Talanta 130 (2014) 288–293

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Capillary electrophoresis coupled with automated fraction collection

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ARTICLE INFO

Article history:
Received 11 April 2014
Received in revised form
2 July 2014
Accepted 7 July 2014
Available online 15 July 2014

Keywords:
Capillary electrophoresis
Fraction collection
CE-SELEX
Aptamer

ABSTRACT

A fraction collector based on a drop-on-demand ink-jet printer was developed to interface capillary zone electrophoresis with a 96 well microtiter plate. We first evaluated the performance of the collector by using capillary zone electrophoresis to analyze a 1 mM solution of tetramethylrhodamine; a fluorescent microtiter plate reader was then used to detect the analyte and characterize fraction carryover between wells. Relative standard deviation in peak height was 20% and the relative standard deviation in migration time was 1%. The mean and standard deviation of the tetramethylrhodamine peak width was 5 ± 1 s and likely limited by the 4-s period between droplet deposition. We next injected a complex mixture of DNA fragments and used real-time PCR to quantify the product in a CE-SELEX experiment. The reconstructed electrophoretic peak was 27 s in duration. Finally, we repeated the experiment in the presence of a 30-μM thrombin solution under CE-SELEX conditions; fractions were collected and next-generation sequencing was used to characterize the DNA binders. Over 25,000 sequences were identified with close matches to known thrombin binding aptamers.

1. Introduction

Capillary electrophoresis is a useful tool for the analysis of biological samples such as polypeptides, lipids, DNA sequencing fragments, and metabolites [1–9]. Capillary electrophoresis instruments typically employ absorbance, laser-induced fluorescence, or mass spectrometry for on-line detection and analysis. While on-line detection is essential for most analyses, sample fractionation and recovery are necessary for use of capillary electrophoresis for preparative separations.

There have been few reports of capillary electrophoresis for preparative separations. Gannaro and Salas-Solano reported the use of a commercial capillary electrophoresis instrument that deposits fractions in a 96 well microtiter plate for characterization of deaminated peptide variants [10]. The technique first measures the migration time of a target molecule. In subsequent runs, the separation voltage is set to zero at the migration time of a target compound; the distal end of the capillary is then placed into a well of a microtiter plate that contains running buffer. The voltage is reapplied for a period of time corresponding to the analyte peak width to deposit the target molecule within the well. Potential is again set to zero, and the capillary tip is returned to the normal outlet buffer. Timing of subsequent fraction collection is reported to be difficult, and a separate run appears to be typically used for each fraction that is collected. This protocol requires a number of capillary manipulations, requires knowledge of the migration time of the components to be collected, and appears to allow collection of only one fraction from an injection.

It would be desirable to deposit fractions in succession into the wells of a microtiter plate without stopping the separation or careful calibration of migration time of analyte. In 1985 Hjertén and Zhu demonstrated the first fraction collection using capillary electrophoresis (CE) as the separation method [11]. Since then, fraction collection has primarily been used to couple CE with matrix assisted laser desorption/ionization (MALDI) mass spectrometry. Owing to the difficulty of performing on-line CE-MALDI-MS, off-line coupling systems are preferred [12,13]. Several approaches have been reported for off-line preparative separation and fractionation that mainly differ in the delivery of analyte to the MALDI plate [14–16].

We reported a CE-MALDI interface design that employs a drop-on-demand matrix sheath flow controlled by a high-speed ink jet printer valve [17]. That instrument placed the distal end of the separation capillary within an ink-jet nozzle, which was held at ground potential. MALDI matrix solution was introduced through a Tee fitting attached to the nozzle; the MALDI solution was pumped by nitrogen pressure. A high-speed miniaturized valve controlled deposition to the MALDI plate. In this paper, we modified our MALDI plate spotter for use as a 96 well fraction collector in preparative capillary zone electrophoresis. We demonstrate the fraction collector for analysis of a dye using a fluorescent plate

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http://dx.doi.org/10.1016/j.talanta.2014.07.018
0039-9140/

reader, for analysis of an oligonucleotide using real-time PCR, and for generation of aptamers using CE-SELEX [18,19].

2. Materials and methods

2.1. Materials and reagents

Fused silica capillary (50 μm ID and 150 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ USA). The fluorescent standard 5-carboxytetramethylrhodamine SE (TAMRA) was purchased from AnaSpec (San Jose, CA USA). Other reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO USA). All solutions were prepared from deionized-distilled water obtained from a Barnstead Nanopure System (Thermo-Fisher Scientific, Waltham, MA USA).

2.2. Laser-induced fluorescence detection

Two systems were employed. The first, described in this section, employed laser-induced fluorescence detection and was used to provide a reference separation. The instrument was similar to others reported by our group [20–22]. Briefly, fluorescence was excited using a CW 532 nm diode-pumped laser (CrystaLaser Model CL532-025), which was focused into the sample stream at the center of a sheath-flow cuvette. Fluorescence was collected at right angles and detected by a cascade of single-photon counting avalanche photodiode modules (PerkinElmer, Montreal, PQ Canada) with a sampling frequency of 50 Hz [23,24].

A bare fused silica capillary (50 μm ID, 150 μm OD, 40-cm length) was used for analysis. The first sample was 1 μM TAMRA prepared in a 15 mM sodium tetraborate running buffer. The sample was injected for 5 s at 5 kV and electrophoresis was performed at 10 kV, supplied by a Spellman High Voltage power supply (CZE1000R, Newark, NJ USA). Samples were analyzed in triplicate.

A second sample consisted of a random single-stranded DNA library (Sigma-Aldrich, St. Louis, MO USA). The 80 base sequence, 5′-AGCAGCACAGAGGTCAGATG-N(40)-CCTATGCGTGCTACCGTGAA-3′, was designed with priming regions for PCR amplification at both the 5′- and 3′-ends flanking a random region allowing up to 4^{40} sequences within the pool. A TAMRA fluorescent tag was incorporated at the 5′-end for laser-induced fluorescence detection. The library was injected for 5 s at 5 kV onto a preconditioned 45 cm capillary. The separation running buffer was 10 mM sodium tetraborate/HEPES (Sigma-Aldrich, St. Louis, MO USA), and the separation was performed at 15 kV.

A third sample consisted of a 10 μL aliquot of the 100 μM stock oligonucleotide library solution added to 10 μL of binding buffer (50 mM TRIS, 100 mM NaCl, 1 mM $CaCl_2$). The mixture was heated to 94 °C to destroy secondary structures that may have formed during storage. The solution was cooled by 0.5 °C/second to a final temperature of 20 °C in a thermal cycler (PTC-100, MJ Research). The heat-treated 10 μM random ssDNA library solution was incubated at room temperature with 1 mg/mL human α-thrombin protein (Hematologic Technologies, Inc., Vermont USA) for a minimum of 15 min to allow binding.

2.3. Fraction collector

A second system employed fraction collection and is diagramed in Fig. 1. The distal end of the separation capillary was threaded through a Tee fitting using a capillary sleeve and ferrule from Upchurch Scientific (Oak Harbor, WA USA). The valve, tee, nozzle, and inline filter were the same as used in ref 17 and were from The Lee Company (Westbrook, CT USA).

The capillary tip was positioned at the exit of the nozzle to avoid contamination within the spotting apparatus. The capillary and nozzle were secured ~2 mm above the surface of the plate to ensure proper sample delivery. The metal nozzle was held at ground potential. The deposition buffer was held under nitrogen pressure at ~5 psi. The collection plate was positioned below the instrument on a Prior Scientific microscope stage (Rockland, MA USA). The stage was mounted to an aluminum breadboard by a Plexiglas block. Instrument control was programmed in Labview (National Instruments, Austin, TX USA).

The motion of the stage was matched to a 96 well microtiter plate with 9 mm well spacing in the *X* and *Y* dimensions in a 12 × 8 pattern. The stage was moved in the *X* direction in odd rows and the $-X$ direction in even rows, creating a serpentine pattern. Fraction width, which controls time between depositions, was determined from reference data obtained by the fluorescence detector. Under typical conditions, each droplet consists of 10 μL sheath liquid and a few picoliters of solution from the capillary.

2.4. TAMRA fraction collection

The separation conditions and the capillary length were identical to those used for laser-induced fluorescence detection. Due to the dilution of analyte in the fraction collector and the poor sensitivity of the fluorescent plate reader, we used a very high concentration TAMRA solution for this characterization experiment. A 1 mM TAMRA solution was prepared in the 15 mM sodium tetraborate separation buffer and injected for 5 s at 5 kV.

The capillary was preconditioned for 5 min with NaOH, ddH_2O, and separation buffer before each analysis. Following each preconditioning step, the valve was primed for 2 s with either ddH_2O or separation buffer in the valve reservoir.

Half-area, black with clear bottom, 96-well plates (Corning, Corning, NY USA) were used for collection. A fraction width of 4 s was determined from the reference data obtained by capillary electrophoresis with fluorescence detection. The valve pulse width was set to 0.05 s, dispensing 15 mM sodium tetraborate buffer through the valve. Fraction collection and electrophoresis began simultaneously. Fluorescence intensity within each of the 96 wells was measured with a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA USA) using SoftMax Pro software (Table 1).

2.5. Oligonucleotide fraction collection

A random oligonucleotide library (Sigma-Aldrich, St. Louis, MO USA) served as a ssDNA pool. The 80 base sequence, 5′-AGCAGCACAGAGGTCAGATG-N(40)-CCTATGCGTGCTACCGTGAA-3′, was designed with priming regions for PCR amplification at both the 5′- and 3′-ends flanking a random region allowing up to 4^{40} sequences within the pool. No fluorescent tag was attached to this DNA pool.

The library was injected for 5 s at 5 kV onto a preconditioned 45 cm capillary. The separation running buffer was 10 mM sodium tetraborate/HEPES (Sigma-Aldrich, St. Louis, MO USA), and the separation was performed at 15 kV.

Hard-shell, white well, 96-well PCR plates (Bio-Rad) were used for fraction collection. A fraction width of 7 s was determined by reference data obtained by laser-induced fluorescence detection. The valve pulse width was set to 0.05 s, precisely dispensing valve buffer. To limit sample handling and sample loss, the valve buffer reservoir contained all necessary real-time PCR reagents per the manufacturers protocol; reagents and final concentrations for 10 μL reactions were iTaq Universal SYBR Green supermix (1 ×) and forward and reverse primers (300 nM each). We deviated from the recommended protocol and diluted the PCR reagents in

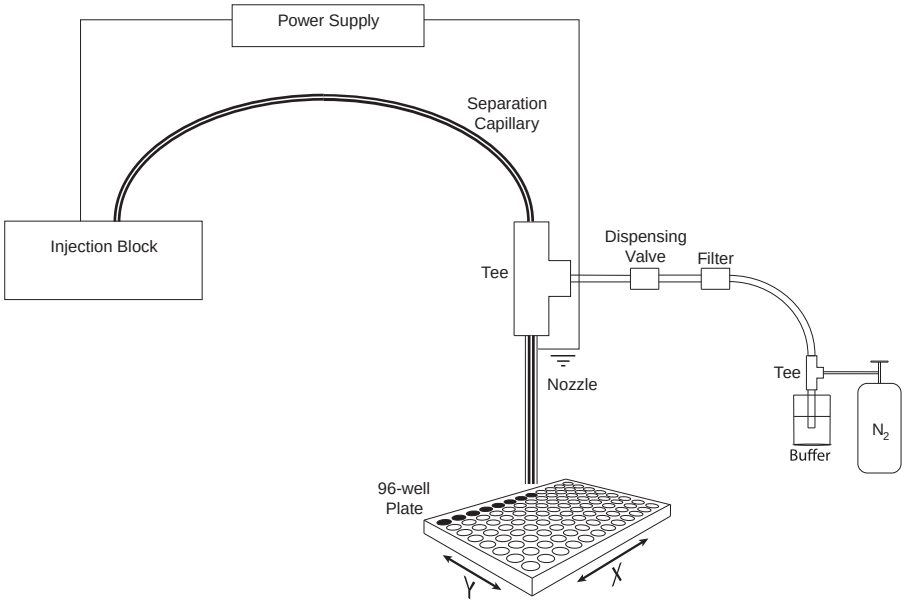

Fig. 1. Schematic of the CE-high efficiency fraction-collection instrument. The schematic illustrates the distal end of the capillary threaded through a tee and resting at the tip of the nozzle. Buffer flow is controlled at the dispensing valve and washes the fraction exiting the capillary into a plate well. The 96-well plate is fixed to a motorized microscope *X*–*Y* stage.

Table 1
Settings applied by SoftMax Pro software for TAMRA fluorescence readings across a 96-well plate employing a SpectraMax M5 Microplate Reader.

Read type	Spectrum
Read mode	Fluorescence Bottom read
Wavelengths	Ex: 530 nm Em start: 570 nm Em stop: 590 nm Step: 5 nm Cutoff: 570 nm
Sensitivity	Readings: 10 PMT: Auto
Automix	Off
Calibrate	On
Assay plate type	96-well half-area, clear bottom
Wells to read	Read entire plate
Settling time	Off

separation buffer instead of nuclease free water to achieve uninterrupted separation. Fraction collection and electrophoresis began simultaneously.

After fraction collection, the 96-well PCR plate was sealed and centrifuged, bringing the deposited fractions to the bottom of the wells for amplification. The PCR protocol was designed to optimize the reaction based on the annealing temperatures of the forward (5′-AGCAGCACAGAGGTCAGATG-3′) and reverse (5′-TTCACGGTAGCACGCATAGG-3′) primers and the pre-mixed components of iTaq Universal SYBR Green Supermix (Bio-rad); 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s (denature), 56.7 °C for 30 s (anneal), and 72 °C for 30 s (extend). Following each extension, real-time fluorescence was measured in each well using a CFX96 Touch Real-Time PCR Detection System (Bio-rad).

2.6. Aptamer generation

A 10 μL aliquot of the 100 μM stock oligonucleotide library solution was added to 10 μL of binding buffer (50 mM TRIS, 100 mM NaCl, 1 mM $CaCl_2$). The mixture was heated to 94 °C to destroy secondary structures that may have formed during storage. The solution was cooled by 0.5 °C/s to a final temperature of 20 °C in a thermal cycler (PTC-100, MJ Research). The heat-treated 10 μM random ssDNA library solution was incubated at room temperature with 1 mg/mL human α-thrombin protein (Hematologic Technologies, Inc., Vermont USA) for a minimum of 15 min to allow binding.

Fractions were collected and amplified as in Section 2.5. Post-amplification, pooled wells containing the target were submitted to the Genomics & Bioinformatics Core Facility at the University of Notre Dame (Notre Dame, IN USA). In preparation for deep sequencing analysis, an Illumina library was constructed on the submitted sample. Sample quality was verified via a bioanalyzer trace; the majority of the material present was 214 bp. We use Illumina's TruSeq Universal Adapter 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC TTCCGATCT and TruSeq Adapter, Index 6 5′-GATCGGAAGAGCACACGTCTGAACT CCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG. Sequencing was performed using a MiSeq nano flow cell for a single 140 bp read, and the sample was spiked with 25% PhiX control to generate library diversity. The MiSeq run generated over 1 million high quality reads. The sequences were compared with the established thrombin-binding aptamer sequence.

3. Results and Discussion

3.1. Evaluation of the fraction collector using a fluorescent plate reader to detect TAMRA and its impurities separated by capillary zone electrophoresis

We first evaluated the system by separating TAMRA from trace-level impurities, Fig. 2. We employed an ultrasensitive laser-induced fluorescence detector, which was sampled at 50 Hz (blue trace). In a separate experiment, the same capillary was coupled to our fraction collector for separation of a much higher concentration (1 mM) sample; fractions were collected in a 96 well microtiter plate at four second intervals and fluorescence intensity was measured off-line using a fluorescent plate reader (green trace).

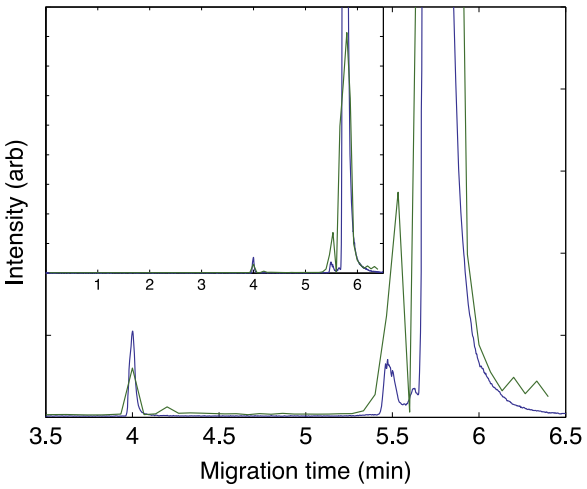

Fig. 2. Electropherograms produced by the plate reader (green) and laser-induced fluorescence (blue) for the analysis of TAMRA solutions. Insert shows the full-scale electropherograms. A two-point algorithm was used to bring the laser-induced fluorescence electropherogram into alignment with the plate-reader electropherogram (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

High TAMRA concentration was required because of the low sensitivity plate reader used in this experiment, and saturation of the detector limited its dynamic range.

A simple two-point algorithm was used to bring the traces into alignment [25]; the offset between the traces was minor and likely reflects electric field distortions caused by the high concentration TAMRA peak.

The electropherograms consists of a strong peak generated by TAMRA and two much lower intensity peaks due to fluorescent impurities present in the dye. The signal generated from the microtiter plate reader nicely matches that of the laser-induced fluorescence signal.

We fit a Gaussian function to the TAMRA peak

$$Gaussian(t) = a \times e^{-0.5(t-t_0)^2/sigma^2}$$

where t is time, a is peak amplitude, t_0 is the migration time, and sigma is the peak width. The fraction collector was reasonably reproducible; the relative standard deviation in peak amplitude was 20% and the relative standard deviation in migration time was 1% ($N=3$), which are typical values in capillary zone electrophoresis using manual sample injection. The mean and standard deviation of the tetramethylrhodamine peak width was 5 ± 1 s and was likely limited by our 4-s spot deposition period.

3.2. Evaluation of the system using real time PCR to detect oligonucleotides

We injected a random pool of oligonucleotides into the CZE system and captured fractions in a microtiter plate. We placed enzyme and primers in the sheath fluid of the fraction collector, which eliminated the need for subsequent addition of the real-time PCR reagents to the microtiter plate. Real-time PCR was used to quantify the amount of oligonucleotide deposited in each well, Fig. 3.

Fig. 3 presents two sets of traces. The first set consists of the converted C_T values, where the signal$=1/2^{C_t}$, and provides an estimate of the total amount of DNA deposited in each well. The traces for the oligonucleotide-thrombin mixture and the control sample are quite similar. This similarity is expected because only a small fraction of the oligonucleotide pool will complex with thrombin and generate a mobility shift.

A much more powerful tool for identifying the presence of DNA-thrombin complexes employs the endpoint signal after 40 cycles; this amount of amplification is sufficient to detect a very small number of molecules of the oligonucleotide pool. Real-time PCR signals reach an endpoint signal of reasonable intensity if any amplifiable DNA is present.

Fig. 3 results in two important observations. First, the essentially zero signal before 6 min in the oligonucleotide-thrombin mixture, and before 7.5 min in the control mixture demonstrates the extraordinarily low background signal generated by this interface, which is essentially contamination free.

Second, there is a ~1-minute window between 6.5 and 7.5 min that generates appreciable endpoint signal for the oligonucleotide-thrombin mixture but no signal for the oligonucleotide pool itself. It is these oligonucleotides that are expected to contain aptamers that bind to the target molecule.

3.3. Thrombin aptamers generated using CE-SELEX.

Electropherograms were generated by capillary electrophoresis and laser-induced fluorescence detection of both the fluorescently labeled ssDNA library (blue trace) and the library bound to thrombin (green trace), Fig. 4. A two-point algorithm was used to bring the traces in alignment [25]. The uncomplexed library begins to migrate at ~8 min. The binding of an aptamer to a human α-thrombin protein molecule produces a migration shift that deviates from the unbound library sequences; the most prominent display of this effect occurs at 7 min.

We repeated the separation of the DNA-thrombin mixture using an unlabeled library and fraction collection in a 96-well plate. To simplify subsequent analysis, PCR reagents were employed as the sheath liquid. After fraction collection, the plate was sealed and centrifuged before placing it into the real-time PCR instrument. Fig. 3 is a reconstruction of the real-time PCR data. In contrast to our control data (blue trace), we observe by end-point fluorescence signal that trace amounts of sequences have shifted in migration in the DNA-thrombin mixture (green trace). Based on the real-time PCR data combined with the CE-LIF data of Fig. 4, we pooled the contents of the wells spanning ~1 min prior to the uncomplexed library peak; the pooled contents were then submitted for deep sequencing.

3.4. Aptamer sequencing and validation

Preprocessing extracted roughly 800,000 reads from the original dataset of 1,082,975 sequences. Using Perl program code, the ligated adapter sequences were trimmed, followed by the priming regions. The desired sequences of lengths 38–42 bases, representing the random region, were selected for analysis; this subset encompasses approximately 98% of the high quality reads. We assume 4 or more instances of neighboring guanines constitute an exact or near-exact match based on the three-dimensional structure of the thrombin-binding DNA aptamer [26]; ~48,000 sequences contain four or more 'GG'. The data were transformed into FASTA format and mapped against the established 15-mer [27] and 29-mer [28] thrombin-binding aptamer (TBA) sequences utilizing the Burrows-Wheeler alignment tool (BWA) [29]. BWA fastmap analysis returned 10,534 sequence matches to the 15-mer TBA and 17,606 matches to the 29-mer TBA, omitting duplicate matches found on a single read. MEME motif discovery tool (version 4.9.1) was also used to detect enriched sequences/motifs to confirm results [30].

Roughly 6% of the sequences are consistent with known thrombin-binding aptamers. Higher stringency fraction collection, additional rounds of PCR amplification, and additional selection

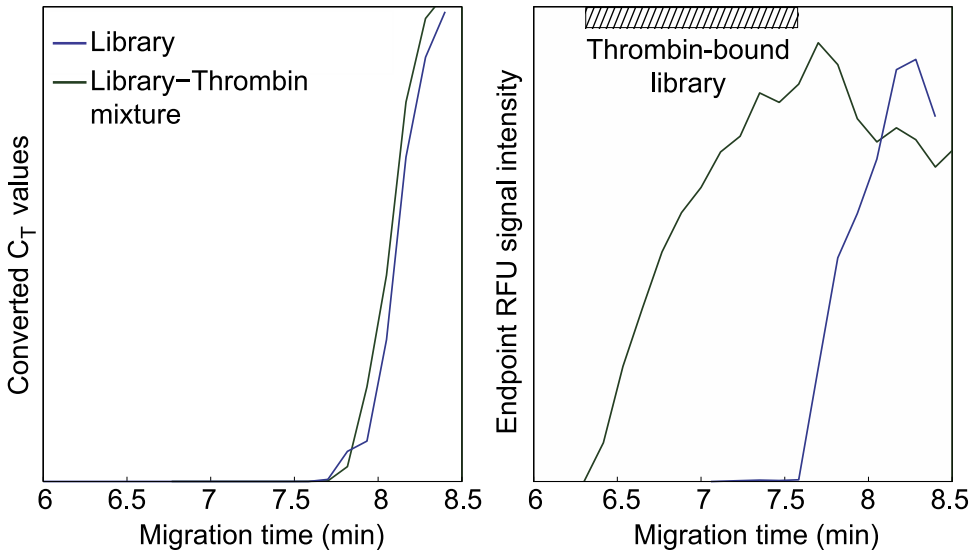

Fig. 3. Electropherograms from real-time PCR analysis of an 80-mer oligonucleotide mixture that contains a random 40-mer insert (blue), and the 80-mer oligonucleotide mixture pre-incubated with thrombin to promote aptamer binding (green). Left panel: The C_T values reconstruct the electropherogram and should map the DNA content. C_T values are reported for each well that contains amplified template greater than the software-determined threshold. This threshold can cause wells containing only trace amounts of amplified template to go unreported. Right panel: The endpoint signal reports the relative fluorescence signal (Endpoint RFU Signal Intensity) after 40 cycles of PCR amplification. The endpoint signal is a powerful test for the presence of any of the oligonucleotide mixture, irrespective of concentration (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

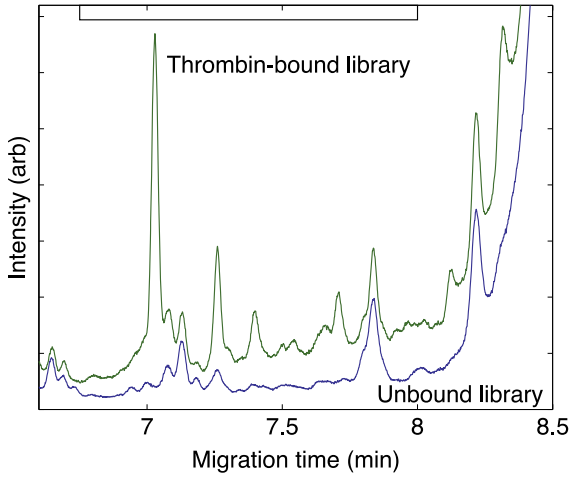

Fig. 4. Capillary electrophoresis-laser-induced fluorescence detection of unbound library and library bound to thrombin. The thrombin-bound library shows several new peaks migrating in the 7–8 min region corresponding to CE-SELEX aptamers bound to thrombin. Green trace is the thrombin bound library, blue trace is the unbound library. The bar at the top of the trace indicates the collection window used for sample collection in the CE-SELEX experiment (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cycles would undoubtedly lead to a larger fraction of target-binding aptamers generated.

4. Conclusions

We report a flexible and low-cost fraction collector for preparative capillary zone electrophoresis. We demonstrated its application to the isolation of putative aptamers against thrombin. The fraction collector provides reasonable separation efficiency and modest sample carryover. However, it has two obvious limitations. First, sample is subjected to over 1000-fold dilution during fraction generation. Second, the use of a 96 well microtiter plate limits the number of fractions that can be collected and the temporal resolution of fraction collection. Fortunately, it is trivial to adjust the spotter to match the footprint of higher density microtiter plates, such as those containing 384 or 1536 wells.

Acknowledgments

We gratefully acknowledge support from the National Institutes of Health (5R01GM096767). We thank the University of Notre Dame's Genomics Core Facility for sequencing services and Dr. Jun Li in the Department of Applied and Computational Mathematics and Statistics for assistance with data preprocessing. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under Grant no. DGE-1313583. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors(s) and do not necessarily reflect the views of the National Science Foundation.

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