

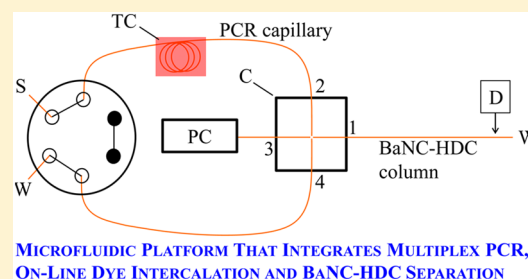
# Charging YOYO-1 on Capillary Wall for Online DNA Intercalation and Integrating This Approach with Multiplex PCR and Bare Narrow Capillary–Hydrodynamic Chromatography for Online DNA Analysis

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## S Supporting Information

**ABSTRACT:** Multiplex polymerase chain reaction (PCR) has been widely utilized for high-throughput pathogen identification. Often, a dye is used to intercalate the amplified DNA fragments, and identifications of the pathogens are carried out by DNA melting curve analysis or gel electrophoresis. Integrating DNA amplification and identification is a logic path toward maximizing the benefit of multiplex PCR. Although PCR and gel electrophoresis have been integrated, replenishing the gels after each run is tedious and time-consuming. In this technical note, we develop an approach to address this issue. We perform multiplex PCR inside a capillary, transfer the amplified fragments to a bare narrow capillary, and measure their lengths online using bare narrow capillary–hydrodynamic chromatography (BaNC-HDC), a new technique recently developed in our laboratory for free-solution DNA separation. To intercalate the DNA with YOYO-1 (a fluorescent dye) for BaNC-HDC, we flush the capillary column with a YOYO-1 solution; positively charged YOYO-1 is adsorbed (or charged) onto the negatively charged capillary wall. As DNA molecules are driven down the column for separation, they react with the YOYO-1 stored on the capillary wall and are online-intercalated with the dye. With a single YOYO-1 charging, the column can be used for more than 40 runs, although the fluorescence signal intensities of the DNA peaks decrease gradually. Although the dye–DNA intercalation occurs during the separation, it does not affect the retention times, separation efficiencies, or resolutions.



Polymerase chain reaction (PCR) is widely used for analysis of a specific segment of DNA, or a DNA variation. Multiplex PCR is a variant of conventional PCR in which two or more loci are simultaneously amplified in the same reaction. Since its introduction in 1988,<sup>1</sup> this technique has been utilized successfully in various areas of DNA analyses such as DNA deletion,<sup>2,3</sup> mutation<sup>4</sup> and polymorphism,<sup>5</sup> DNA quantitative assays,<sup>6</sup> etc. Multiplex PCR can also be utilized to boost the sample throughput<sup>7–12</sup> for strain identification. Analysis of the products of multiplex PCR is often carried out by electrophoretic separation<sup>8–10,12</sup> or DNA melting curve analysis.<sup>13</sup> PCR and the following electrophoretic separation have been integrated;<sup>14–17</sup> however, because the gels used in the above integrated devices need to be replenished after each run, to prevent the gels from being blocked by large DNA templates, performing the separations is tedious and time-consuming. In this Technical Note, we describe a new approach to address this issue. We perform multiplex PCR inside a capillary tube, transfer the amplified fragments to a narrow capillary online, and measure their lengths by bare narrow capillary – hydrodynamic chromatography (BaNC-HDC),<sup>18,19</sup> which is a new technique developed in our laboratory for free-solution DNA separation. BaNC-HDC is capable of resolving DNA with a wide size range, and the separation is performed in a solution without any sieving matrices. PCR products can be injected directly into a BaNC-HDC column for separation without any

purification (such as DNA template/enzyme removal, desalting, etc.).

To prepare a sample for BaNC-HDC separation, the PCR products must be fluorescently labeled. Labeling these DNA can be accomplished by throwing a fluorescent intercalating dye in the solution either during the reactions if the dye (e.g., SYBR Green I) is compatible with the PCR, or after the reactions if the dye (e.g., YOYO-1) is incompatible with the PCR. In this experiment, we focus on the latter. To intercalate the DNA with YOYO-1, we flush the bare narrow capillary with a YOYO-1 solution; positively charged YOYO-1 is adsorbed (or charged) onto the negatively charged capillary wall. As DNA molecules are driven down the capillary column for separation, they react with YOYO-1 stored on the capillary wall and are online-intercalated with the dye. With a single YOYO-1 charging, the column can be used for more than 40 runs, although the fluorescence signal intensities of the DNA peaks decrease gradually. Although the dye–DNA intercalation occurs during the separation, it does not affect the retention times, separation efficiencies, or resolutions.

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## EXPERIMENTAL SECTION

**Materials and Reagents.** YOYO-1 was purchased from Life Technologies (Grand Island, NY). A DNA ladder (1-kb plus), tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), and other common reagents were purchased from Fisher Scientific (Pittsburgh, PA). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ). 10.0 mM tris-EDTA (TE) buffer consisted of 10.0 mM Tris and 1.00 mM EDTA, and its pH was adjusted to 8.0. All solutions were prepared using ultrapure water (Nanopure Ultrapure Water System, Barnstead, Dubuque, IA), filtered through a 0.22  $\mu\text{m}$  filter (VWR Scientific, Sugar Land, TX), and vacuum-degassed before use.

**Apparatus.** Figure 1A presents the apparatus used in this experiment to perform multiplex PCR, online DNA–YOYO-1 intercalation, and BaNC-HDC separation. The system

consisted of a pressure chamber (PC), a chip injector (C), a PCR capillary and a thermocycler (TC), a six-port valve, a BaNC-HDC column, and a confocal laser-induced fluorescence detector (D). The detailed descriptions of the pressure chamber, chip injector, and detector can be seen in the Supporting Information (SI). The PCR capillary had a length of 2 m, and an inner diameter (i.d.) of 75  $\mu\text{m}$ , and it was treated with dichlorodimethylsilane and bovine serum albumin; the detailed treatment procedure is described in the SI. The thermocycler (TC) used is a MJ Research PTC-200 Peltier Thermal Cycler. A six-port valve was utilized in conjunction with the chip injector to facilitate sample injection for BaNC-HDC separation. The valve could be switched between an “open” position (as the PCR capillary from position 2 and auxiliary capillary from position 4 on the chip injector were connected to sample (S) and waste (W)) and a “closed” position (as the above two capillaries were connected to blocked ports). The BaNC-HDC column was a fused silica capillary having a length of 55 cm and an i.d. of 2  $\mu\text{m}$ .

**Performing Multiplex PCR, Online YOYO-1 Intercalation, and BaNC-HDC Separation.**

After the apparatus was assembled as shown in Figure 1,  $\sim 4$  nL of 7.0  $\mu\text{M}$  YOYO-1 in 1 $\times$  TE buffer (10.0 mM Tris-HCl and 1.0 mM EDTA at pH 8.0), and then  $\sim 4$  nL of 1 $\times$  TE buffer were flushed through the BaNC-HDC column from the waste side under a pressure of  $\sim 350$  psi. The system was initialized after a vacuum was applied to the waste side (W) on the six-port valve to clean the YOYO-1 in the chip injector. The operating procedures depicted in Figure 1 can be described as follows:

**Step 1 (Figure 1B):** A multiplex PCR solution was first aspirated into the PCR capillary by applying a vacuum to the waste side (W) on the six-port valve while the valve was set at the “open” position. At this time, an ambient pressure or a pressure ( $\sim 10$  psi) slightly higher than the ambient pressure was applied to the pressure chamber (PC).

**Step 2 (Figure 1C):** The valve was switched to the “closed” position, and thermal cycle reactions were performed (see the SI for details).

**Step 3 (Figure 1D):** The valve was switched back to the “open” position while a vacuum was applied to W to pull the PCR products into the cross section of the chip injector.

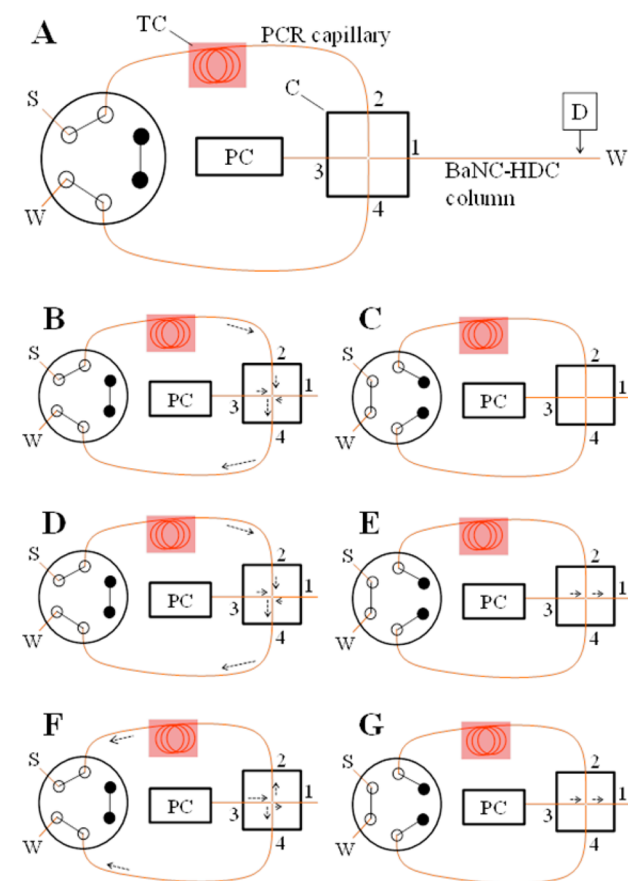
**Step 4 (Figure 1E):** The valve was switched to the “closed” position, and a predetermined pressure (e.g., 40 psi) was applied to PC for a given period of time (e.g., 10 s); a portion of the sample in the cross section of the chip injector was driven to the BaNC-HDC column. The amount of the injected sample was controlled by the pressure and the injection time.

**Step 5 (Figure 1F):** The valve was switched to the “open” position while a pressure (e.g., 40 psi) was applied to PC; the residue sample in the cross section of the chip injector was flushed away from the inlet of the BaNC-HDC column.

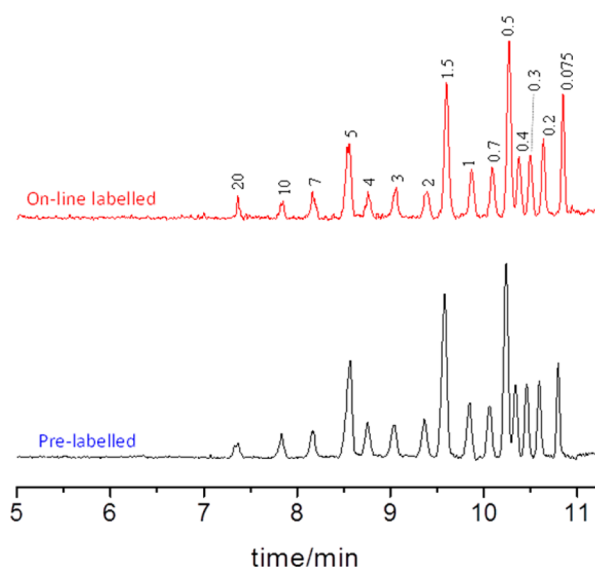
**Step 6 (Figure 1G):** The valve was switched to the “closed” position, and a predetermined pressure (e.g., 200 psi) was applied to PC to effect BaNC-HDC separation.

## RESULTS AND DISCUSSION

To characterize our approach for online DNA–YOYO-1 intercalation, we used an experimental setup that was reported previously<sup>18</sup> (see Figure S1 in the SI for details). In one experiment, we used a bare capillary column and separated a 1-kbp-plus DNA ladder preintercalated with YOYO-1. The bottom trace of Figure 2 presents the separation results. In a separate experiment, we charged YOYO-1 on the interior



**Figure 1.** Schematic diagram of experimental setup for PCR-BaNC-HDC. Panel (A) illustrates the experimental setup. (Legend: S, sample; W, waste; C, microchip injector; PC, pressure chamber; and TC, thermocycler.) The solid dots depicted on the six-port injection valve indicate ports that are blocked. Capillaries are connected to positions 1, 2, 3, and 4 on microchip injector are separation capillary, PCR capillary, pressure capillary, and waste capillary, respectively. The separation capillary had a length of 55 cm (35 cm effective), an outer diameter (o.d.) of 150  $\mu\text{m}$ , and an i.d. of 2  $\mu\text{m}$ . The PCR capillary had a length of 2 m, an o.d. of 150  $\mu\text{m}$ , and an i.d. of 75  $\mu\text{m}$ . The pressure capillary had a length of 10 cm, an o.d. of 150  $\mu\text{m}$ , and an i.d. of 20  $\mu\text{m}$ . The waste capillary had a length of 30 cm, an o.d. of 150  $\mu\text{m}$ , and an i.d. of 75  $\mu\text{m}$ . Panels (B)–(G) depict schematic diagrams for illustrating major operating procedures; the arrows indicate the flow directions (see details in text).

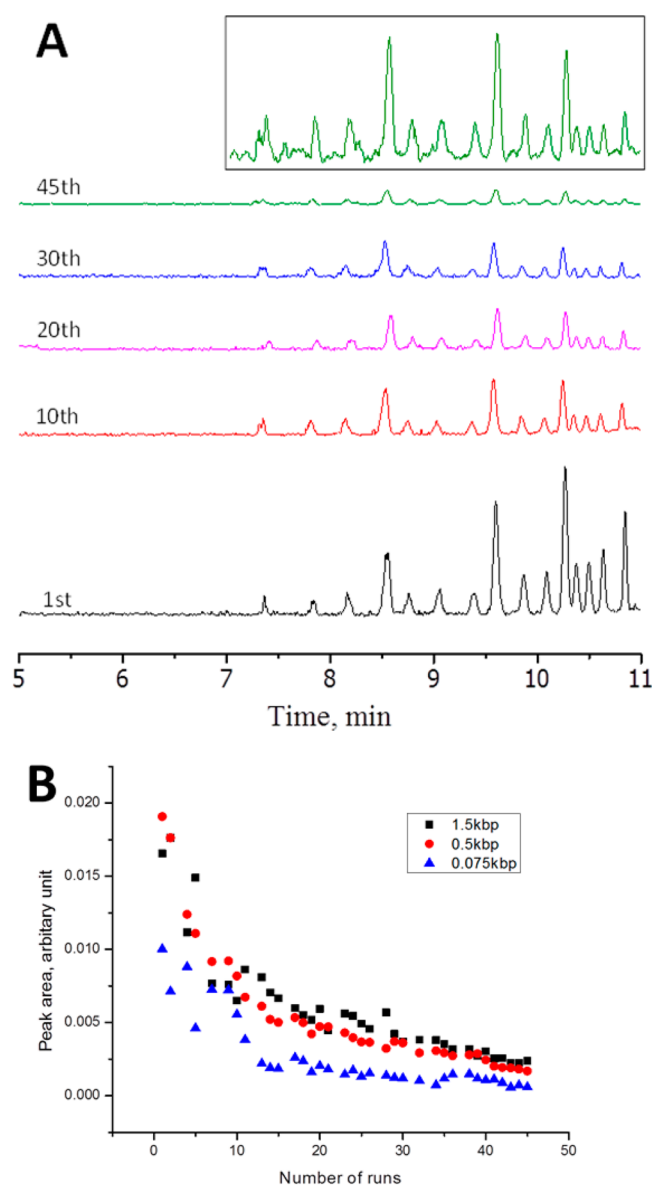


**Figure 2.** Comparison of the online-labeled methodology and the pre-labeled methodology. The top trace shows the BaNC-HDC separation of an online-labeled DNA ladder (0.075, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.0, 10, and 20 kbp), while the bottom trace presents the separation of a pre-labeled DNA ladder. The nanocapillary had a total length of 50 cm (40 cm effective) and a radius of 1  $\mu\text{m}$ . The 1-kb-plus DNA ladder (total DNA = 4  $\text{ng}/\mu\text{L}$ ) was injected at 40 psi for 10 s, and the separation was carried out at 350 psi.

surface of the capillary column and separated the same DNA ladder but without YOYO-1 intercalation. The top trace of Figure 2 presents the separation results; all DNA fragments were nicely labeled and detected, and comparable resolutions and similar retention times were obtained. That is, we did not sacrifice any performance by using the online intercalation approach.

Importantly, we do not need to recharge YOYO-1 for every BaNC-HDC separation. Figure 3A presents a few typical traces (the 1st, 10th, 20th, 30th, and 45th runs) as we injected the same sample repetitively after we charged the BaNC-HDC column once. Apparently, as the repetitive-injection test proceeded, the YOYO-1 storage on the capillary wall shrank. As a result, the number of YOYO-1 molecules intercalated with each DNA fragment diminished, and, consequently, the fluorescence intensities decreased (Figure 3B). An excellent feature of this approach is that, although fluorescence intensities decreased gradually, the resolution, efficiency, and retention time remained virtually unchanged (see Figure S2 in the SI for details). All DNA fragments could be positively identified and their sizes be accurately measured even after dozens of injections (e.g., see the inset of Figure 3A for an expanded view of the BaNC-HDC trace of the 45th run).

To demonstrate the utility of this approach for practical uses, we assembled a system (Figure 1) that integrated a capillary PCR device, a microchip injector, and a BaNC-HDC column in a microfluidic format for multiplex PCR, online injection, BaNC-HDC separation, and online DNA-YOYO-1 intercalation. The resolved DNA bands were monitored via a laser-induced fluorescence (LIF) detector. Figure 4A presents the separation results of multiplex PCR products. The sizes of three products were estimated (see the SI for details) to be 1.080, 0.895, and 0.398 kbp, which is in excellent agreement with their theoretical values (1.074, 0.874, and 0.400, respectively). Figure

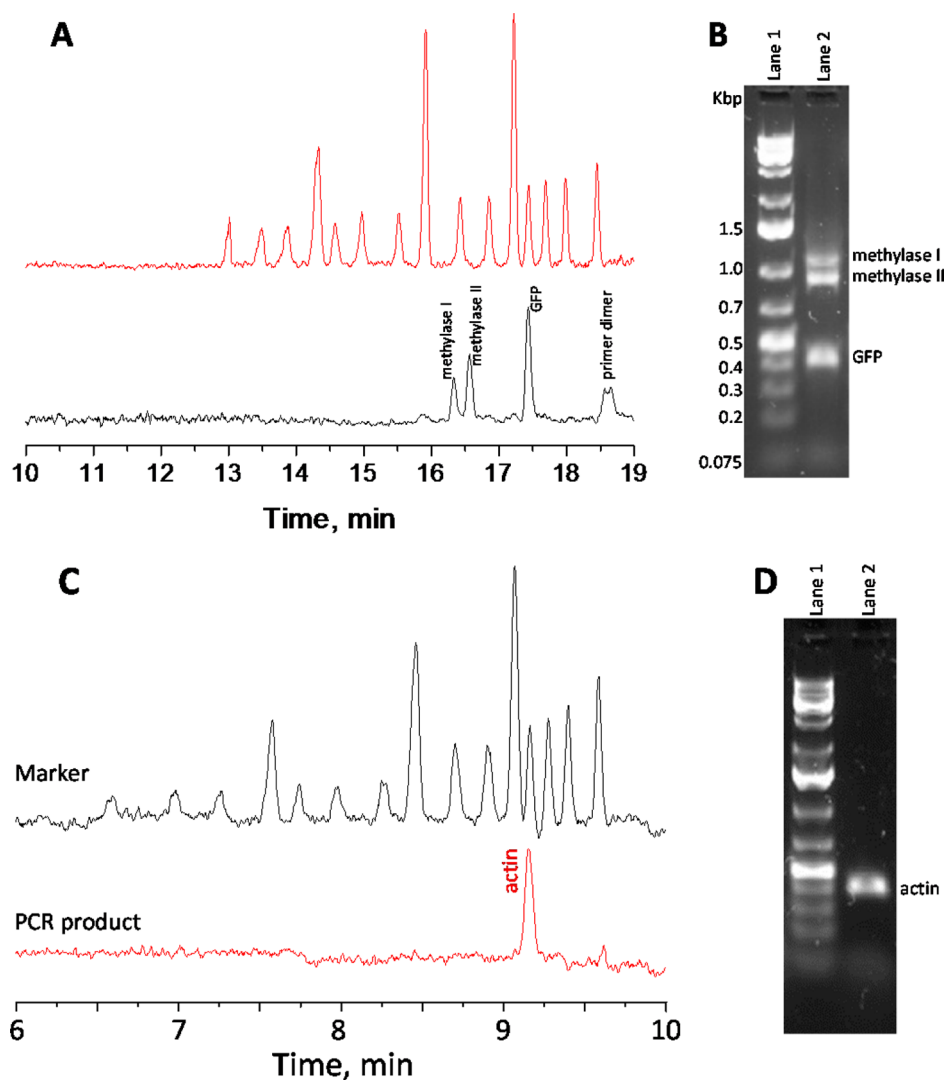


**Figure 3.** Repetitive run after single-time YOYO-1 charging: (A) typical separation traces of DNA ladder (4  $\text{ng}/\mu\text{L}$  total DNA) (the inset presents an expanded view of the 45th run); and (B) peak areas varying with the number of repetitive runs. All separation conditions were the same as those presented in Figure 2.

4B presents the slab-gel separation results; the multiplex PCR was performed using a conventional Eppendorf tube and a MJ Research thermocycler. Figure 4C presents the separation of a segment of a rice genomic DNA amplified in the PCR capillary. Because BaNC-HDC can tolerate large DNA template, the amplified fragment was online-transferred to the BaNC-HDC column for separation. The BaNC-HDC separations were executed under  $\sim 400$  psi, and DNA-YOYO-1 intercalation was accomplished during the separation. The size of the fragment was measured to be 404 bp, compared to its theoretical value of 405 bp. Figure 4D presents the slab-gel separation result of the conventionally amplified product.

## CONCLUSIONS

We have developed an innovative and simple approach for the online intercalation of YOYO-1 with DNA. The method



**Figure 4.** Performing multiplex PCR, BaNC-HDC separation, and online dye intercalation on an integrated microfluidic platform. Panel (A) shows BaNC-HDC separation traces; the top trace was obtained from separating a 1-kbp-plus DNA ladder, and the bottom trace was obtained from separating the multiplex PCR products amplified in the capillary thermocycler (30 cycles; see Capillary PCR in the SI for details). The BaNC-HDC separations were executed under  $\sim 200$  psi. Panel (B) shows the slab-gel electrophoresis of the crude multiplex PCR products amplified by a MJ Research thermocycler (30 cycles). ("Lane 1" shows data from the DNA ladder; "Lane 2" shows data from crude PCR multiplex products.) Panel (C) shows BaNC-HDC separation of a PCR-amplified genomic region (30 cycles; see Capillary PCR in the SI for details). The top trace represents the separation of a 1-kbp-plus DNA ladder, and the bottom trace is the result of the separation of an actin sequence from a rice genomic DNA that was amplified by the capillary PCR and online-intercalated with YOYO-1. The BaNC-HDC separations were executed under  $\sim 400$  psi. Other conditions were similar to those described for Panel (A). Panel (D) shows the slab-gel electrophoresis of a conventional PCR-amplified actin sequence. ("Lane 1" shows data from the DNA ladder, and "Lane 2" shows data from the actin.) Other conditions were similar to those described for Panel (B).

consumed only  $\sim 4$  nL of YOYO-1 solution to charge the BaNC-HDC column, and the YOYO-1 adsorbed on the capillary wall allows for dozens of BaNC-HDC runs without recharging. Taking advantage of an excellent feature of BaNC-HDC for tolerating high salt contents, large DNA templates, and big enzyme molecules required for PCR, we have integrated a capillary PCR with BaNC-HDC and this online intercalation approach onto a microfluidic platform. We finally demonstrated the feasibility of using this platform for multiplexed PCR, online intercalation of the amplified products, BaNC-HDC separation, and LIF detection. With the advancement of microfluidic and nanofluidic devices for bioanalysis, single-DNA molecules have been entrapped in nanopores for enzyme digestions,<sup>20</sup> microfluidic chips have

been developed for PCR,<sup>21,22</sup> and a miniaturized LIF detector<sup>23</sup> has also been constructed for detection on microfluidic chips. The reported microfluidic platform, combining with the above technologies, holds great promises for point-of-care applications.

## ■ ASSOCIATED CONTENT

### § Supporting Information

Apparatus and protocol for characterizing online DNA and YOYO-1 intercalation; resolution, efficiency, and retention time varying with number of runs; preparation of chip injector; capillary surface modification for capillary PCR; capillary PCR; incompatibility of YOYO-1 with PCR; and calculation of the three multiplex products length are provided as Supporting



Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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