

# High-Throughput Separations Using Multiplexed Capillary Electrophoresis with Laser-Induced Fluorescence Detection



The authors evaluated the performance of multiplexed capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for high-throughput separation-based assays using fluorescein isothiocyanate as a model compound and fluorescein as an internal standard. They assessed performance areas, including linearity of response, precision of migration time, peak area and peak height response with and without internal standard correction, limit of detection, cross-talk between adjacent capillaries, and reproducibility of performance over time. In this article, they also discuss representative data for the application of multiplexed CE-LIF to screening enzyme inhibitors and measuring endogenous enzyme levels.

Capillary electrophoresis (CE) is a high-efficiency separation technique capable of analyzing a wide variety of compounds using extremely small sample volumes in a short period of time (1). Scientists have used CE to analyze compound classes ranging from small inorganic ions to large biomolecules such as proteins and deoxyribonucleic acid (DNA). Since its introduction, CE has been performed serially using a single capillary; however, the Mathies (2,3), Yeung (4,5), and Kambara (6,7) groups introduced parallel or multiplexed CE in the early 1990s. The use of multiplexed (96-lane) capillary arrays offers the potential of increasing the throughput of sample analysis without a tremendous increase in instrumentation complexity or cost. Initial efforts with multiplexed arrays focused on capillary gel electrophoresis for DNA sequencing because of the interest in the Human Genome Project (2–21). Several commercial DNA sequencers based upon multiplexed capillary gel electrophoresis are available, including the Prism 3700 DNA analyzer (Applied Biosystems, Foster City, California), the MegaBace 1000 system (Molecular Dynamics, Sunnyvale, California), and the SCE9600 genetic analyzer (SpectruMedix,

State College, Pennsylvania). A few reports have addressed the development, characterization, and application of multiplexed CE for enzyme analysis, combinatorial screening of catalysis reaction conditions, the analysis of small organic molecules, and peptide mapping (22–26).

We evaluated the performance of multiplexed CE with laser-induced fluorescence detection (LIF) using fluorescein isothiocyanate (FITC) as a model compound and fluorescein as an internal standard. We used these compounds to characterize the linearity, precision, limit of detection, day-to-day reproducibility, and cross-talk of the multiplexed CE-LIF system. In addition, we performed multiplexed CE-LIF separations to determine endogenous enzyme levels and to screen for enzyme inhibitors.

## Experimental

**Chemicals and reagents:** We purchased the disodium salt of fluorescein from ICN (Aurora, Ohio) and FITC from MicroSolv (Long Branch, New Jersey). The bovine serum albumin (98.5%), boric acid, borax (sodium tetraborate decahydrate, 99.0–105.0%), 3-[*N*-morpholino]-propane-sulfonic acid, magnesium chloride, adenosine-5'-phosphate, and  $\beta$ -glycerophosphate

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were bought from Sigma (St. Louis, Missouri). We purchased sodium orthovanadate (99.98%), ethylene-glycol-bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid, and dithiothreitol from Aldrich Chemical Co. (Milwaukee, Wisconsin). The reagent-grade sodium hydroxide (0.1 N and 1 N) and high performance liquid chromatography-grade methanol were bought from JT Baker (Phillipsburg, New Jersey).

We generated distilled, deionized water using a Milli-Q water-purification system (Millipore, Bedford, Massachusetts). The matrix metalloprotease-13 enzyme and proprietary metalloprotease-13 inhibitors were prepared at Procter & Gamble Pharmaceuticals (Mason, Ohio). We purchased zymogram developing buffer from Novex (Carlsbad, California). We bought the mitogen-activated protein kinase 2 enzyme from Upstate Biotechnology (Lake Placid, New York) in an initial enzyme concentration of 100  $\mu\text{g}/\text{mL}$ . Peptides labeled on the *N*-terminus with FITC included Pro-Leu-Gly-Leu-Ala-Arg (metalloprotease-13 substrate), Pro-Leu (metalloprotease-13 product), Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg (mitogen-activated protein kinase 2 substrate), Ala-Pro-Arg-Thr( $\text{PO}_3^{2-}$ )-Pro-Gly-Gly-Arg-Arg (mitogen-activated protein kinase 2 product), and Leu-Arg-Arg-Ala-Ser( $\text{PO}_3^{2-}$ )-Leu-Gly (mitogen-activated protein kinase 2 internal standard). These peptides were synthesized by SynPep (Dublin, California).

**Preparation of FITC standards:** We prepared initial solutions of FITC and fluorescein in methanol at the 0.39- and 0.33-mg/mL levels, respectively. We serially diluted an aliquot of the 0.39-mg/mL FITC solution with 25 mM sodium tetraborate (pH 9.3) to prepare a series of eight FITC stock solutions that covered a 0.95–95 ng/mL concentration range. The 0.33-mg/mL fluorescein solution was diluted with 25 mM sodium tetraborate (pH 9.3) to yield a 2.5- $\mu\text{g}/\text{mL}$  internal standard spiking solution. We prepared working FITC standard solutions by adding a 1-mL aliquot of each FITC stock solution into vials that already contained 20  $\mu\text{L}$  of the 2.5- $\mu\text{g}/\text{mL}$  internal standard spiking solution. The working standards had a 0.95–95 ng/mL FITC concentration range, and each contained 50 ng/mL of the internal standard. We divided each of the resulting working standards solutions into small aliquots and stored them in polypropylene vials with polytetrafluoroethylene-lined caps at  $-70^\circ\text{C}$ . On each analysis day, we removed a fresh vial of each working standard from storage,

thawed it, used it for analysis, and discarded it.

**Performance characterization:** We used the FITC working standards to examine the linearity, precision, reproducibility, and cross-talk of the multiplexed CE-LIF instrumentation. Each of the eight standards was loaded into individual rows of a 96-well plate so each standard would be injected into 12 separate but consecutive capillaries. We determined the average values for peak area, peak height, peak area ratio (FITC-to-fluorescein peak areas), and peak height ratio (FITC-to-fluorescein peak heights) for each standard. We used the average peak height and peak area ratios for each standard ( $n \leq 12$ ) to construct a nonweighted linear regression curve. We generated replicate ( $n = 2-3$ ) standard curves each day for three days and calculated the slope, intercept, and correlation coefficient for each curve.

We evaluated the precision of the response and migration time for the standards by examining the average values for the migration time, corrected migration time (ratio of FITC to fluorescein migration times), peak area, peak height, peak height ratio, and peak area ratio for each FITC standard in a representative standard curve. Additionally, we compared these values for selected standards across standard curves obtained during three days.

The reproducibility of the instrument performance was determined by performing 20 consecutive injections from a 96-well microtiter plate filled with the 95-ng/mL FITC standard. We examined the relative standard deviation (RSD) values for the corrected migration time and peak area ratio.

The cross-talk between adjacent capillaries was determined by injecting a blank buffer on either side of the capillaries that contained the 95-ng/mL FITC standard. With this approach, each blank capillary reflected cross-talk from only one adjacent capillary. We calculated the cross-talk by dividing the peak area of the FITC peak in the blank buffer capillary by the FITC peak area from the adjacent capillary with the 95-ng/mL FITC standard.

**Metalloprotease-13 enzyme inhibition study:** We prepared a stock solution of the metalloprotease-13 enzyme (0.5 nM) in the zymogram developing buffer. We initially dissolved the peptide substrate for the metalloprotease-13 enzyme — FITC-Pro-Leu-Gly-Leu-Ala-Arg — in methanol and diluted an aliquot of this solution with the zymogram developing buffer, which contained 3% dimethyl sulfoxide, to obtain a 4  $\mu\text{M}$  substrate working solution. We also

prepared proprietary metalloprotease-13 inhibitors at the 16.7 mM level in the zymogram developing buffer with 3% dimethyl sulfoxide. Enzyme reaction mixtures were prepared by mixing 100  $\mu$ L of the metalloprotease substrate solution, 50  $\mu$ L of the metalloprotease enzyme solution, and 50  $\mu$ L of the various proprietary inhibitors in small polypropylene tubes. We kept the reaction mixtures at ambient temperature for 30 min and then diluted them 10-fold with 10 mM boric acid that was adjusted to pH 10 with 1 N sodium hydroxide. The resulting samples were analyzed by CE-LIF, as described below.

**Mitogen-activated protein kinase 2 enzyme standard curve:** We prepared an assay dilution buffer that contained 20 mM 3-[*N*-morpholino]-propanesulfonic acid, 25 mM  $\beta$ -glycerophosphate, 5 mM ethylene-glycol-bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid, 1 mM sodium orthovanadate, and 1 mM dithiothreitol; it was adjusted to pH 7.2 using 1 N sodium hydroxide. After the pH adjustment, we added bovine serum albumin at the 1-mg/mL level. We prepared a 2.5 mM adenosine-5'-phosphate and 75 mM magnesium chloride combined stock solution by dissolving the appropriate amount of each compound in assay dilution buffer. The mitogen-activated protein kinase 2 peptide substrate (FITC-Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg) was prepared at the 333- $\mu$ g/mL level by dissolving an appropriate weight of the peptide in assay dilution buffer. We prepared mitogen-activated protein kinase 2 enzyme stock standard solutions ranging from 7.8 to 250 ng/mL by serial dilution of the initial 100- $\mu$ g/mL mitogen-activated protein kinase 2 enzyme solution with assay dilution buffer. A 250-ng/mL internal standard spiking solution was prepared by dissolving an appropriate amount of the FITC-labeled internal standard (FITC-Leu-Arg-Arg-Ala-Ser[PO<sub>3</sub><sup>2-</sup>]-Leu-Gly) in assay dilution buffer. We prepared mitogen-activated protein kinase 2 enzyme working standards by aliquoting 45  $\mu$ L of the 333- $\mu$ g/mL peptide substrate solution, 15  $\mu$ L of the combined adenosine-5'-phosphate-magnesium chloride stock solution, and 15  $\mu$ L of each mitogen-activated protein kinase 2 enzyme stock standard solution into a series of microcentrifuge tubes. The resulting mixtures provided mitogen-activated protein kinase 2 enzyme working standards that covered a 1.5–50 ng/mL enzyme concentration range.

The mitogen-activated protein kinase 2 enzyme working standard solutions were

vortexed and incubated at 37 °C for 15 min. The enzyme reaction was stopped by diluting 10  $\mu$ L of each mitogen-activated protein kinase 2 enzyme working standard solution with 90  $\mu$ L of the 250-ng/mL internal standard solution and vortexing. Each working standard was prepared in triplicate and analyzed by CE-LIF, as described below. The average peak height ratio for each standard was plotted versus the mitogen-activated protein kinase 2 enzyme concentration to generate a standard curve.

**CE instrumentation:** For analysis, we used an HTS9610 CE system (Spectru-Medix) equipped with 96 separate 52 cm  $\times$  50  $\mu$ m uncoated fused-silica capillaries (35-cm effective length); a 25-A, three-phase, air-cooled all-line argon ion laser with 300-mW output power for wavelengths 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 charge-coupled device camera. The inlet end of the CE system was arranged in a standard 96-well microtiter plate format with 96 separate inlet electrodes. All the capillaries were in a parallel format at the detection window, and a common outlet was used for the exit of the capillaries. The charge-coupled device camera was used with an *f*/4 stop, bin 2, and 500-ms exposure time to obtain the separation image. Data were collected using Checkmate software and processed with SpectruPro 1.80 software (both from SpectruMedix). All instrumental conditioning, separation, and data-acquisition parameters were controlled automatically by the software.

**CE conditions:** For all studies, we conditioned the capillaries before each run by sequentially rinsing them with 0.1 N sodium hydroxide; distilled, deionized water; and running buffer for 2 min each using a 5-mL/min flow rate. All separations were performed at ambient temperature.

The separation conditions for FITC used a hydrodynamic injection (–0.5 psi for 10 s), a 25 mM sodium tetraborate running buffer (pH 9.3), a 5-kV separation voltage, and a 25-min run time. For the limit of detection study, we changed the hydrodynamic injection to a –1.0 psi injection for 10 s.

The separation conditions for the metalloprotease enzyme work included a hydrodynamic injection (–0.5 psi for 10 s), a 10 mM boric acid run buffer adjusted to pH 10 using 1 N sodium hydroxide, an 11-kV separation voltage, and a 7.5-min run time.

The mitogen-activated protein kinase 2

enzyme analysis used a hydrodynamic injection (–0.5 psi for 10 s), a 50 mM sodium tetraborate running buffer (pH 9.3), a 2.8-kV separation voltage, and a 45-min run time.

## Results and Discussion

**Multiplexed CE-LIF performance characterization: CE separation:** Figure 1a shows a typical charge-coupled device image of all 96 capillaries for the analysis of an FITC standard curve. Figures 1b–1e show the resulting electropherograms obtained from the charge-coupled device image for several representative standards. Each standard was injected into 12 sequential capillaries with concentration increasing from the bottom to the top of the capillary array (Figure 1a).

The spots correspond to the individual components in the standards, and the frame number translates into the migration time for each peak. The more defined and intense the spot, the sharper the peak shape and the larger the peak area and peak height. The intensity of the FITC peak increases from the bottom to the top of the array as the concentration of FITC increases, and the intensity of the fluorescein internal standard remains constant. An impurity in the FITC becomes visible between the FITC and fluorescein spots as the concentration of FITC increases. The migration times were relatively constant across the array. The streaking observed in some of the capillaries is caused by a slight tailing of the analytes being accentuated by the high contrast used to generate the picture. We used the high contrast to allow the lowest standards to be seen easily.

**Linearity and limit of detection:** We injected FITC standards in order of increasing concentration across the capillary array, with each standard being injected into 12 consecutive capillaries. We used the average peak area and peak height ratios for each standard to construct a nonweighted linear regression curve. Table I lists the statistical figures of merit for replicate standard curves collected during three days. The standards were linear for two orders of magnitude with correlation coefficient values of at least 0.999. The slope remained constant during the three-day period, and the intercept remained near zero.

The limit of detection for FITC under our operating conditions was 165 pg/mL ( $5 \times 10^{-10}$  M) using a nonstacking hydrodynamic injection in which the samples were dissolved in the running buffer. The signal-to-noise ratio (S/N) obtained under these conditions ranged from 2 to 4 across the

capillary array and was comparable to the limit of detection obtained with single-lane CE-LIF instruments. Several options exist for lowering the limit of detection, including using a stacking injection, increasing the laser power from half intensity used in this study to full intensity, and optimizing the exposure time and bin number for sensitivity.

**Precision:** We examined the precision of the average values for the migration time, corrected migration time, peak area, peak area ratio, peak height, and peak height ratio using data obtained from a representative standard curve (Table II). Table III lists the RSD values for the internal standard-corrected fluorescence values for several standards obtained from the first standard curve run on each of the three days. The RSDs obtained for the average values of the migration time, peak areas, and peak heights were 0.56–1.2%, 10.2–23.9%, and 7.4–20.4%, respectively, across the standards (Table II). Using an internal standard to correct each of these parameters resulted in dramatic improvement in the RSDs for the average values of the corrected migration times, peak area ratios, and peak height ratios, with values of 0.064–0.11%, 1.6–6.3%, and 1.8–4.2%, respectively, across the capillaries (Table II). We found similar results for the RSDs of the peak area and peak height ratios for several standard levels during three days (Table III).

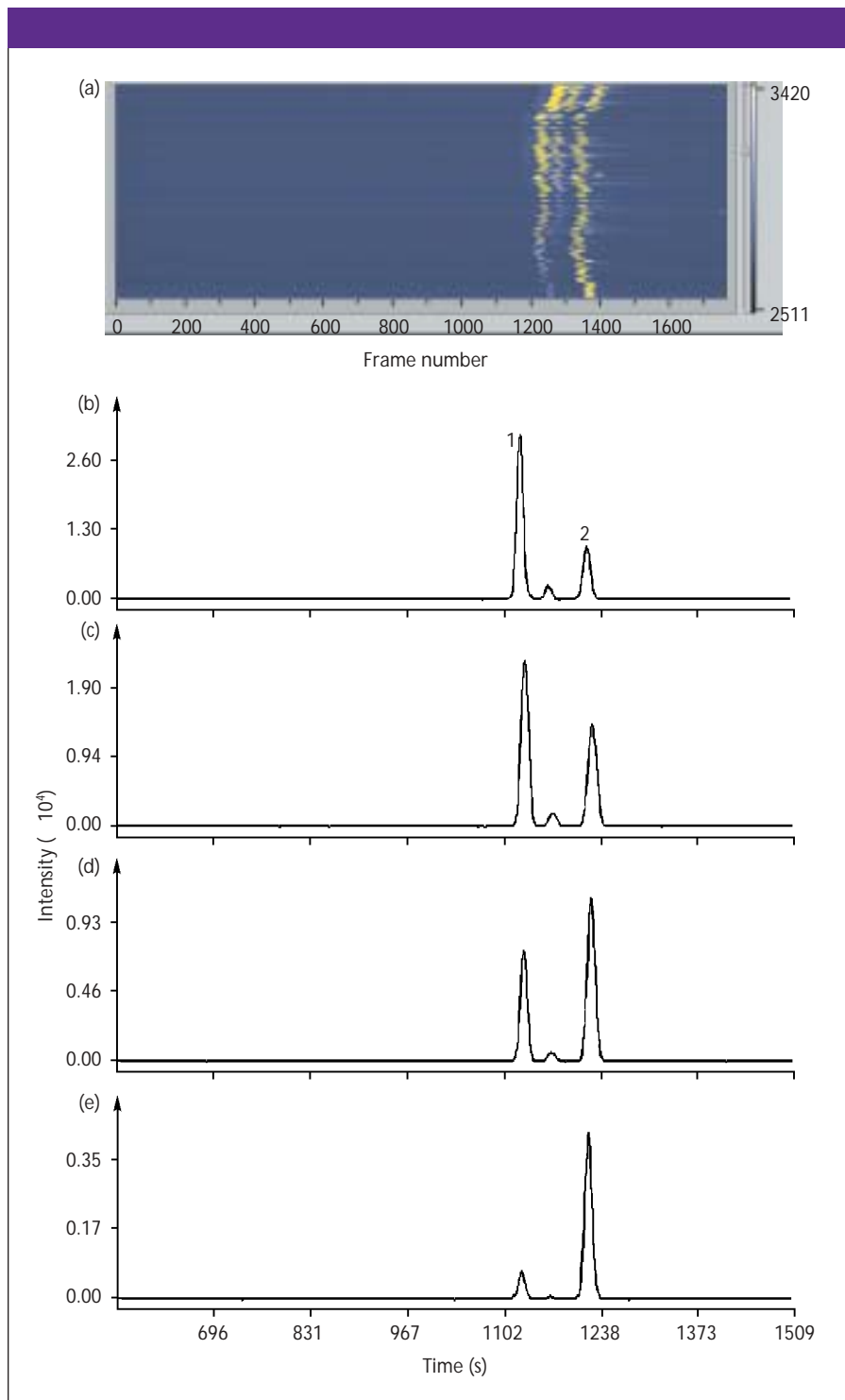
We expected the variation in the average values of the peak areas and peak heights resulting from variation in camera digitization error, differences in the inner diameters of the capillaries, small differences in the vacuum experienced by the capillaries during injection, and slight variations in the intensity of the laser excitation throughout the entire array and optical collection system. Similarly, we anticipated slight variations in the average value of the migration times across the capillaries because of differences in the surface properties of individual capillaries and the potential for slight temperature differences across the capillary array. Using an internal standard to generate corrected migration times and peak area and peak height ratios dramatically improved the precision of our data.

Our results are in agreement with those of Yeung and co-workers (19,20), who used two internal standards to correct variations in migration time and peak area for the separation of four kinds of fluoresceins. They found RSD values for uncorrected migration times and peak areas were 6.3–9.7% and 39–55%, respectively. After the nor-

malization with two internal standards, their RSDs for migration time and peak areas dropped to 0.6–1.4% and 2.6–12%, respectively.

**Reproducibility and cross-talk:** We examined the reproducibility of the instrument performance by running 20 consecutive injections from a microtiter plate filled with

the 95-ng/mL FITC standard. Data from every third injection, starting with the second injection, were processed for a capillary from the middle of each row to examine the reproducibility of the corrected migration time (Table IV) and the peak area ratio (Table V). The corrected migration time remained constant throughout the 20 runs



**Figure 1:** CE separation of FITC standards. Shown are (a) a charge-coupled device image collected for the analysis of a typical FITC standard curve covering a concentration range from 0.95 to 95 ng/mL and representative electropherograms obtained from the analysis of FITC standards at (b) 47.7-, (c) 23.8-, (d) 9.53-, and (e) 2.38-ng/mL concentrations. Peaks: 1 = FITC, 2 = fluorescein.

as reflected by the low RSD values, which ranged from 0.04% to 0.14%. The peak area ratio for FITC obtained within a capillary

also exhibited good reproducibility, with RSD values of 3.5–7.5%. As previously mentioned, the results obtained for

the analysis of replicate standard curves during three days also demonstrated the stable performance of the system over time.

Cross-talk between adjacent capillaries is a concern because of the close proximity of capillaries in the multiplexed array. To examine the cross-talk, we flanked capillaries injected with a high concentration of FITC standard (95 ng/mL) with two capillaries injected with blank running buffer. We determined cross-talk caused by fluorescence scattering from capillaries with the FITC standard by dividing the FITC peak area observed in the blank capillaries by the corresponding peak area from the adjacent FITC-containing capillary.

The average cross-talk across the entire array was 0.46%, with a range of 0.22–0.87%. The observed range in the cross-talk likely was the result of several factors, including uneven spacing of some capillaries in the detection window, variation in the diameter of each capillary, variation in vacuum injection, and optical distortion of the image system. In many cases, the small amount of cross-talk observed between the capillaries may present no problem; however, judicious choice of sample and standard arrangement in the 96-well microtiter plate could help alleviate any problems created by a small amount of cross-talk.

**Application of multiplexed CE-LIF to enzyme assays: Enzyme inhibitor screening:** Endogenous enzymes are involved in a variety of disease states and often are targets for therapeutic drug development. Scientists routinely develop high-throughput screening assays in homogeneous formats using 96- or 384-well microtiter plates to identify small molecule enzyme inhibitors. Frequently, peptides tagged strategically with a fluorescent label and a quencher are used as the substrate for the enzymes. After cleavage of the substrate peptide by the enzyme, the quencher is removed, and the peptide product fragment with the fluorescent label can be detected. Analysts use the amount of fluorescent product generated by the enzyme to determine the extent of inhibition by test compounds. However, false positive or negative results can occur in homogeneous assay systems if the test inhibitors either fluoresce at the chosen excitation wavelength or quench the fluorescence of the enzyme product.

Multiplexed CE-LIF offers a rapid method to obtain high-quality data, and it avoids the above-mentioned potential problems because CE provides a separation step before the detection of the enzyme product. In contrast to a homogeneous microtiter

**Table I:** Figures of merit for FITC standard curves

| Day | Run Number | Peak Area Ratio         |                        |                         | Peak Height Ratio       |                        |                         |
|-----|------------|-------------------------|------------------------|-------------------------|-------------------------|------------------------|-------------------------|
|     |            | Slope ( $\times 10^7$ ) | Intercept              | Correlation Coefficient | Slope ( $\times 10^7$ ) | Intercept              | Correlation Coefficient |
| 1   | 1          | 2.63                    | $3.06 \times 10^{-5}$  | 0.9995                  | 2.32                    | $-7.55 \times 10^{-3}$ | 0.9994                  |
|     | 2          | 2.62                    | $8.50 \times 10^{-4}$  | 0.9996                  | 2.33                    | $-3.52 \times 10^{-3}$ | 0.9995                  |
|     | 3          | 2.57                    | $5.74 \times 10^{-4}$  | 0.9995                  | 2.28                    | $1.54 \times 10^{-3}$  | 0.9995                  |
| 2   | 1          | 2.61                    | $1.25 \times 10^{-2}$  | 0.9994                  | 2.28                    | $6.11 \times 10^{-3}$  | 0.9995                  |
|     | 2          | 2.65                    | $1.22 \times 10^{-2}$  | 0.9999                  | 2.32                    | $7.05 \times 10^{-3}$  | 0.9998                  |
|     | 3          | 2.63                    | $1.78 \times 10^{-2}$  | 0.9998                  | 2.30                    | $1.58 \times 10^{-3}$  | 0.9998                  |
| 3   | 1          | 2.56                    | $-2.62 \times 10^{-3}$ | 0.9996                  | 2.26                    | $-5.00 \times 10^{-3}$ | 0.9996                  |
|     | 2          | 2.56                    | $1.52 \times 10^{-3}$  | 0.9995                  | 2.24                    | $3.38 \times 10^{-4}$  | 0.9995                  |

**Table II:** Migration time and response precision percent RSD data from representative standard curve\*

| FITC Concentration (ng/mL) | Migration Time | Corrected Migration Time | Peak Area | Peak Area Ratio | Peak Height | Peak Height Ratio |
|----------------------------|----------------|--------------------------|-----------|-----------------|-------------|-------------------|
| 0.95                       | 0.87           | 0.11                     | 23.9      | 6.3             | 17.5        | 4.2               |
| 2.38                       | 0.78           | 0.083                    | 19.6      | 3.0             | 18.8        | 2.6               |
| 4.78                       | 0.67           | 0.065                    | 18.8      | 3.2             | 11.7        | 2.6               |
| 9.53                       | 0.50           | 0.064                    | 12.1      | 2.6             | 7.4         | 2.7               |
| 23.8                       | 1.20           | 0.11                     | 10.2      | 1.6             | 9.6         | 1.8               |
| 47.7                       | 0.56           | 0.076                    | 11.8      | 1.8             | 9.4         | 1.8               |
| 71.5                       | 0.64           | 0.068                    | 21.9      | 2.3             | 18.5        | 1.9               |
| 95.3                       | 0.82           | 0.089                    | 20.9      | 4.1             | 20.4        | 4.0               |

\*  $n \leq 12$  for each standard.

**Table III:** Response precision percent RSD data from standard curves over time\*

| FITC (ng/mL) | Peak Height Ratio |       |       | Peak Area Ratio |       |       |
|--------------|-------------------|-------|-------|-----------------|-------|-------|
|              | Day 1             | Day 2 | Day 3 | Day 1           | Day 2 | Day 3 |
| 0.95         | 4.2               | 2.8   | 3.4   | 6.3             | 3.9   | 4.6   |
| 9.5          | 2.7               | 3.0   | 0.9   | 2.6             | 5.0   | 0.9   |
| 95           | 4.0               | 2.4   | 2.1   | 4.1             | 2.3   | 2.7   |

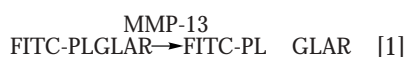
\* Results taken for the first standard curve performed each day;  $n \leq 12$  for each standard.

**Table IV:** Migration precision for multiple runs

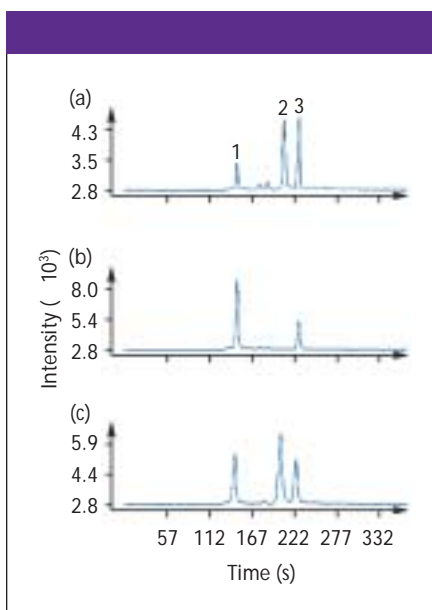
| Capillary Number | Corrected Migration Time |       |       |        |        |        |       | Average | RSD (%) |
|------------------|--------------------------|-------|-------|--------|--------|--------|-------|---------|---------|
|                  | Run 2                    | Run 5 | Run 8 | Run 11 | Run 14 | Run 17 |       |         |         |
| 6                | 0.920                    | 0.921 | 0.921 | 0.921  | 0.923  | 0.922  | 0.921 | 0.11    |         |
| 18               | 0.921                    | 0.922 | 0.921 | 0.922  | 0.922  | 0.922  | 0.922 | 0.06    |         |
| 30               | 0.921                    | 0.919 | 0.920 | 0.919  | 0.919  | 0.922  | 0.920 | 0.14    |         |
| 42               | 0.920                    | 0.921 | 0.921 | 0.921  | 0.921  | 0.921  | 0.921 | 0.04    |         |
| 54               | 0.921                    | 0.921 | 0.921 | 0.921  | 0.922  | 0.922  | 0.921 | 0.06    |         |
| 66               | 0.920                    | 0.922 | 0.921 | 0.923  | 0.923  | 0.922  | 0.922 | 0.13    |         |
| 78               | 0.920                    | 0.922 | 0.922 | 0.922  | 0.923  | 0.923  | 0.992 | 0.12    |         |
| 90               | 0.920                    | 0.920 | 0.919 | 0.921  | 0.922  | 0.921  | 0.921 | 0.11    |         |
| Average          | 0.920                    | 0.921 | 0.921 | 0.921  | 0.922  | 0.922  | —     | —       |         |
| RSD (%)          | 0.06                     | 0.12  | 0.10  | 0.13   | 0.15   | 0.07   | —     | —       |         |

plate-based enzyme inhibition assay that requires the incorporation of a quencher and a fluorescent label in the peptide substrate, multiplexed CE is more simple because only a fluorescent label is required. The addition of the fluorescent tag still can affect the interaction of a peptide substrate with the enzyme, so users should exercise care in positioning the fluorescent label.

We used metalloprotease-13 (Collagenase-3), a protease involved in cartilage remodeling, as a model system for our studies and a peptide that contained a fluorescent label (FITC) as the substrate. As equation 1 shows, the cleavage of the substrate by the metalloprotease-13 enzyme yields two product fragments, one of which contains the FITC label.



We performed a series of enzyme incubations using proprietary metalloprotease-13 inhibitors that possessed a range of inhibitory potencies and used CE-LIF for the separation and detection of the resulting enzyme product. Figure 2 shows representative electropherograms obtained for a standard solution, an enzyme reaction mixture with a highly potent metalloprotease-13



**Figure 2:** Electropherograms obtained for (a) the standard mix containing substrate, product, and internal standard for the metalloprotease-13 assay; (b) the incubation of metalloprotease-13 enzyme with substrate in the presence of a potent metalloprotease-13 inhibitor; and (c) the incubation of metalloprotease-13 enzyme with substrate in the presence of a weak metalloprotease-13 inhibitor. Peaks: 1 = substrate, 2 = product, 3 = fluorescein.

**Table V:** Response precision during multiple runs

| Peak Area Ratio  |       |       |       |        |        |        |         |         |
|------------------|-------|-------|-------|--------|--------|--------|---------|---------|
| Capillary Number | Run 2 | Run 5 | Run 8 | Run 11 | Run 14 | Run 17 | Average | RSD (%) |
| 6                | 0.38  | 0.40  | 0.38  | 0.38   | 0.34   | 0.36   | 0.37    | 5.5     |
| 18               | 0.42  | 0.40  | 0.40  | 0.35   | 0.38   | 0.35   | 0.38    | 7.5     |
| 30               | 0.39  | 0.38  | 0.36  | 0.37   | 0.37   | 0.33   | 0.37    | 5.6     |
| 42               | 0.39  | 0.39  | 0.39  | 0.38   | 0.34   | 0.35   | 0.37    | 6.0     |
| 54               | 0.38  | 0.40  | 0.37  | 0.36   | 0.37   | 0.35   | 0.37    | 4.6     |
| 66               | 0.39  | 0.39  | 0.39  | 0.37   | 0.37   | 0.36   | 0.38    | 3.5     |
| 78               | 0.40  | 0.41  | 0.39  | 0.36   | 0.38   | 0.35   | 0.38    | 6.1     |
| 90               | 0.41  | 0.40  | 0.38  | 0.38   | 0.37   | 0.35   | 0.38    | 5.6     |
| Average          | 0.40  | 0.40  | 0.39  | 0.37   | 0.37   | 0.35   | —       | —       |
| RSD (%)          | 3.6   | 2.3   | 3.7   | 3.1    | 4.6    | 3.3    | —       | —       |

inhibitor ( $IC_{50} = 1.5$  nM), and an enzyme reaction with a weak metalloprotease-13 inhibitor ( $IC_{50} = 11,687$  nM). The substrate, product, and internal standard all were resolved for the standard solution within 5 min (Figure 2a). The potent inhibitor completely blocked the conversion of substrate to product (Figure 2b), and the weak inhibitor was unable to prevent the conversion (Figure 2c).

Analysts can use multiplexed CE-LIF to screen a large number of inhibitors at one concentration on a plate or to construct dose-response curves for a smaller number of compounds. The multiplexed CE approach requires no quencher in the substrate peptide because the substrate and product are separated within the capillary.

**Measurement of endogenous enzyme levels:** Kinase enzymes are part of the cellular communication network and serve as intracellular signal transduction mediators. Members of this broad enzyme family catalyze the transfer of a phosphate group from adenosine-5'-phosphate to various polypeptide and protein substrates. Mitogen-activated protein kinase enzymes are members of the

serine-threonine kinase family and catalyze the transfer of a phosphate group to a serine or threonine residue on target proteins (27). Kinase enzymes typically are measured using radioenzymatic assays based upon the transfer of the terminal phosphate group of  $^{32}P$ - $\alpha$ -adenosine-5'-phosphate to a kinase-specific peptide substrate (28,29). Analysts use a selective membrane to isolate the radiolabeled peptide product before detection.

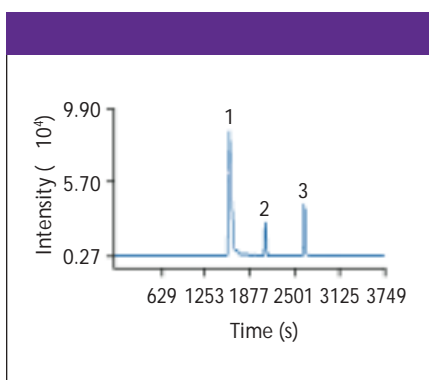
An alternative approach for measuring the levels of a given kinase is to use a fluorescently labeled peptide substrate with CE-LIF (30,31). CE is ideally suited to separate the substrate and product peptide because of the charge difference introduced by the addition of the phosphate group into the product peptide. We performed the evaluation using an FITC-labeled peptide substrate and multiplexed CE-LIF for analyzing mitogen-activated protein kinase 2 enzyme levels (equation 2).



We incorporated a fluorescently labeled peptide internal standard in the assay to improve the accuracy and precision of our results.

Figure 3 is a typical electropherogram of a mitogen-activated protein kinase 2 enzyme incubation mixture. Problems with severe peak tailing of the substrate required us to use a relatively high ionic strength running buffer (50 mM borate), which necessitated the use of a relatively low separation voltage (2.8 kV) to avoid excessive Joule heating. The low separation voltage resulted in a relatively long run time (45 min) for the separation; however, the ability to run 96 capillaries simultaneously compensated for the long run time.

A typical standard curve for mitogen-activated protein kinase 2 enzyme was linear



**Figure 3:** Electropherogram showing the separation of the substrate, product, and internal standard used for the assay of mitogen-activated protein kinase 2 enzyme. Peaks: 1 = substrate, 2 = product, 3 = internal standard.

from 1.5–50 ng/mL with a 15-min reaction time (Figure 4). We also were able to reduce the limit of detection to 0.2 ng/mL by using a 60-min incubation time (data not shown).

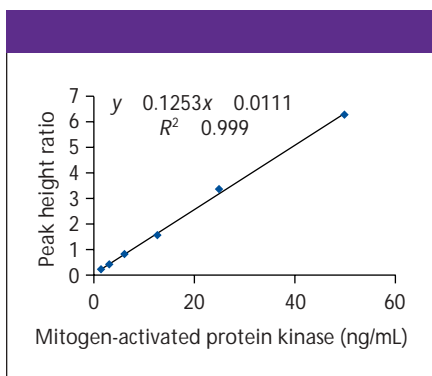
## Conclusion

Multiplexed CE–LIF provided accurate, precise, and stable performance for high-throughput separation-based assays. We were able to enhance linearity, precision, and reproducibility by using an internal standard to correct for slight differences across the capillary array. The multiplexed capability enables the rapid analysis of samples and provides the potential for greatly increasing the throughput of separation-based assays. We demonstrated the application of the multiplexed CE–LIF for screening enzyme inhibitors and for determining trace levels of endogenous enzymes.

The 96-capillary CE system has a broad range of potential pharmaceutical applications, including the examination of the combinatorial libraries' purity, high-throughput  $pK_a$  and log  $P$  determinations using indirect fluorescence detection, the study of drug–protein and protein–protein interactions, the development of rapid two-dimensional protein maps for proteomics, and the trace analysis of small molecules and biological compounds using affinity CE.

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**Figure 4:** Typical standard curve for mitogen-activated protein kinase 2 enzyme obtained from a multiplexed CE–LIF analysis. Each standard represents the average value obtained from three capillaries.