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Short communication

Highly sensitive DNA detection with a combination of 2 DNA-intercalating dyes for microchip electrophoresis

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ABSTRACT

A highly sensitive DNA detection method using a combination of ethidium bromide (EtBr) and SYBR Green II (SG II) for microchip electrophoresis was developed. By use of the combination of these intercalating DNA-staining dyes for microchip electrophoresis with Hitachi SV1100 system, the fluorescence intensities corresponding to DNA fragments were obviously increased over those obtained with EtBr only, with accuracy of DNA sizing and quantification. The detection limit with EtBr and the combination of EtBr and SG II were 0.048 and 0.007 ng/μl, respectively. This highly sensitive DNA detection just using the combination of these dyes offering high resolution in a short time will be useful for various biological analyses.

1. Introduction

DNA analysis by agarose electrophoresis is one of the most basic methods in molecular biology. In the agarose gel electrophoresis, a run on the agarose gel requires 30–60 min and requires 10–100 ng amounts of DNA samples. After the electrophoresis, a separate step of imaging by densitometric scanning of a photograph or CCD image of the stained gel is necessary [1]. Ethidium bromide (EtBr) is frequently employed for DNA staining for the purpose of DNA sizing and semiquantification. Recently, some other fluorescent dyes such as SYBR Green I (SG I) and SYBR Green II (SG II) have been used for highly sensitive nucleic acid detection instead of EtBr [2].

Microchip electrophoresis has recently attracted much attention for DNA analysis due to its high efficiency, high throughput, time-saving ability, and cheap reagents [3]. For the commercial instrument, Hitachi SV1100, a loading gel containing EtBr is used; and the lower detection limit of DNA is 0.1 ng/μl. In the present study, we examined the DNA detection limit of a Hitachi SV1100 and an EtBr and SG II mixture instead of only EtBr. The accuracies of DNA sizing and quantification were also examined.

2. Materials and methods

2.1. Sample preparation and reagents

Sample DNA fragments of 322 bp derived from pUC118 (3162 bp) were digested with *Pvu*II (TOYOBO, Tokyo, Japan), separated by gel electrophoresis on agarose, and then purified with a QIAquick Gel Extraction Kit (QIAGEN, Tokyo, Japan). PCR markers (50 bp, 150 bp, 300 bp, 500 bp, and 766 bp) and a low-molecular-weight DNA ladder (25 bp, 50 bp, 75 bp, 100 bp, 150 bp, 200 bp, 250 bp, 300 bp, 350 bp, 500 bp, and 766 bp) were purchased from NEB (Ipswich, MA). EtBr, SG I, and II (concentration not given) were obtained from Molecular Probes, Inc. (Eugene, OR). The sample DNA fragments of 322 bp (10 ng, 5 ng, 1 ng, 0.5 ng, and 0.25 ng) were subjected to gel electrophoresis on 2% agarose gels followed by staining with 1.0 μg/ml EtBr for 1 h. For SG II staining, a 200,000× dilution of a commercial SG II stock solution or an EtBr and SG II mixture (EtBr + SG II) was used. The stained gel was excited with a 254 nm trans-illuminator, after which a CCD (ATTO Co., Tokyo, Japan) image was obtained.

2.2. Instrumentations and microchip preparation

Experiments were performed on a Hitachi SV1100 microchip electrophoresis instrument (Hitachi High-Technologies Co., Tokyo, Japan) with a light-emitting diode confocal fluorescence detector (excitation at 470 nm and measurement of fluorescence at 580 nm). The instrument consists of a bench-top device (chip

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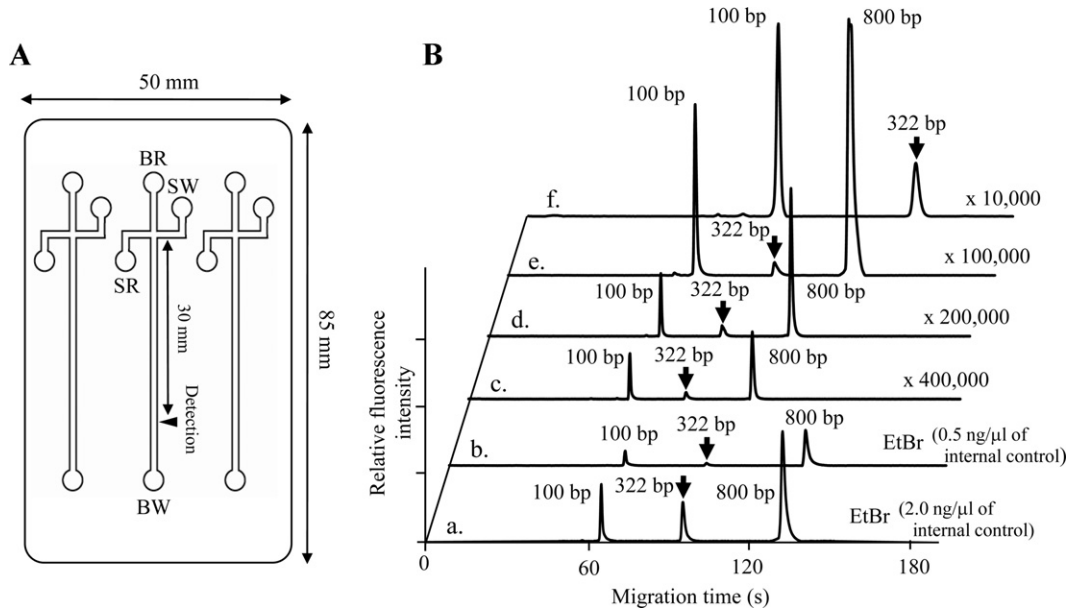

Fig. 1. Design of the *i*-chip used of Hitachi SV1100 microchip electrophoresis and analysis of 322-bp DNA fragment after microchip electrophoresis with EtBr or SG II. (A) Schematic diagram of the microchannel structure. The loading gel was infused from the buffer waste (BW) into the microchannel of the *i*-chip by using a syringe, and buffer reservoir (BR) and sample waste (SW) were filled with 10 μl of gel by using a pipette. A pipette was used to fill the sample reservoir (SR) with 1.0 μl of internal control and 9.0 μl of sample. (B) Electropherograms from analysis of the 322-bp DNA fragment with EtBr staining or SG II staining. The bottom electropherogram was obtained with 2.0 ng/μl of the internal control and 1.0 ng/μl of the 322-bp DNA fragment stained with EtBr (a). The second electropherogram from the bottom was obtained with 0.5 ng/μl of the internal control and 100 pg/μl of the 322-bp DNA fragment stained with EtBr (b). The other electropherograms were obtained with 0.5 ng/μl of the internal control and 100 pg/μl of the 322-bp DNA fragment stained with SG II diluted as indicated (c–f).

reader) that is connected to a personal computer. The SV1100b software includes data collection, presentation, and interpretation functions. Disposable *i*-chips (Hitachi High-Technologies Co.), fabricated from polymethylmethacrylate, and comprising an interconnected network of fluid reservoirs and microchannels, were used for all the experiments (Fig. 1A). An *i*-DNA 12 kit (Hitachi High-Technologies Co.) was employed for microchip electrophoresis instead of the original loading gel in an *i*-chip DNA kit (Hitachi High-Technologies Co.) because of the presence of EtBr in the original loading gel. For microchip electrophoresis, EtBr was added to the loading gel to the final concentration at 1.0 μg/ml. For the examination of the accuracies of DNA sizing and quantification after microchip electrophoresis with the *i*-DNA 12 kit, 1.0 μl of an internal control comprising 20 ng/μl each of 100- and 800-bp dsDNA fragments, 1.0 μl of 10 ng/μl of 322-bp DNA fragment, and 8.0 μl of double-distilled water (ddw) were mixed and then subjected to electrophoresis. For the microchip electrophoresis with the original *i*-chip DNA, 1.0 μl of an internal control comprising 20 ng/μl each of 100- and 800-bp dsDNA fragments, 1.0 μl of 1.0 ng/μl of 322-bp DNA fragment, and 8.0 μl of ddw were mixed and then electrophoresed. As DNA markers and 322-bp DNA fragment for DNA sizing and quantification, 1.0 μl of an internal control comprising 5.0 ng/μl each of 100- and 800-bp dsDNA fragments and 9.0 μl of sample were mixed and subjected to electrophoresis. For the examination of the effects of SG I or II on the accuracy of DNA sizing and quantification on microchip electrophoresis, 10,000–400,000× dilutions of each dye were added to the loading gel without EtBr.

2.3. Microfluidic separation

Samples were loaded by electrokinetic injection, which was achieved by applying 300 V for 60 s to the sample waste (SW) well while grounding the other 3 wells. The separation procedure was performed by applying a fixed 130 V to both the sample reservoir (SR) and SW wells, while the buffer reservoir (BR) well was kept grounded. Simultaneously, 750 V was applied to the buffer waste (BW) well. Each sample could be analyzed in parallel within 4 min.

3. Results and discussion

The 322-bp DNA fragment was analyzed to evaluate the ability of the Hitachi SV1100 with the *i*-DNA 12 kit to generate consistent results with respect to DNA sizing and quantification like the original loading gel in the *i*-chip DNA kit. The Hitachi SV1100 is capable of estimating DNA size between 100 and 800 bp with the original loading gel [3]. The concentration of the internal control DNA fragments measured manually was 2.0 ng/μl, and we used 20 ng/μl. A single peak corresponding to 322-bp DNA was observed between the peaks of the internal controls (Fig. 1B-a). The estimated DNA size differed from the predicted size of 322 bp by only 3 bp (data not shown). Also, the estimated DNA concentration of the 322-bp fragment differed from the predicted concentration (1.0 ng/μl) by 4.0% (data not shown). In the microchip electrophoresis with the original loading gel, accuracy in DNA sizing in the range between 100 bp and 800 bp and accuracy of quantification at <1.8 ng/μl DNA were reported earlier [3]. These results indicate that the accuracies of DNA sizing and quantification were similar to those obtained with the original loading gel.

SG I or II was added to the loading gel from the *i*-DNA 12 kit to a final concentration corresponding to a 10,000×, 100,000×, 200,000× or 400,000× dilution of the commercially available stock solution. On analysis of 100 pg/μl of the 322-bp DNA fragment with SG I, obvious errors in DNA sizing and quantification, compared with those obtained with SG II, were observed (data not shown). Although there was a tendency for dose-dependent increases in the fluorescence intensities of DNA fragments, the migration times were also extended in a dose-dependent manner for SG II; and the 800-bp fragment at the 10,000× dilution was out of range for DNA sizing (Fig. 1B-f). The size and quantity of the 322-bp DNA fragment were estimated to be 317 ± 3 bp and 111.6.0 ± 11.5 pg/μl (n = 3) with the 400,000× dilution, 331 ± 4 bp and 98.0 ± 6.0 pg/μl (n = 3)

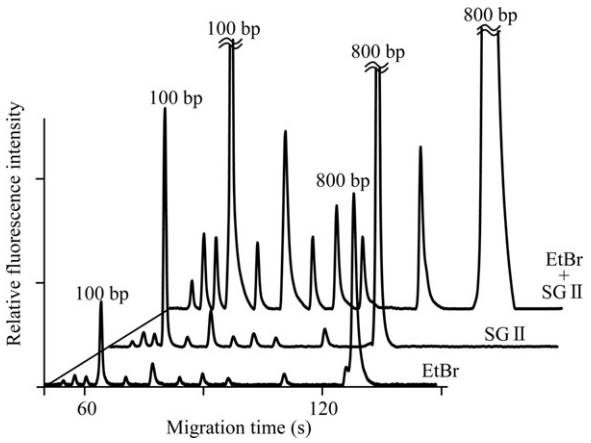

Fig. 2. Effect of dye on the accuracy of DNA sizing by microchip electrophoresis. Electropherograms obtained with 1.0 ng/μl of the low-molecular-weight DNA ladder and the internal control stained with EtBr, SG II or EtBr + SG II are shown.

with the 200,000× dilution, and 347 ± 13 bp and 92.6 ± 13.0 pg/μl (n = 3) with the 100,000× dilution; not detection but these parameters could not be measured with the 10,000× dilution (data not shown). So we employed 200,000× diluted SG II (i.e., 20 times below the manufacturer's recommended dilution) for DNA staining in subsequent experiments. The reproducibility of the microchip electrophoresis in different channels and in a single channel with successive electrophoreses with EtBr and/or 200,000× diluted SG II staining was examined. The relative standard deviation in every case for the migration times of the 100- and 800-bp fragments was less than 3% (data not shown). These results indicate that the reproducibility of microchip electrophoresis was retained with SG II and EtBr + SG II DNA staining.

Electropherograms of a low-molecular-weight DNA ladder and internal control are shown in Fig. 2. Although peaks corresponding to molecular weight with EtBr or SG II were very weak, 12 peaks corresponding to 25–800-bp fragments were obtained for every DNA staining. A linear relationship was observed between the estimated DNA size and the applied DNA size with all the dyes, i.e., EtBr (y = 0.9873x − 0.0224, R^2 = 0.9993), SG II (y = 0.9829x + 2.966, R^2 = 0.9992), and EtBr + SG II (y = 0.9846x + 1.6674, R^2 = 0.9994). These results demonstrate that SG II or EtBr + SG II DNA staining as well as EtBr staining is suitable for DNA sizing between 25 and 800 bp. The fluorescence intensity corresponding to each DNA fragment with SG II or EtBr + SG II was obviously increased compared with that obtained with EtBr staining (EtBr + SG II > SG II > EtBr).

To examine the DNA concentration detection limit with EtBr, SG II, and EtBr + SG II, we analyzed 0.005–1.0 ng/μl 322-bp DNA fragments (Fig. 3). Peaks corresponding to 0.1 ng/μl and 0.033 ng/μl 322-bp DNA fragment were slightly detectable upon EtBr (Fig. 3A) and SG II staining (Fig. 3C), respectively. Upon EtBr + SG II staining, the 0.01 ng/μl DNA fragment was detectable by microchip electrophoresis (Fig. 3E). The accuracy of quantification of the 322-bp DNA fragment was examined next. The accuracy of quantification observed with EtBr staining was at <1.0 ng/μl (y = 0.954x + 0.0477, R^2 = 0.983). Although the error of quantification increased with SG II staining at >0.4 ng/μl, the accuracy of quantification observed with EtBr + SG II staining was at <1.0 ng/μl (y = 1.0522x + 0.0007, R^2 = 0.9826). The lower detection limit of the DNA concentration was calculated graphically: 0.048 ng/μl for EtBr, 0.025 ng/μl for SG II, and 0.007 ng/μl for EtBr + SG II staining. Thus, the lower detection limit for the DNA was 7 times lower for EtBr + SG II staining than for EtBr staining. Upon agarose gel electrophoresis with EtBr staining, 5.0 ng DNA could be detected (Fig. 3B). On the other hand, 5.0 ng DNA was hardly detected by SG II staining (Fig. 3D). As we employed 200,000× diluted SG II (i.e., 20 times below the manufacturer's recommended dilution) for DNA staining, this diluted concentration was apparently too low for agarose gel electrophoresis CCD image analysis. Judging from the results of DNA staining with EtBr (Fig. 3B) and EtBr + SG II (Fig. 3F), the amounts of EtBr intercalated DNA were similar. Since the absorption spectrum of EtBr overlaps with the fluorescence spectrum of SG II [2], the excitation energy of SG II is transferred to ground-state EtBr by the effect of Forster-type energy transfer [4]. Because the fluorescence quantum yield of EtBr is apparently lower than that of SG II, as shown in Fig. 2, the fluorescence intensity may be decreased relative to the SG II staining. However, the fluorescence intensity in the case of EtBr + SG II staining was apparently higher than the sum of the indi-

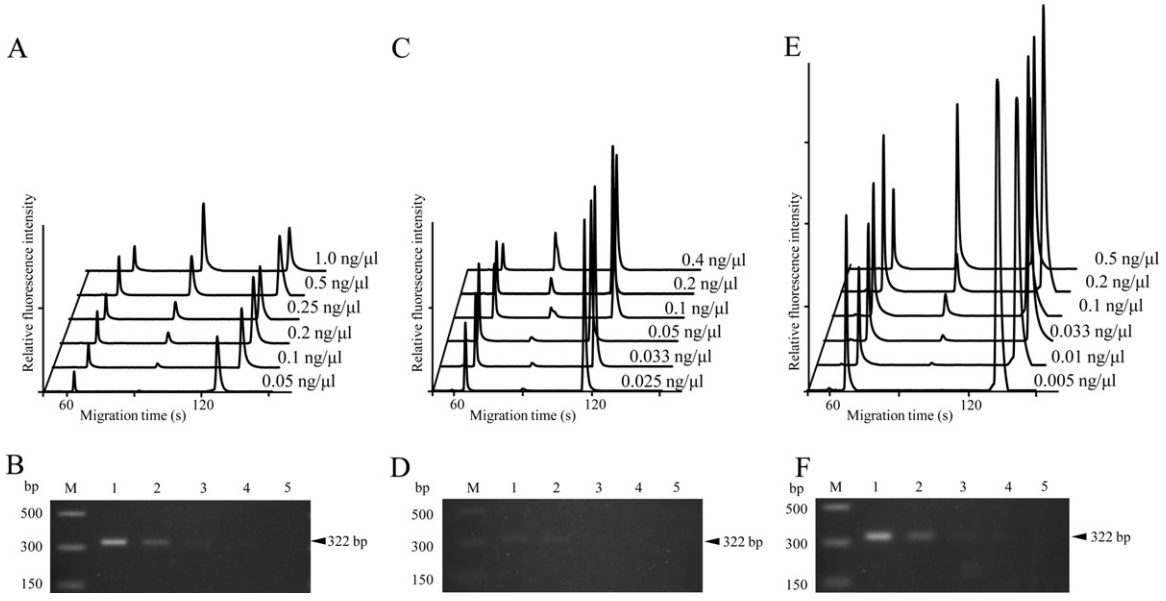

Fig. 3. Lower detection limit of Hitachi SV1100 microchip electrophoresis. Electropherograms of sample DNA fragments at different concentrations stained with EtBr (A), SG II (C) or EtBr + SG II (E) are shown. The 322-bp DNA fragment was diluted to 0.005–1.0 ng/μl with ddw; and 9.0 μl of a sample and 1.0 μl of the internal control were added to the SR. The results of 2% agarose gel electrophoresis for staining with 1.0 μg/ml of EtBr (B), 200,000× diluted SG II stock solution (D) or both EtBr and SG II (F) for 1 h are also shown. Lanes M and 1–5 correspond to PCR markers and 10, 5, 1, 0.5, and 0.25 ng of 322-bp DNA fragment, respectively.

vidual fluorescence intensities corresponding to the DNA stained with EtBr or SG II (Fig. 2). Furthermore, shifts occurred in the migration times for EtBr + SG II staining compared with those for EtBr or SG II staining. EtBr and SG II are known as intercalating dyes for DNA staining, and the intercalator–DNA interaction induces shifts in migration times by changing the weights and charges of the DNA fragments [5]. Therefore, the amount of intercalated SG II in DNA with EtBr + SG II may be much greater than that with individual intercalation. It is known that the binding of EtBr to closed circular DNA causes unwinding of the duplex and the superhelix structure [6]. This unwinding of the DNA structure may lead to easier intercalation of SG II with the DNA than that of SG II alone. Therefore, the fluorescence intensity apparently increased, which made highly sensitive DNA detection.

Our presented simple method for highly sensitive DNA detection with good accuracy as to DNA sizing and quantification involving a commercially available microchip electrophoresis system will be useful for a variety of biological and biochemical analyses.

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