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Application of multiplexed capillary electrophoresis with laser-induced fluorescence (MCE–LIF) detection for the rapid measurement of endogenous extracellular signal-regulated protein kinase (ERK) levels in cell extracts

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Abstract

Multiplexed (96-lane) capillary electrophoresis with laser-induced fluorescence (MCE–LIF) detection was used for the rapid analysis of extracellular signal-regulated protein kinase (ERK) levels from in vitro cell extracts. The levels of ERK enzyme in cell extracts were determined by monitoring the conversion of a fluorescent-labeled peptide substrate to a phosphorylated fluorescent-labeled peptide product using MCE–LIF. The incorporation of a fluorescent internal standard was found to improve the precision of the analysis. The enzyme assay conditions including substrate concentration, reaction time and enzyme linear range were rapidly optimized using the MCE–LIF approach for both direct and immunoprecipitation-based ERK assays. The levels of ERK from in vitro cell extracts stimulated with angiopoietin 1 (Ang1*) were determined using the MCE–LIF approach. The advantages of MCE–LIF for developing and applying enzyme assays, as well as the figures of merit for the direct and immunoprecipitation ERK assays, are discussed.

Keywords: Multiplexed capillary electrophoresis; Protein kinase; Enzymes

1. Introduction

Mitogen-activated protein kinases (MAPK) are a super family of serine/threonine kinases that play a key role in intracellular signal transduction in all eukaryotic cells [1–3]. Activation of receptor tyrosine kinases and G protein-coupled receptors by a variety of extracellular signals, such as growth factors, cytokines and stress, activate the various MAPK subfamilies [1–3]. One subfamily, the extracellular signal-regulated protein kinases (ERKs) are activated by a variety of growth factors including vascular endothelial growth factor (VEGF) and angiopoietins (Ang) [4,5]. Two isoforms, ERK1 (also known as p44 MAPK) and ERK2 (also known as p42 MAPK), are activated via dual phosphorylation

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of threonine and tyrosine residues contained in their catalytic activation loop by upstream kinases [2,3]. The activated ERKs subsequently phosphorylate a variety of intracellular proteins including cytosolic 70/90-kDa ribosomal S6 kinase, c-Raf, EGF receptor, cytosolic phospolipase A2, myelin basic protein and transcription factors c-*myc*, c-*jun* and c-*fos* [6–9]. Modulation of transcription factors by the ERK pathway regulates expression of genes essential for cellular processes such as growth, differentiation, and proliferation. Abnormalities in signal transduction via the ERK pathway may lead to diseases and proliferative disorders, most notably cancer [10]. Elucidating the role of the ERK pathway, as well as other MAPK pathways, in cellular response and disease requires methodology for monitoring cellular levels of the active kinases.

Two widely employed approaches for measuring active MAPK levels are radioenzymatic and Western assays. In the radioenzymatic method, the amount of active MAPK is determined by measuring the transfer of a radioactive γ-phosphate (^{33}P or ^{32}P) group from adenosine-5′-triphosphate (ATP) to a protein or peptide target [11–17]. Myelin basic protein (MBP), or a synthetic peptide representing the consensus sequence of MBP, is most commonly used as a substrate for assaying the ERK isoforms. Quantitation of the phosphorylated peptide product via scintillation counting or phosphoimaging is done after separation of the product from excess radiolabeled ATP using phosphocellulose P81 paper or gel electrophoresis. The radioenzymatic-based approach suffers from drawbacks such as environmental concerns with radioisotope use, multiple washing steps, and off-line detection. In the Western approach, anti-phospho-MAPK antibodies are used to probe the levels of active MAPKs either directly in the gel or after transfer of the MAPKs to nitrocellulose films. The Western approach is limited by relatively long incubation times, the need for multiple antibodies, multiple wash steps and quantitative accuracy.

Alternative assay methods have been pursued recently to overcome the shortcomings of conventional assay techniques. An enzyme-linked immunosorbent assay was done for several kinases [18] and an immunoassay using europium-chelate labeled antibodies was developed for protein tyrosine kinases [19]. Two immunoassays were developed for tyrosine kinases that employed fluorescence polarization for detection [20,21].

Capillary electrophoresis (CE) can be used to assay active kinase enzymes since phosphorylated and nonphosphorylated peptides are easily separable based on differences in charge-to-size ratio imparted by the addition of the phosphate group. CE provides inherent advantages such as reduced sample consumption, separation of background interferents and on-line detection. Several CE applications have been developed for assaying a variety of protein kinases [22–28]. Conventional CE and microchip-based CE assays for measuring protein kinase A levels using fluorescent-labeled peptide substrates have been reported [22,23]. Another microchip-based assay was developed to monitor multiple kinase reactions in a single run [24]. CE-based assays were developed using fluorescent-labeled substrates for calcium/calmodulin-binding protein kinase [26] and protein kinase C [27]. Additionally, an assay based on the use of CE with UV detection for GST-tyrosine kinase [28] has been reported. All of these CE-based approaches were performed serially using a single capillary.

In the early 1990s, parallel or multiplexed capillary electrophoresis (MCE) was introduced by the Mathies [29,30], Yeung [31–33] and Kambara [34,35] groups. The use of multiplexed capillary arrays offer the potential of increasing the sample throughput without a tremendous increase in instrumentation complexity or cost. Initial efforts with multiplexed arrays focused on capillary gel electrophoresis (CGE) for DNA sequencing due to the interest in the Human Genome Project [29–46]. A few reports have also addressed the development, characterization and application of MCE for enzyme analysis, combinatorial screening of catalysis reaction conditions, the analysis of small organic molecules and peptide mapping [47–52]. We report on the application of MCE with laser-induced fluorescence (MCE–LIF) detection for the accurate, precise and rapid determination of active ERK enzyme levels. Both direct and immunoprecipitation MCE–LIF-based assays were developed and applied to the analysis of ERK levels from human umbilical

vein endothelial cells (HUVEC) following stimulation with angiopoietins 1 (Ang1*), a ligand for the TIE family of receptor tyrosine kinases.

2. Experimental

2.1. Chemicals and materials

3-[*N*-Morpholino] propanesulfonic acid (MOPS), $MgCl_2$, adenosine 5′-triphosphate (ATP), borax (sodium tetraborate decahydrate, 99.0–105.0%), β-glycerophosphate and bovine serum albumin (BSA, 98.5%) were from Sigma (St. Louis, MO, USA). Ethylene-glycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was purchased from Fluck Chemie (Buchs, Switzerland). Sodium *ortho*-vanadate (99.98%) and dithiothreitol (DTT) were obtained from Aldrich (Milwaukee, WI, USA). Reagent grade sodium hydroxide (0.1 and 1 N) and HPLC-grade methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Protease inhibitor cocktail tablet (complete, EDTA-free) was from Roche Diagnostics (Mannheim, Germany). Peptides labeled on the N-terminus with fluorescein isothiocyanate (FITC) included APRTPGGRR-COOH, APRT-(PO_3^{2-})PGGRR-COOH and FITC-LRRAS(PO_3^{2-})-LG and were purchased from SynPep (Dublin, CA, USA). Active EKR2 (100 μg/ml) was from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal phospho-p44/p42 MAP kinase antibody (p-Ab) directed against the phosphorylated forms of ERK1 and ERK2 was obtained from Cell Signaling Technology (Beverly, MA, USA). Distilled-deionized water (dd-H_2O) was from a Milli-Q system (Millipore, Bedford, MA, USA). Magnetic Porous Glass (MPG) beads coated with protein A and a 3-in-1 magnetic particle separator (MPS) were obtained from CPG (Lincoln Park, NJ, USA). Ang1* was a generous gift from Regeneron Pharmaceuticals (Tarrytown, NY). Ang1* is a genetically engineered variant of angiopoietin-1 generated by replacing the first 77 residues of angiopoietin-1 with the first 73 residues of angiopoietin-2 (Ang-2). In addition, a non-conserved cysteine residue in the Ang-2 sequence is mutated to serine.

2.2. Buffers and reagents

2.2.1. Buffers

2.2.1.1. Assay dilution buffer (ADB). ADB contained 20 m*M* MOPS, 25 m*M* β-glycerophosphate, 5 m*M* EGTA, 1 m*M* sodium *ortho*-vanadate and 1 m*M* DTT and was adjusted to pH 7.2 using 1 N sodium hydroxide. Following the pH adjustment, BSA was added at the 1 mg/ml level. Immediately before use, one protease inhibitor cocktail tablet was added per 50 ml of the ADB.

2.2.1.2. Cell lysis buffer. Triton lysis buffer consisted of 20 m*M* Tris–HCl, pH 8.0, 137 m*M* NaCl, 2 m*M* EDTA, 1 m*M* Na_3OV_4, 1 m*M* sodium fluoride, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 10% glycerol and 1% Triton X-100.

2.2.1.3. Water–BSA (H_2O–BSA) diluent. Bovine serum albumin (BSA) was dissolved in distilled-deionized water at the 1 mg/ml level.

2.2.2. Growth media

Endothelial growth media (EGM, Clonetics, San Diego, CA, USA) consisted of 10 μg/ml human recombinant epidermal growth factor, 1.0 mg/ml hydrocortisone, 50 mg/ml gentamicin, 50 μg/ml amphotericin B, 3 mg/ml bovine brain extract, 2% fetal bovine serum (Clonetics) and 1% antibiotic/antimycotic from Gibco (Grand Island, NY, USA).

2.2.3. Combined ATP/$MgCl_2$ stock

A 6 m*M* ATP and 153 m*M* $MgCl_2$ combined stock solution (ATP/$MgCl_2$) was prepared by dissolving the appropriate amount of each compound in ADB.

2.2.4. Internal standard (I.S.) stock solution

FITC-LRRAS(PO_3^{2-})LG was dissolved in H_2O–BSA diluent at the 0.2 mg/ml level and was used as the I.S. stock solution. I.S. spiking solutions were prepared from this stock solution by appropriate dilution in H_2O–BSA diluent.

2.2.5. Preparation of MPG protein a beads

MPG protein A beads (50 mg in 10 ml solution) for immunoprecipitation experiments were prepared following the instructions provided by the vendor to give a series of 1.6-ml microtubes each containing 0.25 mg of MPG protein A beads.

2.2.6. MCE background electrolyte

A 50 m*M* borate buffer (pH 9.3) was prepared by dissolving 38.14 g of borax in 2 l of distilled-deioinzed water without adjusting pH. The solution was filtered through a 0.22-μm filter and degassed under vacuum for 1 h.

2.3. Methods

2.3.1. ERK2 product standard curve

A 0.8-mg/ml stock solution of product peptide (FITC-APRT(PO_3^{2-})PGGRR) was prepared in ADB. The stock solution was used to prepare product standard solutions covering a concentration range from 40 to 8000 ng/ml by serial dilution with ADB. An aliquot (10 μl) of each product standard solution was added to a series of 1.6-ml polypropylene microtubes already containing 10 μl of a 2.1 μg/ml I.S. solution, 5 μl of a 108 μg/ml substrate peptide (FITC-APRTPGGRR) solution and 75 μl of the BSA–H_2O solution. The working ERK2 product standards covered a concentration range from 4 to 800 ng/ml. Aliquots (10 μl) of each ERK2 working standard solution were placed into 11 consecutive wells of a 96-well plate and analyzed by MCE–LIF as described below. For each capillary, the peak height for the product peptide was divided by the peak height of the I.S. to generate a peak height ratio (PHR) for that standard. The average PHR for each standard was calculated and plotted versus the product concentration to yield the product standard curve.

2.3.2. Michaelis–Menton curve

ERK2 substrate peptide (FITC-APRTPGGRR) solutions were prepared in H_2O–BSA solution over a concentration range from 18 μg/ml to 2.4 mg/ml. An aliquot (60 μl) of each substrate solution was added to a series of polypropylene microtubes already containing 30 μl of the ATP–$MgCl_2$ solution and 30 μl of ADB and mixed. An aliquot (30 μl) of a 200-ng/ml ERK2 enzyme solution in ADB was added to each microtube, the contents of each tube mixed and incubated at 37 °C. The final concentrations of ERK2 substrate in the incubation mixtures were 7.2, 15, 30, 60, 120, 240, 480 and 960 μg/ml while the final ERK2 enzyme concentration was 40 ng/ml. During the incubation, aliquots (10 μl) were removed from each tube at several time points (140, 310 and 440 s) and added to individual polypropylene microtubes already containing 90 μl of a 230 ng/ml I.S. solution. The dilution step effectively stopped the enzyme reaction. Each of the resulting diluted incubation mixtures was analyzed by MCE–LIF in triplet as described below. The average PHR for the product peptide was obtained for each substrate concentration and was used to calculate the concentration of the product peptide by interpolation from a product standard curve prepared as described above. A plot of product concentration versus reaction time was generated for each substrate concentration and linear regression of each plot was used to obtain the initial reaction rate velocity for each substrate concentration. Only data collected before 10% substrate conversion was used for linear regression. The initial rates were plotted versus the peptide concentration to generate the Micheals–Menton plot using GraFit Version 4.0 (Erithacus Software, Staines, UK).

2.3.3. Enzyme progression plot

Aliquots (60 μl) of a series ERK2 enzyme stock solutions (10, 100 and 250 ng/ml) in ADB were added to a series of polypropylene microtubes already containing 180 μl of peptide substrate solution (333 μg/ml) and 60 μl of the ATP–$MgCl_2$ solution. The resulting solutions were mixed and incubated at 37 °C. At given time intervals, a 10-μl aliquot of each incubation mixture was removed and pipetted into a plastic microtube already containing 90 μl of a 230 ng/ml I.S. solution to stop the reaction. The final concentrations of ERK2 in the incubation mixtures were 2, 20 and 50 ng/ml and the peptide substrate concentration was 200 μg/ml. Each solution was analyzed in triplicate by MCE–LIF as described below. The average PHR was determined

for the generated peptide product for each ERK2 enzyme concentration and the PHR was plotted versus incubation time to generate the enzyme progression curves.

2.3.4. Immunoprecipitation of ERK2

Aliquots (100 μl) of ERK2 solutions, either standards or cell lysate samples, were added to polypropylene microtubes containing 170 μl of ADB and 45 μl of polyclonal ERK antibody, vortexed and incubated overnight at 4 °C with gentle rocking. After incubation, the solutions were transferred to tubes containing 0.25 mg MPG protein A beads for immunoprecipitation. The mixtures were incubated for another hour with gentle rocking at 4 °C. The beads were then collected using the MPS device to trap the magnetic beads against the sides of the tubes. The supernatants were removed and stored at 4 °C for the determination of ERK2 levels remaining in solution. The beads containing the antibody-bound ERK2 were washed sequentially with 1 ml of ADB, 1 ml of 1 mg/ml BSA in PBS and twice with 1 ml of PBS. For each washing the tubes were placed in the MPS device and the supernatant was carefully removed and discarded. The beads were then suspended in 15 μl of ADB prior to assaying for ERK2 levels via reaction with the enzyme substrate followed by analysis using MCE–LIF. In some studies, the immunoprecipitation was performed twice on the same supernatant to determine the efficiency of the immunoprecipitation procedure.

2.3.5. ERK2 enzyme standard curves

2.3.5.1. Direct assay ERK2 standard. Aliquots (15 μl) of ERK2 stock solutions (7.8–250 ng/ml) in ADB were added to separate polypropylene microtubes already containing 45 μl of a 333 mg/ml peptide substrate solution and 15 μl of the ATP–$MgCl_2$ solution, vortexed and incubated at 37 °C for 15 min. The final concentration of ERK2 enzyme in the reaction mixtures ranged from 1.6 to 50 ng/ml. After incubation, the reaction was stopped by adding 10 μl of the enzyme reaction mixture to 90 μl of a 230 ng/ml I.S. solution. The resulting solutions were analyzed in triplicate by MCE–LIF as described below. The average PHR for the generated product was obtained for each enzyme standard and plotted versus enzyme concentration to generate a linear regression standard curve. Similarly, an ERK2 enzyme standard curve ranging from 0.25 to 10 ng/ml was prepared and the incubation time increased to 60 min.

2.3.5.2. Immunoprecipitation ERK2 standard curve. An ERK2 immunoprecipitation standard curve, covering a concentration range from 1 to 318 ng/ml, was prepared by mixing 100-μl aliquots of individual ERK2 standards in ADB (7.8–1178 ng/ml) with 170 μl of ADB in polypropylene microtubes and immunoprecipitating as described above. The isolated beads were suspended in 15 μl of ADB and added to separate polypropylene microcentrifuge tubes already containing 45 μl of a 333 μg/ml peptide substrate solution and 15 μl of ATP–$MgCl_2$ solution. The resulting mixtures were vortexed and incubated at 37 °C for 15 min with vortexing every 5 min to suspend the beads. Following the incubation, the beads were removed using the MPS device and 10 μl of each supernatant was diluted with 90 μl of a 230 ng/ml I.S. solution and analyzed by MCE–LIF in triplicate as described below. The average PHR for the product for each standard was plotted versus the ERK2 concentration to generate a linear regression ERK2 immunoprecipitation standard curve.

2.3.6. In vitro human umbilical vein endothelial cell (HUVEC) studies

HUVECs were obtained as primary cells from Clonetics. HUVECs (passage <4) were maintained in endothelial growth medium (EGM) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were handled in accordance with manufacturer's recommendations. HUVECs were plated in tissue culture dishes at 1×10^6 cells per 100-mm dish and grown to 90% confluency in EGM (Clonetics). Prior to stimulation, cultured HUVEC were serum-starved for 2 h by replacing EGM with phosphate-buffered saline to reduce basal ERK activity. ERK was activated by adding various concentrations of Ang1* in PBS–0.2% BSA for 7 min. Cells were

lysed directly in the plates by the addition of 1 ml of cell lysis buffer (see above). The plates were then incubated at 4 °C for 20 min with shaking. The supernatants were transferred to a clean polypropylene tubes and stored frozen at −80 °C until analysis.

2.3.7. Analysis of cell lysate samples by MCE–LIF

2.3.7.1. Direct analysis of cell lysates. Aliquots (15 μl) of the HUVEC cell lysates described above were added into separate polypropylene microtubes already containing 45 μl of 333 μg/ml ERK2 substrate and 15 μl of ATP–$MgCl_2$ solution and incubated at 37 °C for 15 min. A 10-μl aliquot of each incubation mixture was removed and added to polypropylene microtubes already containing 90 μl of a 230 ng/ml I.S. solution and analyzed in triplicate by MCE–LIF as described below. The average PHR for the product obtained for each sample was used to determine the concentration of ERK2 in the cell-lysate sample by interpolation from a linear regression standard curve prepared by the direct assay approach described previously.

2.3.7.2. Immunoprecipitation analysis of cell lysates. Aliquots (100 μl) of the cell lysate samples were added to microtubes containing 170 μl of ADB. The resulting solutions were immunoprecipitated with ERK2 polyclonal antibody and MPG protein A beads as described previously. The isolated beads for each sample were resuspended in 15-μl aliquots of ADB and added to separate polypropylene microtubes already containing 45 μl of 333 μg/ml ERK2 substrate and 15 μl of ATP–$MgCl_2$ solution. The resulting mixtures were vortexed and incubated at 37 °C for 15 min with vortexing every 5 min to resuspend the beads. Following incubation, the beads were removed using the MPS device and aliquots (10 μl) of each incubation mixture were added to polypropylene microtubes already containing 90 μl of a 230 ng/ml I.S. solution and analyzed in triplicate by MCE–LIF as described below. The average PHR for the product obtained for each sample was used to determine the concentration of ERK2 in the cell-lysate sample by interpolation from a linear regression standard curve prepared by the immunoprecipitation approach as described above.

2.3.8. Accuracy and precision of ERK2 analysis in the cell lysate

2.3.8.1. Direct assay spiking studies. Aliquots (180 μl) of unstimulated HUVEC cell lysate were spiked with 20 μl of either ADB or various ERK2 standard stock solutions (0.125, 1.25 and 5 μg/ml) to give lysates with 0, 12.5, 125 and 500 ng/ml ERK2 enzyme. Aliquots (7.5 μl) of each spiked lysate were added into polypropylene microtubes already containing 7.5 μl of ADB, 45 μl of a 333 μg/ml peptide substrate solution and 15 μl of ATP–$MgCl_2$ solution and mixed by vortexing. The resulting solutions were incubated at 37 °C for 15 min. Following incubation, 10 μl of each reaction mixture was added into polypropylene microtubes already containing 90 μl of a 230 ng/ml I.S. solution, vortexed and analyzed in triplicate by CE–LIF as described below. The average PHR of the product for each spiked sample was used to calculate the concentration of the ERK2 enzyme by interpolation from a linear regression ERK2 standard curve prepared by the direct assay approach as described previously. Recoveries of ERK2 from the spiked lysates were calculated by dividing the ERK2 concentration found in the samples by the expected ERK2 concentration and multiplying by 100.

2.3.8.2. Immunoprecipitation spiking studies. Aliquots (100 μl) of spiked cell lysates prepared above were studied by immunoprecipitation as described previously. The isolated beads containing the bound ERK2 enzyme were suspended in 15-μl aliquots of ADB and added to separate polypropylene microtubes already containing 45 μl of 333 μg/ml ERK2 substrate and 15 μl of ATP–$MgCl_2$ solution. The resulting mixtures were vortexed and incubated at 37 °C for 15 min with vortexing every 5 min to suspend the beads. Then aliquots (10 μl) of each mixture were added to polypropylene microtubes already containing 90 μl of a 230 ng/ml I.S. solution and analyzed in triplicate by MCE–LIF as described below. The average PHR for the product of each sample was used to determine the

concentration of ERK2 in the spiked cell-lysate sample by interpolation from a linear regression ERK2 standard curve constructed using the immunoprecipitation approach described previously. Recoveries of ERK2 from the spiked lysates were calculated by dividing the ERK2 concentration found in each sample by the expected ERK2 concentration and multiplying by 100.

2.4. MCE conditions

The MCE–LIF analysis was done with an HTS9610 electrophoresis system from SpectruMedix (State College, PA, USA). The system was equipped with 96 uncoated fused-silica capillaries (52 cm×50 μm I.D., 35 cm effective length), a 25-A three-phase air-cooled all-line argon ion laser with output power (300 mV) for the wavelength from 458 to 514 nm (457.9 nm, 4%; 476.5 nm, 12%; 488 nm, 22%; 496.5 nm, 13%; 501.7 nm, 7%; and 514.5 nm, 42%) and a CCD camera. A standard 96-well plate format was used at the inlet end with 96 individual inlet electrodes. At the detection window, capillaries were arranged in a parallel format. The CCD camera was used for signal detection with an f4 stop, bin 3 and 570 ms exposure time. Data was collected using Checkmate software and processed with SpectroPro1.80 software (SepectruMedix).

For all studies, the capillaries were conditioned prior to each run by rinsing with 0.1 N NaOH, distilled-deionized water and running buffer for 2 min each at 5 ml/min flow-rate. All electrophoresis was performed at ambient temperature. Samples were injected by pressure (−0.35 p.s.i. for 10 s) and separation was conducted at 2.8 kV for 45 min. The running buffer was 50 m*M* sodium tetraborate (pH 9.3).

3. Results and discussion

3.1. Separation of substrate, product and internal standard by MCE–LIF

A typical CCD image of the 96-capillary array is shown for the ERK2 product standard curve in Fig. 1A. The standards were injected sequentially into 11 consecutive capillaries with the product concentration increasing from the top to the bottom of the array. A water sample was injected into a single capillary between each set of product concentrations. The dots within a given capillary correspond to individual components and the intensity of the dots is proportional to the concentration of the components. The intensity of the ERK2 product can be seen to increase as you move down the array, while the concentration of the substrate and the I.S. remain constant. Additionally, impurities from the substrate

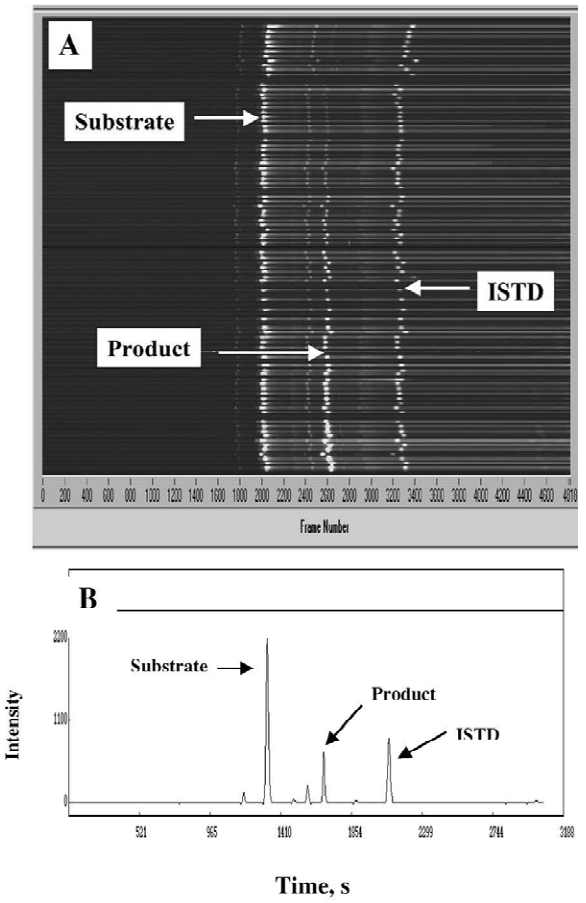

Fig. 1. CE separation of ERK2 product standards: (A) a charge-coupled device image collected for the analysis of a typical ERK2 product standard curve covering peptide product concentrations from 4 to 800 ng/ml, internal standard concentration of 230 ng/ml and a peptide substrate concentration of 5.4 μg/ml and (B) a representative electropherogram for the 160 ng/ml product peptide standard.

and I.S. can also be seen in the CCD image. The streaking observed in some of the capillaries is due to a slight tailing of the analytes being accentuated by the high contrast used to generate the picture. High contrast was employed to allow the lowest standards to be easily visualized. A representative electropherogram constructed from the CCD image is shown in Fig. 1B.

The migration time of each component can be seen to be relatively constant across the array and the intensity for the components within a product standard concentration are relatively constant. Slight variations in the migration time are expected due to slight differences in the capillary surface chemistry between capillaries and the potential for thermal gradients across the capillaries. Similarly, slight variations in the intensities within a standard concentration are expected due to variation in camera digitization error, minor differences in capillary diameters, variations in laser intensity across the capillary and differences in the vacuum injection pressure experienced by individual capillaries during the injection. The incorporation of the I.S. allows for correction of the non-uniformities introduced by the capillary array. The migration time and peak height for the product in a given capillary are corrected by dividing these values by the corresponding migration time and peak height obtained for the I.S. to give the corrected migration time and the PHR. The percent relative standard deviation (%RSD) obtained within-day for the migration time, corrected migration time, peak height and the PHR are shown in Table 1 for several ERK2 product standards and similar results were obtained across days (data not shown). The %RSD values were significantly improved by the incorporation of the I.S. correction. The long-term performance and reproducibility of the MCE–LIF instrumentation has been reported previously [52]. A plot of the PHR for the product peptide was linear over two orders of magnitude with a correlation coefficient of 0.999 (data not shown).

Table 1
Injection precision of ERK2 product standards

[Product standard] (ng/ml)	Percent relative standard deviation ($n=6$)			
	Migration time (s)	Corrected migration time (s)	Peak height	Peak height ratio
4	1.19	0.26	17	8.8
40	0.33	0.07	10	3.0
400	3.79	0.91	11	5.1

3.2. Michaelis–Menton plot and enzyme progression curve for ERK2 enzyme

In developing the ERK2 enzyme assay conditions it was important to determine the optimal substrate concentration for the enzyme reaction. Ideally, the substrate concentration should be on the flat part of the Michaelis–Menton plot to avoid dramatic changes in the reaction rate due to small errors in the substrate concentration and to provide for the maximum reaction rate. A Michaelis–Menton plot was constructed by holding the ERK2 enzyme level constant and varying the substrate concentration from 7 to 960 μg/ml. The reaction rate was determined for each concentration of substrate and the resulting reaction rates were plotted versus the corresponding substrate concentrations to obtain the Michaelis–Menton plot (Fig. 2). A K_{max} value of

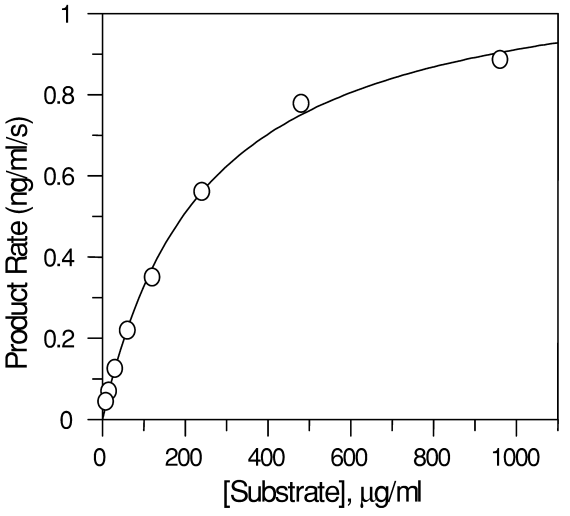

Fig. 2. Michaelis–Menten plot. Plots of reaction rates versus FITC-labeled peptide substrate concentration. Incubations performed using 40 ng/ml ERK2 with various FITC-labeled peptide substrate concentrations (7.5–960 μg/ml) at 37 °C for 15 min ($n=6$ for each substrate concentration).

247±17 μg/ml (168±2 μ*M*) was found for the substrate. However, a substrate concentration of 200 μg/ml was chosen as a compromise between rapid enzyme reaction rate without excessive use of substrate during the enzyme assays. The high throughput capability of the MCE–LIF system allowed the data for the Michaelis–Menton plot to be generated in a single run. It should be noted that the introduction of a fluorescent group into a substrate peptide sequence can change the K_{max} and reaction kinetics relative to the unlabeled substrate, however, for our quantitative purposes this was not of concern.

The time period over which the enzyme incubation yields a linear increase in product concentration was determined in a single run at three ERK2 enzyme levels (2, 25 and 50 ng/ml) using a fixed substrate concentration of 200 μg/ml (Fig. 3). The generation of product was a linear function of time at all enzyme concentrations for at least 20 min. At longer incubation points there was a deviation from linearity at all ERK2 concentrations, however, the 2 ng/ml standard provided linear results for at least 60 min.

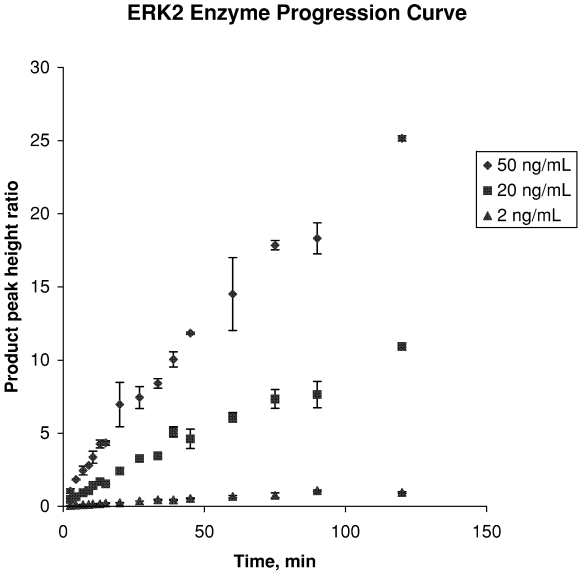

Fig. 3. ERK2 product progression curve. Various ERK2 enzyme concentrations (2, 20 and 50 ng/ml) were incubated with FITC-labeled peptide substrate (200 μg/ml) at 37 °C and samples were taken at various time points. Peak height represents average ($n=4$) for replicate injections.

For routine analysis, the incubation was performed for 15 min at 37 °C.

3.3. Direct ERK2 enzyme standard curve

In the direct assay, the standard curve for ERK2 was constructed in ADB by adding the peptide substrate to a series of tubes containing various ERK2 concentrations, incubating at 37 °C for 15 min, diluting an aliquot of the incubated standard solution with the I.S. solution and analyzing the amount of product formed by multiplexed MCE–LIF. The standard curve for the direct assay was found to be linear over ERK2 concentrations from 4 to 250 ng/ml using a 15-min incubation period (Fig. 4A). The linear range could be lowered to 0.2–10 ng/ml using a 60-min incubation interval (Fig. 4B). In general, the RSD for replicate injections ($n=3$) of the standards ranged from 3 to 15%.

3.4. Immunoprecipitation ERK2 standard curve

Direct assay of ERK2 levels in cell lysate has the advantage of convenience and speed. However, the HUVEC cell lysate may contain additional kinases that could utilize the FITC-labeled peptides as substrates. To evaluate this possibility an immunoprecipitation assay was developed to isolate ERK2 from the cell lysates prior to analysis. Lysates from HUVEC cells stimulated with Ang1* were treated with a polyclonal antibody selective for the active form of ERK2. The resulting ERK2-antibody complex was isolated from the solution using MPG protein A beads. The efficiency of the isolation step was evaluated by performing two consecutive immunoprecipitation reactions on the same cell lysate. Additionally, control and nonspecific binding samples were obtained by doing no treatment to the lysate and by treating the lysate with protein A beads in the absence of antibody, respectively. The amount of ERK2 remaining in the cell lysate samples and attached to the beads after each treatment was determined by incubation with the peptide substrate followed by MCE–LIF analysis. The PHR obtained for the various cell lysate supernatants and bead samples are shown in Fig. 5A,B, respectively. The control samples maintained a constant level of ERK2 over both incubation periods indicating the enzyme

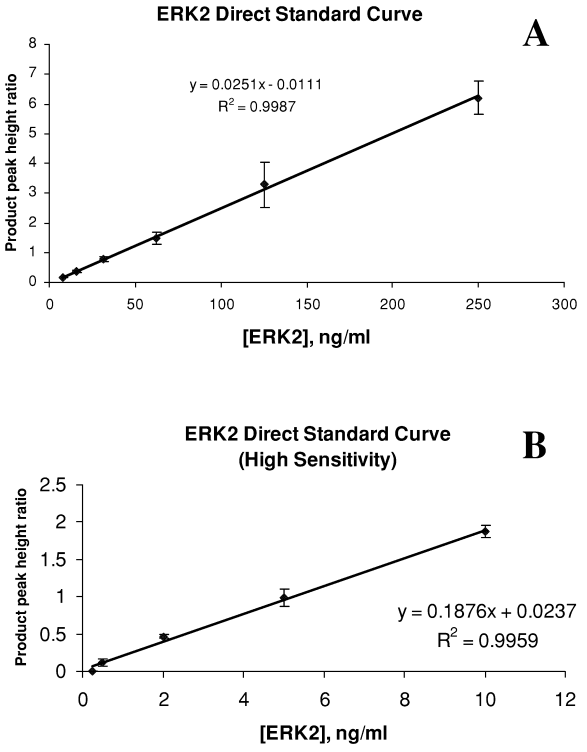

Fig. 4. ERK2 direct standard curves obtained by incubation of: (A) ERK2 standards with 200 μg/ml FITC-labeled peptide substrate at 37 °C for 15 min and (B) ERK2 standards incubated with 200 μg/ml peptide substrate at 37 °C for 60 min. An FITC-labeled peptide internal standard was spiked into each standard prior to analysis. All standards were injected in replicate ($n=4$) and the average peak height was plotted versus ERK2 concentration.

was stable under the study conditions (Fig. 5A). The slight decrease in the ERK2 enzyme level in the cell lysate (Fig. 5A) and the increase in ERK2 levels on the beads (Fig. 5B) following treatment of the lysate with only beads indicates a small amount of non-specific binding. The level of ERK2 was markedly decreased in the cell lysate following the immunoprecipitation treatments (Fig. 5A) and this is reflected by an increased amount of ERK2 found on the beads (Fig. 5B).

An ERK2 immunoprecipitation standard curve was prepared in ADB using the polyclonal antibody and the MPG protein A beads as described above. The ERK2 isolated on the beads was determined by incubating the beads with the peptide substrate and analyzing by MCE–LIF. The immunoprecipitation standard curve was linear over ERK2 concentrations from 1 to 318 ng/ml using a 15-min incubation period (Fig. 6). The RSD for replicate injections ($n=3$) of the standards ranged, in general, from 3 to 15%. As stated previously, the direct assay has the advantage of simplicity and speed while the immunoprecipitation assay offers a higher degree of selectivity by first isolating the ERK2 enzyme from potentially interfering kinases.

3.5. Accuracy and precision of ERK2 assay

The accuracy and precision of measuring ERK2 in cell lysate was evaluated using the direct and immunoprecipitation assay approaches. Cell lysate from unstimulated HUVEC cells was spiked with various amounts of ERK2 (0, 6.25, 62.5 and 250 ng ERK2/ml lysate) and the spiked lysates were analyzed by the direct and the immunoprecipitation approaches. The concentration of ERK2 in the spiked lysates was determined by interpolation from standard curves constructed by the direct and immunoprecipitation approaches. The percent recovery, corrected for the basal levels, for the spiked cell lysates are shown in Table 2. The ERK2 recovery ranged from 103 to 112% and 106 to 120% for the direct and immunoprecipitation assays, respectively. The %RSD for replicate analyses ranged from 2 to 21%. The close agreement between the direct and immunoprecipitation approaches demonstrate the direct approach can be employed for assaying ERK2 levels in HUVEC cells. Therefore, the direct approach was applied to measuring the levels of ERK2 in stimulated HUVECs.

*3.6. Analysis of ERK2 levels in HUVECs stimulated with Ang1**

HUVECs stimulated with various concentrations of Ang1* were analyzed for ERK2 levels by the direct assay approach. Prior to conducting the direct assay, the FITC-labeled substrate and product were shown to be stable in the cell lysate incubation mixture that contained protease and phosphatase inhibitors. An electropherogram obtained from Ang1*-stimulated HUVEC cells is shown in Fig. 7B. In addition to the substrate, product and I.S. peaks a number of additional peaks are present due to the

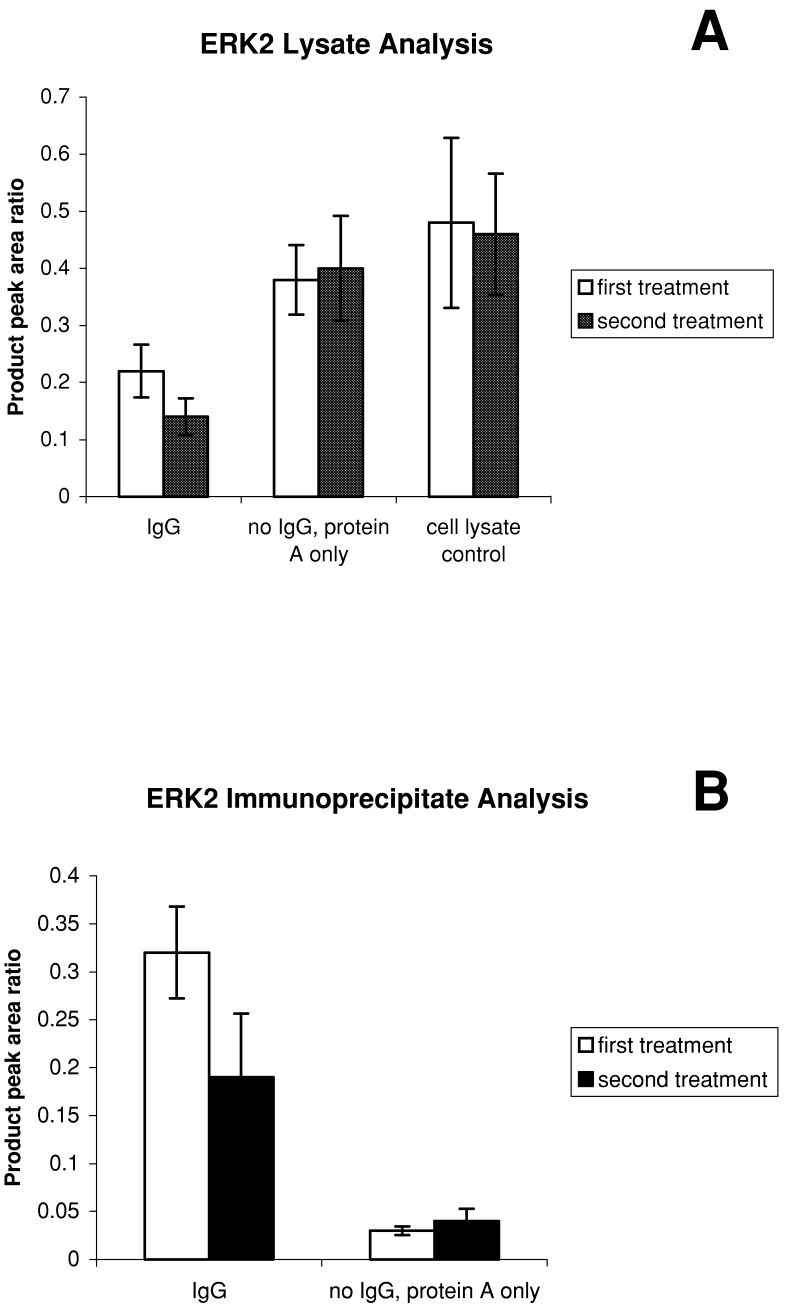

Fig. 5. Immunoprecipitation of ERK2. Plot of peak height ratio obtained for the incubation of FITC-labeled peptide substrate with (A) cell lysates before and after immunoprecipitation with a polyclonal antibody against active ERK2 and (B) GMP beads obtained following immunoprecipitation with polyclonal antibody against active ERK2. Incubations were performed using with 200 μg/ml FITC-labeled peptide substrate at 37 °C for 15 min.

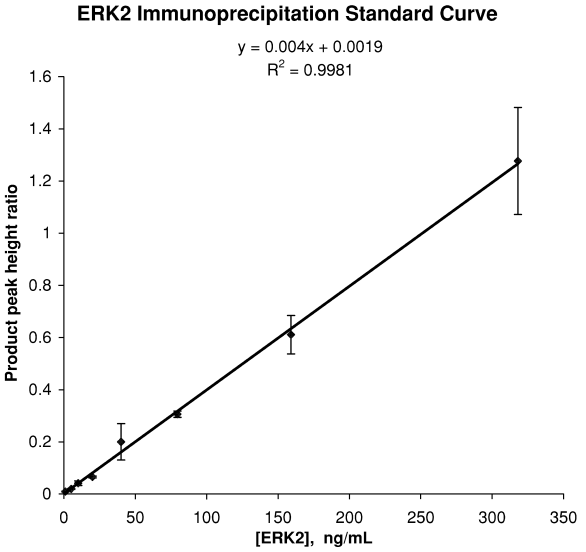

Fig. 6. ERK2 immunoprecipitation-based standard curve obtained by incubating GMP beads obtained from the immunoprecipitation of ERK2 standards with a polyclonal antibody against active ERK2 with 200 μg/ml FITC-labeled peptide substrate for 15 min at 37 °C.

presence of trace impurities in the substrate. A plot of the PHR for the product generated in the stimulated HUVEC samples versus Ang1* concentration is shown in Fig. 7A. The amount of active ERK2 increased, as indicated by the increasing PHR values, with increasing levels of Ang1* used for the stimulation. The MCE–LIF methodology allowed the analysis of 40 samples, in duplicate, to be completed in less than 2 h. Typically, the capillary array was usable for ~100 runs before performance deterioration was noted, usually peak tailing and decreased sensitivity were the major symptoms.

Table 2
Accuracy and precision of ERK2 analysis

Spiked [ERK2] (ng/ml)	% Recovery (% RSD)	
	Direct assay	Immunoprecipitation assay
250	103 (3.5)	106 (7.5)
62.5	102 (9.0)	120 (22)
6.25	112 (20)	112 (10)

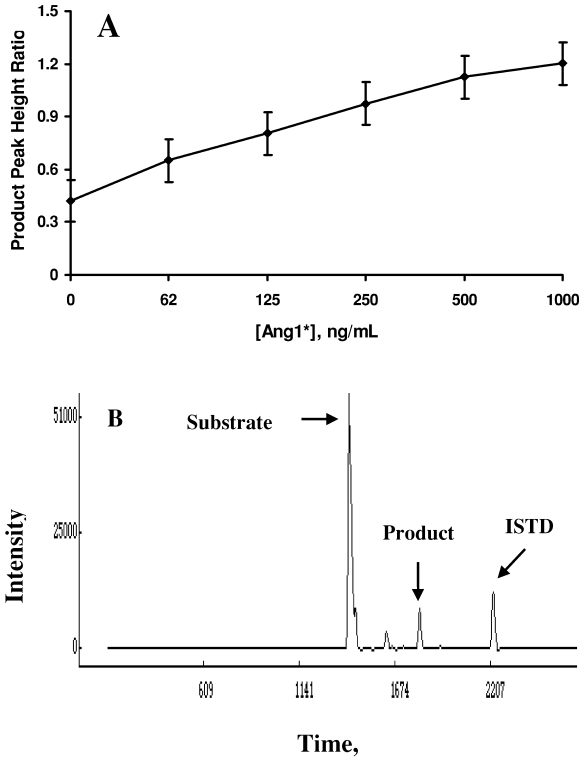

Fig. 7. Stimulation of human umbilical vein endothelial cells (HUVEC) with angiopoietin (Ang 1*): (A) plot of the average peak height ratio versus Ang1* concentration, $n=3$ replicate HUVEC samples for Ang 1* concentration and (B) a representative electropherogram obtained for a HUVEC sample stimulated with 1000 ng/ml Ang1*.

4. Conclusion

The determination of active kinase enzymes levels from in vitro and in vivo settings can provide important information for determining intervention points in disease processes, for monitoring the effect of therapeutic treatments and for studying the mechanism of drug action. We have shown MCE–LIF can provide a rapid approach for monitoring the levels of ERK2 in HUVEC lysates by employing a fluorescently labeled peptide substrate. The methodology was shown to provide accurate and precise quantitation of ERK2 levels using both direct and immunoprecipitation assay formats. The direct assay format has the advantage of simplicity and speed and allows for the analysis of 40 samples in duplicate in less

than 2 h. The MCE–LIF approach should be generally applicable for the determination of variety of kinase enzymes.

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