buffer for 90 min at 200 mA.

### 2.3. DNA construct

The cDNA of rat ERK2 (bases 22–1096) was ligated into *Apa* I and *Xba* I sites downstream of the GFP gene of pEGFP-C1 vector. GFP-ERK2 plasmid was transfected into the PC12 cells using lipofectAMINE 2000 reagent, and the expression of GFP-ERK2 was visually confirmed using a confocal microscope.

### 2.4. Transfection and cell preparation

PC12 cells were cultured in complete DMEM, which contained 10% horse serum, 10% FBS, 100 IU/ml penicillin-G and 50 mg/ml streptomycin solutions. The cells were maintained in T-75 $cm^2$ culture flasks at 37 °C in a humidified incubator in 5% $CO_2$/95% air until harvesting for experiments [19,20]. Prior to plating, culture dishes were treated with 1 mg/ml of collagen and then rinsed with medium. The cells were plated and grown in a 100 mm dish until 70–80% confluent. In each 100 mm dish, GFP-ERK2 plasmid was transfected into the PC12 cells for 32 h using lipofectAMINE 2000, and the cells were serum starved (0.1% fetal bovine serum) for 16 h. After serum starvation, the cells were washed twice with ice-cold PBS and harvested with cell lysis buffer A (50 mM β-glycerophosphate, pH 7.3, 1.5 mM ethylene glycol bis (*b*-aminoethylether) tetraacetic acid (EGTA), 1 mM ethy-

lene diamine tetraacetic acid (EDTA), 0.1 mM sodium vanadate, and protein inhibitor cocktail) then homogenized and centrifuged (15,000 × *g*, 15 min, 4 °C). Supernatants were collected and kept frozen at −20 °C until required. Whole cell lysate proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and were then electrophoretically transferred to nitrocellulose membranes as described previously [21]. The nitrocellulose membrane was probed with a 1:2000 dilution of rabbit anti-GFP polyclonal antibody, anti-ERK polyclonal antibody or anti-phospho-ERK polyclonal antibody and then probed with a 1:2000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase antibody.

### 2.5. Cell fractionation

Transfected PC12 cells were serum-starved (0.1% fetal bovine serum) for 16 h, harvested and lysed as described previously [22]. After stimulation with 100 ng/ml of nerve growth factor (NGF) for 5 min, the cells were washed twice with ice-cold PBS and once with ice-cold buffer A. Cells were then lysed in hypotonic buffer containing 10 mM HEPES(104-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitor cocktail. After centrifugation at 5000 rpm for 15 min, supernatant was collected as cytosolic extracts. Nuclear pellets were resuspended and incubated in nuclear extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, and protease inhibitor cocktail) for 1 h, centrifuged at 13,000 rpm for 20 min, and nuclear fractions collected. Cell fractionated proteins were detected using a Zeiss LSM 510 laser scanning confocal microscope and by CE.

### 2.6. Capillary electrophoresis

CE was carried out with a P/ACE 5000 capillary electrophoresis system with a 5 mW air-cooled argon ion laser detector (Beckman Instrument, Fullerton, CA, USA). Separation was performed using an untreated fused silica capillary column of 50 μm (ID), and length 27 cm. Electropherograms were analyzed using Beckman System Gold software, version 8.1. The running buffer consisted of a 100 mM CAPS buffer containing 2 M betaine, pH 11. Sample injections were performed under pressure (5 s at 0.5 psi) and the applied voltage was 10 kV. To obtain reproducible migration times and peak areas, the capillary tube was washed for 10 min with a washing buffer solution (25 mM sodium phosphate buffer, pH 7.0, 60 mM SDS), 10 min with water, 10 min with 0.5 M NaOH solution and finally flushed with running buffer solution for 10 min, prior to each run. All buffer solutions were sonicated and filtered through a 0.2 μm filter and degassed before use. To determine the phosphorylation of ERK by CE, the total cell lysate, and the cell fractionated lysate were mixed with a 1:200 dilution of anti-phospho ERK polyclonal antibody.

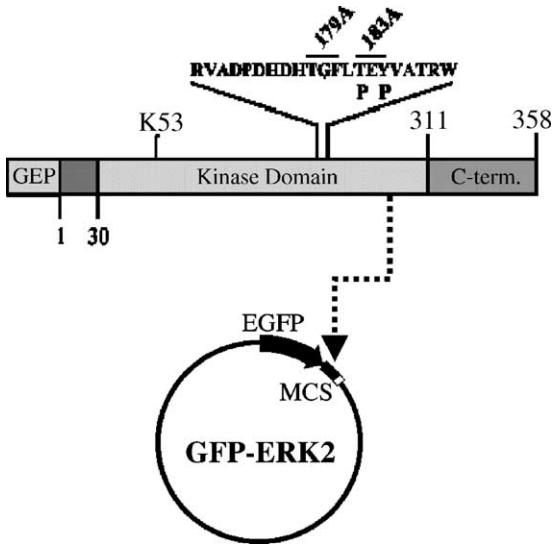


Fig. 1. Scheme of GFP-ERK2 plasmid construct.

## 3. Results and discussion

### 3.1. DNA construct

It is known that the role of the 183–185 residues in the activation loop of ERK2, which include its phosphorylation sites is important [23]. In this study, we investigated the phosphorylation of GFP-ERK2 and GFP-183A (obtained by substituting amino acids from 183 to 185 inclusive with alanines) and compared their roles. The cDNA of rat ERK2 was ligated into the *Apa* I and *Xba* I sites downstream of the green fluorescent gene of pEGFP-C1 vector (Fig. 1).

### 3.2. Determination of GFP-ERK2 and phosphorylated GFP-ERK2 by CE-LIF

We optimized CE-LIF to detect GFP-ERK2 and phosphorylated GFP-ERK2 in PC12 cells. The GFP-ERK2 fusion protein samples obtained from PC12 cells were extracted and analyzed by CE-LIF, without a sample purification stage prior to injection. The application of CE for biological samples such as cell extracts and biofluids is made difficult by the adsorption of sample components to the capillary wall, which affects the reproducibility of the assay. For this reason, we investigated various factors such as pH and buffer type, addictive containing the buffer and washing step between each run.

Since the pH and type of the running buffer are crucial factors for the precise detection of the analytes, the selection of running buffer is important if accurate data is to be obtained. The GFP plasmid construct was found to be pH-sensitive in vivo and in vitro, and the mutant GFP plasmid retained fluorescence in the pH range of 7.0–11.5 [24]. When the pH was reduced below 8.0, GFP-ERK2 was not detected by the CE system. At pHs higher than 8.0, an increase in sensitivity was observed on increasing pH but a decrease in sensitivity

occurred when a pH buffer higher than 11.0 was used. Therefore, we obtained reasonable efficiency and higher sensitivity at pH 11.0. The type of buffer used was a 100 mM CAPS buffer at pH 11.0. This buffer has a higher resolution compared than other buffers (data not shown). Also, the use of CAPS buffer offers some distinct advantages for the zwitterionic compounds, as it inhibits capillary wall and low current at high ionic strength effects as compared to phosphate buffer. These conditions are described in detail elsewhere [25].

In general, proteins in biologic buffer solutions show up as asymmetric peaks and show substantial peak tailing. Some proteins also exhibit broad peaks with unstable migration times. To improve runability, several strategies have been implemented such as extensive washing of the capillary with a base, adding betaine to the electrophoretic buffer and the use of SDS in a micellular electrokinetic chromatography (MEKC) method [26,27]. Based on these strategies and previous study, we optimized conditions for the detection of the phosphorylated and non-phosphorylated forms of the protein. First, we washed the capillary with a base between each run. As a result, the reproducibility of migration times substantially improved and peak tailing decreased. And, by adding betaine to the electrophoretic buffer, the migration time stability improved (data not shown). Finally, we added SDS to the washing buffer to avoid adsorption to the inner wall of the capillary column. The following conditions were found to be optimal: an untreated fused silica capillary (27 cm × 50 m i.d.) with 100 mM CAPS buffer pH 11 containing 2 M betaine at 20 °C, and 10 kV. Washing was performed using a washing buffer solution (25 mM sodium phosphate buffer, pH 7.0, 60 mM SDS),water and 0.5 M NaOH.

Using optimum conditions, we detected GFP-ERK2 and phosphorylated GFP-ERK2 in PC12 cells (Fig. 2). Phophorylated GFP-ERK2 was produced from GFP-ERK2 by treating with NGF. This figure, shows GFP-ERK2 and phosphorylated GFP-ERK2 at 5.3 and 6.7 min, respectively. The relative standard deviation (R.S.D., %) of the migration times of GFP-ERK2, and phosphorylated GFP-ERK2 were 1.8 and 2.8%, respectively.

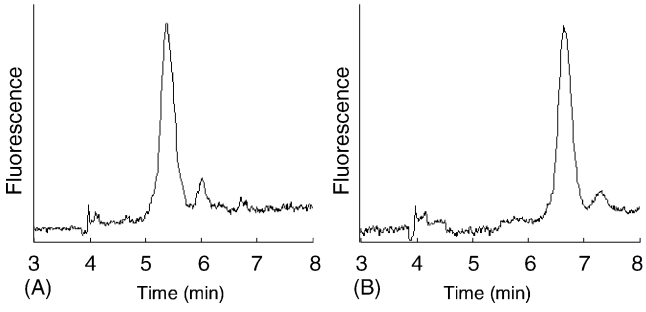


Fig. 2. Electropherogram of GFP-ERK2 (A) and phosphorylated GFP-ERK2 (B) in PC12 cells. CE conditions: 100 mM CAPS buffer containing 2 M betaine; untreated fused silica capillary of 27 cm × 50 μm i.d.; hydrodynamic injection for 5 s; LIF detection (excitation, 488 nm/emission, 520 nm); applied voltage, 10 kV.

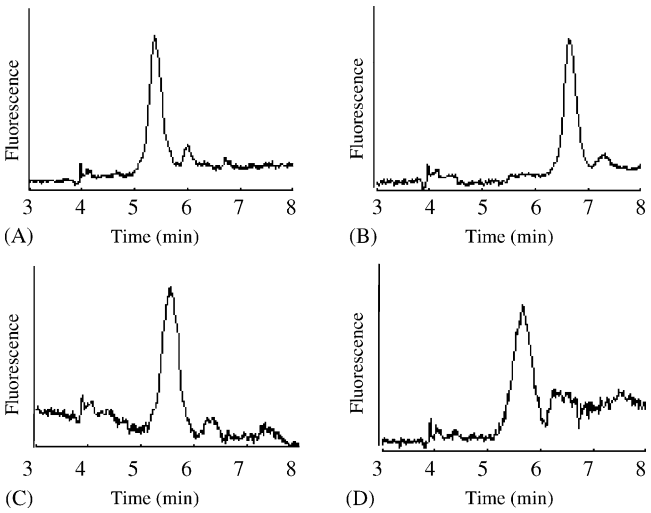


Fig. 3. Electropherograms of GFP fusion proteins by CE-LIF. Transfected PC12 cells stimulated (B and D) or not stimulated (A and C) with 100 ng/ml NGF for 5 min after serum-starved for 16 h, then harvested with cell lysis buffer. (A and B) GFP-ERK2, (C and D) GFP-183A, CE conditions are the same as those shown in Fig. 1.

### 3.3. Detection of the phosphorylation GFP-ERK2 in PC12 cells by CE-LIF and western blotting

Based on the above results, we analyzed differences in the expressions of GFP-ERK2 and GFP-183A after treating with NGF at by CE-LIF. Fig. 3 shows an electropherograms of GFP-ERK2 fusion protein (3A and 3B) and GFP-183A fusion protein (3C and 3D) treated with (3B and 3D) or without NGF (3A and 3C). The peaks were observed within 6 min as shown in Fig. 3A, C and D. However we observed the shifted peak at 6.8 min in Fig. 3B. This result shows that GFP-ERK2 was phosphorylated by NGF whereas GFP-183A was not. This means that GFP-ERK2 is phosphorylated at both threonine185 and tyrosine183.

To investigate the scope of the CE method to GFP-ERK2 fusion protein levels, we compared this method to conventional methods like western blot (Fig. 4). In this study, western blot analysis was carried out with gel electrophoresis using 10% SDS-PAGE and rabbit anti-ERK polyclonal antibody or anti-phospho-ERK polyclonal antibody to analyze GFP-ERK2 fusion protein. Each well was loaded with 30 μl

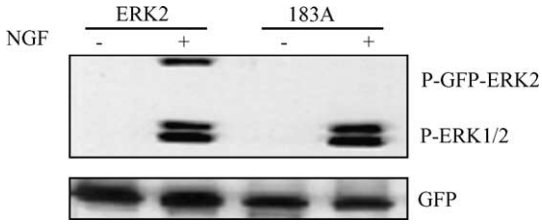


Fig. 4. Expression of the GFP fusion protein in PC12 cells by western blotting with rabbit anti-ERK polyclonal antibody and rabbit anti-phospho-ERK polyclonal antibody. Transfected PC12 cells stimulated with or without 100 ng/ml NGF for 5 min after being serum-starved (0.1% fetal bovine serum) for 16 h.

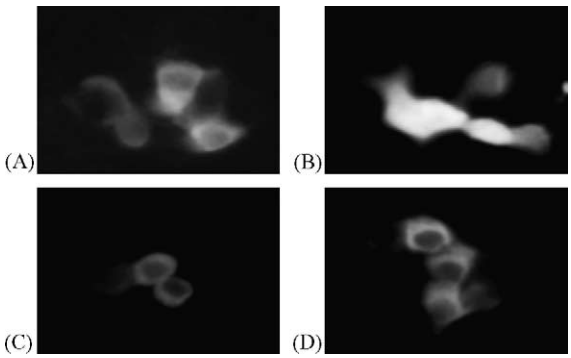


Fig. 5. Confocal laser scanning microscope image of GFP fusion protein. GFP-ERK2 (A and B) or GFP-183A (C and D) transfected into PC12 cells using LipofectAMINE 2000. After transfection, cells were fixed with 3% paraformaldehyde, and then washed several times with PBS. (A and C) Non-stimulated transfected cells, (B and D) transfected cells stimulated with 100 ng/ml NGF for 5 min after being serum-starved for 16 h.

of protein, the loading basis being amount not protein concentration level. Fig. 4 shows that all samples bond to rabbit anti-GFP and to anti-ERK polyclonal antibody. However, only phosphorylated GFP-ERK2 (ERK+) stimulated with NGF bound to anti-phospho-ERK polyclonal antibody. This corresponds with the results obtained with CE (Fig. 3), and demonstrates that CE is a fast and easy method of studying the expression of GFP-ERK2 fusion protein.

### *3.4. Identification of the translocation of phosphorylated GFP-ERK2 using CE-LIF and confocal laser microscopy*

We also investigated the phosphorylation of GFP-ERK2 by confocal laser microscopy to observe its translocation. Results are shown in Fig. 5. To monitor the translocation of ERK in PC12 cells, GFP-ERK2 plasmid, and GFP-183A plasmid were treated with 100 ng/ml of NGF for 5 min. Fig. 5 shows that GFP-ERK2 treated with NGF (B) differed with respect to translocation from the other samples (A, C and D), which is consistent with the results shown in Figs. 3 and 4. Phosphorylated GFP-ERK2 protein was detected in the cytosol and nuclear plasma. Western blotting gave a similar result (data not shown). These results show that phosphorylated GFP-ERK2 translocates from the cytosol to the nucleus.

Fig. 6 shows an electropherogram of GFP-ERK2 protein after cell fractionations of the sample shown in Fig. 5B. In this figure, the phosphorylated GFP-ERK2 protein was detected in the cytosolic fraction and in the nuclear fraction. The relative standard deviation (R.S.D. (%)) of the migration time of phosphorylated GFP-ERK2 was 2.7%. In this result, allows us to conclude that GFP-ERK2 fusion protein translocated from the cytosol to the nucleus.

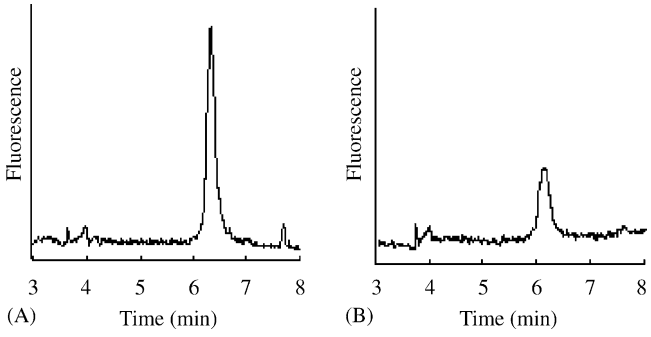


Fig. 6. Electropherograms of GFP fusion proteins by CE-LIF after cell fractionation. Transfected PC12 cells were serum-starved for 16 h and harvested. After stimulation with 100 ng/ml NGF for 5 min cells were lysed in hypotonic buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitor cocktail. After centrifugation, supernatants were collected as cytosolic extracts (A). Nuclear pellets were resuspended and incubated in nuclear extraction buffer for 1 h before centrifugation and collected as nuclear fractions (B). CE conditions are the same as in Fig. 1.

## 4. Conclusion

Here, we describe a method for determining GFP-ERK2 fusion protein phosphorylation in PC12 cell extracts by CE-LIF. To obtain the optimum conditions for detecting the phosphorylation of GFP-ERK2 fusion protein, the influences of the pH and the type of running buffer were investigated. The following optimal conditions were obtained: an untreated fused silica capillary (27 cm × 50 μm i.d.) with 100 mM CAPS buffer of pH 11 containing 2 M betaine at 20 °C, and 10 kV. Under optimized analysis conditions, the R.S.D.s of the migration times of GFP-ERK2 and phosphorylated GFP-ERK2 were 1.8 and 2.8%, respectively. Compared to conventional western blotting, the CE method is rapid, reproducible, allows the analysis of sample volume as low as a few nanoliters, does not require a separate sample purification stage, and does not require radiolabelling.

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