Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 323–328

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Analysis of DNA ligation by microchip electrophoresis

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ARTICLE INFO

Article history:
Received 4 September 2009
Received in revised form
14 December 2009
Accepted 19 December 2009
Available online 29 December 2009

Keywords:
Microchip electrophoresis
DNA ligase
Endonuclease
Alkaline phosphatase
Recombinant DNA

ABSTRACT

We describe the potential of microchip electrophoresis with a Hitachi SV1100, which can be used to determine DNA sizes between 500 and 5000 bp with good quantification (DNA concentration, <8 ng/μl) within 5 min, for the analysis of DNA ligation. On analysis of an electropherogram of a ligation mixture of the pTAC1-T vector and a 789 bp PCR-amplified DNA fragment, the presence of recombinant DNA was easily detected by comparison with an electropherogram obtained without ligase. On analysis of a ligation mixture of pUC19/*Eco* RI without alkaline phosphatase treatment and a 667 bp *Eco* RI-digested fragment of foreign DNA, several peaks observed in the electropherogram corresponded to the formation of monomeric and polymeric insert DNAs, self-ligated vector DNA, and recombinant DNA. On the other hand, several peaks were also observed in the electropherogram of the ligation mixture of pUC19/*Eco* RI with alkaline phosphatase treatment and the 667 bp *Eco* RI-digested fragment of foreign DNA, the fluorescence intensity corresponding to recombinant DNA apparently being increased. These results indicate the potential of microchip electrophoresis for the analysis of DNA ligation, it offering high resolution in a short time.

1. Introduction

DNA ligation is one of the most basic and frequently used methods in molecular biology; for example, for the ligation of a foreign DNA fragment into a vector DNA to construct a recombinant DNA molecule. T4 ligase, which can catalyze the formation of a phosphodiester bond between directly adjacent 5′-phosphate and 3′-hydroxyl termini in duplex DNA with blunt or cohesive-end termini, is used for DNA ligation [1]. For rough confirmation of the presence of an inserted DNA fragment in the recombinant DNA, agarose gel electrophoresis of a ligation mixture is frequently performed, followed by ethidium bromide staining [2]. The resolution of DNA separation on agarose gel electrophoresis is relatively low, so it is difficult to accurately identify the products by means of ligation reaction, e.g., self-ligation of plasmid DNA, monomeric and polymeric circularization of insert DNA, and precise construction of recombinant DNA [3]. These methods are manual and time-consuming, each run on an agarose gel and ethidium bromide staining requiring about 1 h and consuming a microgram amount of a precious ligated DNA sample. After the electrophoresis, a separate step of imaging by densitometric scanning of a photograph or CCD imaging of a stained gel is necessary [1]. Convenient, sensitive, and accurate methods for the analysis of ligated DNA are thus required.

Microchip electrophoresis has received considerable interest as to DNA analysis due to its intrinsic characteristics of high speed, high throughput, low consumption of samples and reagents, miniaturization, and automation [4]. Some commercial instruments, such as the Agilent 2100 Bioanalyzer, Shimadzu MCE2010, and Hitachi SV1100 and SV1210, have been developed, which have greatly promoted the further application of microchip electrophoresis. In microchip electrophoresis, nucleic acid fragments are separated by capillary electrophoresis on a chip with microfabricated channels, with automated detection as well as on-line data evaluation [5]. In the present study, analysis of DNA ligation of pUC19/*Eco* RI with or without alkaline phosphatase treatment and an *Eco* RI-digested segment of foreign DNA, with pTAC1-T as the T vector and PCR product, was performed to examine the feasibility of recombinant DNA analysis using a Hitachi SV1100 with direct application of a DNA ligation mixture comprising unincorporated NTPs, vector and insert DNA, enzyme and buffer components. The potential of microchip electrophoresis for the analysis of DNA ligation was shown, it offering high resolution in a short time.

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0731-7085/$ – see front matter
doi:10.1016/j.jpba.2009.12.023

2. Materials and methods

2.1. Reagents and sample preparation

Restriction endonuclease *Eco* RI was purchased from TOYOBO (Tokyo, Japan). T4 DNA ligase and Calf Intestine Alkaline Phosphatase (CIP) were obtained from NIPPON GENE (Tokyo, Japan). EX Taq Hot Start Version for PCR was purchased from TAKARA Shuzo (Kyoto, Japan). Other reagents were of the highest grade commercially available. Plasmid DNA, DS112-36, which was constructed with a λZAP cDNA library synthesis kit for isolation of the full-length cDNA of rat carnitine palmitoyltransferase I (CPTI) [6], was employed for the ligation reaction. A 667 bp *Eco* RI-digested segment was subcloned into the pUC19 *Eco* RI site with or without CIP treatment, a 3353 bp recombinant plasmid, pYU1, being obtained. In order to amplify the foreign DNA, pUC19/CPTI was used as a template DNA for PCR. Two primers 5′-GTTTTCCCAGTCACGTTGTTGTA-3′ [which binds to nucleotides 359–381 of pUC19] and 5′-GGAAACAGCTATGACCATG-3′ [corresponding to nucleotides 479–461 of pUC19] were used to amplify the foreign DNA, which was inserted into the *Eco* RI site in pUC19 (between nucleotides 396 and 397). The conditions for PCR were denaturation for 30 s at 95 °C, annealing for 30 s at 54 °C, and extension for 30 s at 72 °C, for 35 cycles. The amplified 789 bp DNA fragment was purified with a GENE CLEAN II kit according to the supplied manual (BIO 101, Inc., Vista, CA). For cloning of the PCR product, a DynaExpress TA PCR Cloning Kit was employed (Bio-Dynamics Laboratory Inc., Tokyo, Japan), and the amplified DNA fragment was cloned into the pTAC1-T vector as the T vector, the resulting 3524 bp recombinant DNA being referred to herein as pYU2. Transformation of these ligated DNA into *Escherichia coli* DH5α was performed, and blue-white color selection on an X-gal LB agar plates containing 100 μg/ml of ampicillin was performed to select the clone that contained pYU1 or pYU2.

2.2. Microchip preparation

Disposable *i*-chips (Hitachi Electronics Co., Tokyo, Japan), fabricated from polymethylmethacrylate and comprising an interconnected network of fluid reservoirs and microchannels, were used for all the separation experiments (Fig. 1A). Three samples can be analyzed on one of these chips. The loading gel containing ethidium bromide was infused from the buffer reservoir (wells, 3, 7, and 11) into the microchannels of an *i*-chip by using a syringe, and wells 1, 2, 5, 6, 9, and 10 were filled with 10 μl gel by using a pipette. Wells 4, 8, and 12 were the sample wells, a pipette being used to fill each well with 1.0 μl internal control, including 2.0 ng/μl each of 0.5 and 5.0 kb dsDNA fragments as markers for DNA sizing, and 9.0 μl sample. Each sample could be analyzed in parallel within 5 min.

2.3. Instruments

Experiments were performed with a Hitachi SV1100 microchip electrophoresis instrument (Hitachi Chemicals, Tokyo, Japan), which is capable of rapidly sizing DNA fragments through light-emitting diode confocal fluorescence detection (excitation at 470 nm, measurement of fluorescence at 580 nm) [7]. The instrument consists of a bench-top device (chip reader) connected to a personal computer. The sv1100b software allows data collection, presentation, and interpretation. The data are displayed as both simulated gel images and electropherograms. Electropherograms of the internal controls, i.e., 0.5 and 5.0 kb DNA fragments, are shown in Fig. 1B. Sizing and quantification of DNA fragments can also be presented in a tabular form (Fig. 1C), the estimated DNA size unit being 0.1 kb with the sv1100b software. The chip reader contains programmable high voltage power supplies, each of which is connected to a platinum electrode. These electrodes allow the instrument to perform multiple injections and other fluid manipulations from specific sample wells.

During the sample-loading procedure, 300 V was applied to the sample waste well (2) for 60 s while the other three wells (1, 3, and 4) were grounded. During separation, the buffer reservoir (1) was grounded, and 75 V was applied to both the sample reservoir (4) and sample waste reservoir (2), with 400 V applied thereafter to the analysis reservoir (3).

2.4. Microfluidic separation

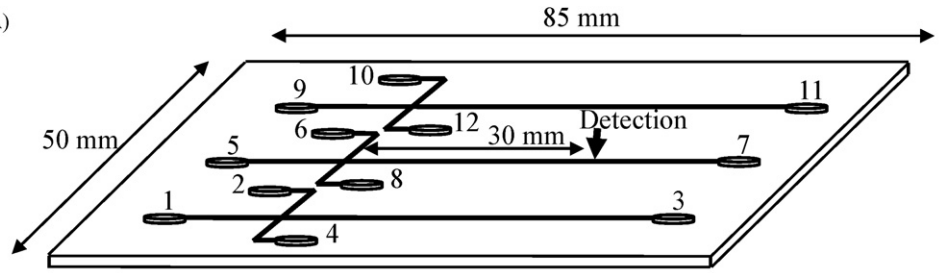
All chips were prepared according to the manufacturer's instructions with the supplied materials including the gel and internal control (*i*-chip LDNA, Hitachi Chemicals). To examine variations in DNA size in different channels, 1.0 μl internal control including 500 and 5000 bp DNA fragments and 9.0 μl TE-buffer instead of a sample were added to a sample well. The sample well was connected through a network of channels to the separation lane, which was used to perform the DNA separation. For estimation of the DNA sizes of pUC19/*Eco* RI and CIP-treated DNAs, samples were purified by phenol/chloroform extraction, precipitated with 2 vol. of cold ethanol, centrifuged, washed once with 70% ethanol, allowed to dry under ambient conditions, and then resuspended in TE-buffer. For analysis of the 667 bp *Eco* RI-digested DNA segment, the 787 bp PCR-amplified DNA fragment was separated by agarose electrophoresis, followed by purification with a GENE CLEAN kit. For analysis of DNA ligation on microchip electrophoresis, 5.0 μl ligation mixture, 4.0 μl TE-buffer, and 1.0 μl internal control were added to the sample well, followed by analysis.

3. Results and discussion

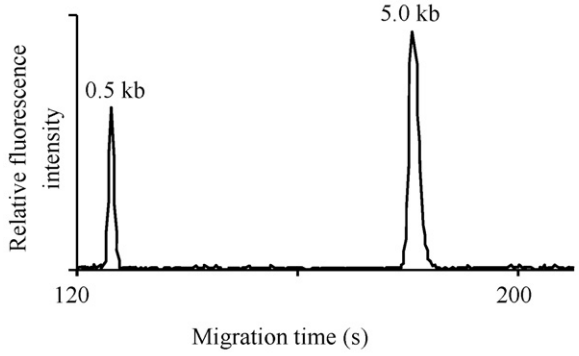
The Hitachi SV1100 performs capillary electrophoresis in each of three different channels, and thus three samples can be analyzed on one chip. To evaluate the reproducibility of electrophoresis in different channels, the migration times in individual channels were examined in comparison with those of internal controls, i.e., 500 and 5000 bp DNA fragments. The relative standard deviation (RSD) in six different channels for the migration times of the 0.5 and 5.0 kb fragments were 1.20% and 0.87%, respectively, indicating the reproducibility of the electrophoresis even in different channels (data not shown). The Hitachi SV1100 with *i*-chip LDNA can be used to estimate DNA sizes between 0.5 and 5.0 kb. 7.2 ng/μl pUC19/*Eco* RI DNA fragment (2686 bp) was analyzed to evaluate the ability of this microchip electrophoresis to generate consistent results with respect to DNA size. The mean ± standard deviation (SD) of DNA size for six different experiments was 2.53 ± 0.003 kb, the error from the predicted size being about 6.1% (data not shown). This error is relative large because the estimated DNA size unit with the sv1100b software is 0.1 kb. To examine the effect of DNA concentration on the accuracy of quantification of a DNA sample, samples containing 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 ng/μl pUC19/*Eco* RI DNA fragment were analyzed with the Hitachi SV1100 (Fig. 2). As shown in Fig. 2, the accuracy of obtained quantification was less than 8.0 ng/μl DNA.

Fig. 3 shows electropherograms of 7.2 ng/μl 789 bp PCR product (A), 7.2 ng/μl PCR product after ligation (B), 5 ng/μl pTAC-1 (2734 bp) (C), 5 ng/μl pTAC-1 after ligation (D), PCR product and pTAC-1 (E), and PCR product and pTAC-1 after ligation (F). The DNA sizes of the PCR product and pTAC-1 were estimated to be 0.7 kb and 2.5 kb, respectively (A, C), the error of DNA sizing being about 10%. A similar phenomenon was observed for the electropherogram of each ligation mixture (Fig. 3B, D). Taq DNA polymerase yields PCR products that have adenine overhangs at each of their 3′

(A)



(B)



(C)

Peak No.	Migration time (s)	Size (kb)	Peak intensity	Concentration (ng/μl)
1	126.4	0.5	642	2.0
2	181.0	5.0	950	2.0

Fig. 1. Design of the *i*-chip and the data output on a microchip. (A) With this chip, capillary electrophoresis can be performed in each of three different channels, and thus three samples can be analyzed on the chip. (B) Analysis of internal controls, using the sv1100b software with the Hitachi SV1100 to present the results in the form of electropherograms. (C) The corresponding analytical results for each internal control DNA fragment were tabulated, and each peak was estimated automatically to be 0.5 and 5.0 kb in DNA size, and 2 ng/μl in concentration, respectively.

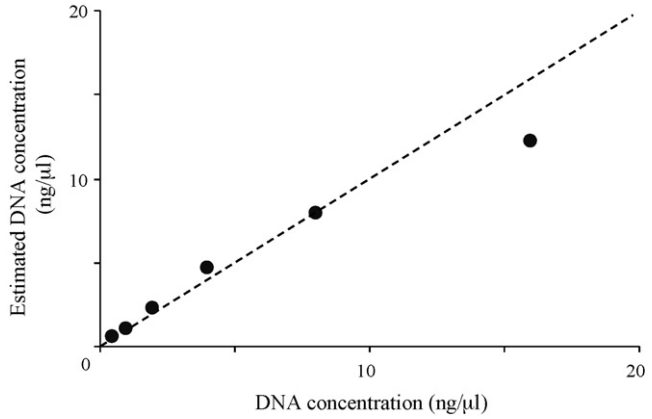


Fig. 2. Effect of DNA concentration on the accuracy of DNA quantification with the Hitachi SV1100. The estimated DNA concentrations with the Hitachi SV1100 are plotted. The filled circles indicate the estimated DNA concentrations of individual samples, and the dashed line indicates the precise relationship between the estimated DNA concentration and the applied sample concentration.

termini [1]. T vectors with a protruding 3′ thymidylate residue at each of their 3′ termini are frequently used to clone PCR-amplified DNA fragments that carry an adenine at their 3′ termini [8,9]. One base pair overhang on each DNA molecule effectively prevents self-ligation (Fig. 3B and D). The ligation mixture without T4 DNA ligase was subjected to microchip electrophoresis, the DNA sizes of the PCR product and pTAC-1 being estimated to be 0.8 kb and 2.4 kb, respectively (Fig. 3E). The estimated DNA sizes were different from when the PCR product and pTAC-1 were analyzed separately. These differences may be due to the presence of $MgCl_2$, DTT, and ATP in the 10× reaction buffer for T4 DNA ligase [10]. A peak (peak 3) appeared just behind the peak corresponding to pTAC-1 (peak 2; estimated DNA size, 2.4 kb) in the electropherogram after the ligation, the material's DNA size being estimated to be 3.3 kb. This peak must correspond to recombinant plasmid DNA pYU2 (predicted DNA size, 3524 bp). After transformation of the ligation mixture, blue-white color selection was performed. Approximately 96.7% of the colonies on an X-gal plate were white (data not shown). Twenty-three white colonies were randomly selected from an X-gal plate and it was confirmed that all of them harbored pYU2.

As shown in Fig. 4, the DNA sizes of pUC19/*Eco* RI (2686 bp) (A), pUC19/*Eco* RI/CIP (2686 bp) (C), and the 667 bp *Eco* RI-digested DNA fragment (E) were estimated to be 2.5 kb, 2.5 kb, and 0.7 kb with the Hitachi SV1100, respectively. On analysis of the ligation

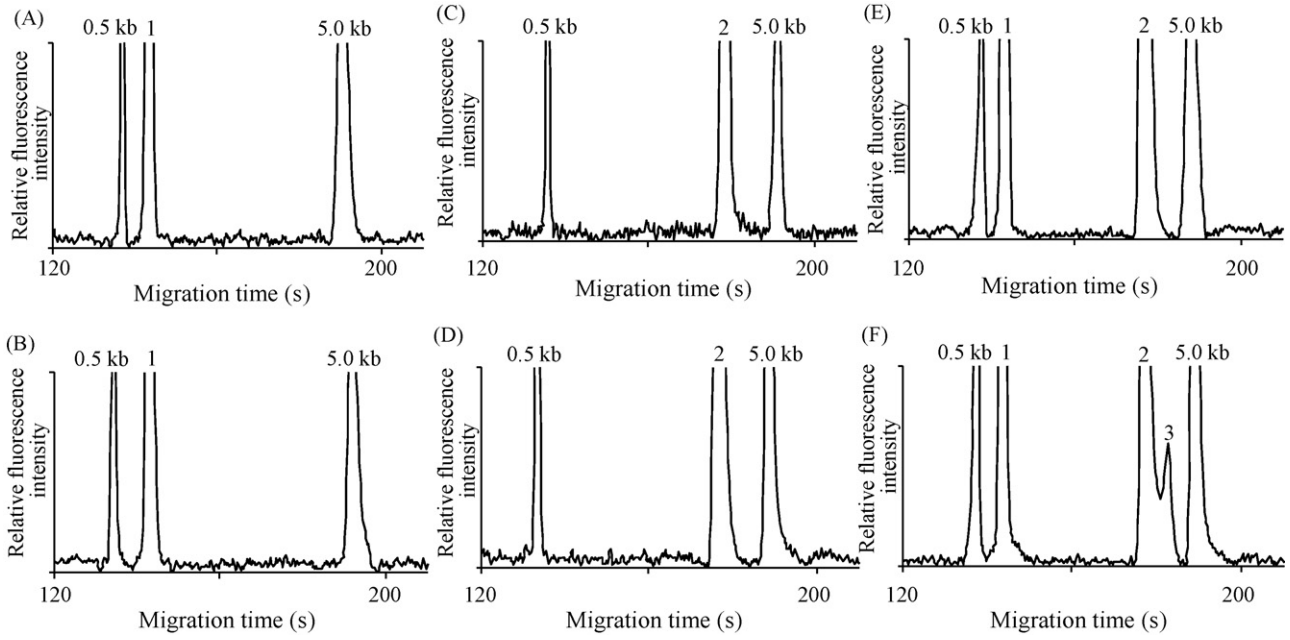


Fig. 3. Electropherograms of the 789 bp PCR product (A), the PCR product after ligation (B), pTAC-1 (C), pTAC-1 after ligation (D), the PCR product and pTAC-1 (E), and the PCR product and pTAC-1 after ligation (F). Peaks 1 and 2 correspond to the PCR product and pTAC-1, respectively. pYU2 is represented by peak 3.

of pUC19/*Eco* RI, two peaks were observed between the internal controls (B). The peak 1 material, corresponding to pUC19/*Eco* RI, had an estimated DNA size of 2.4 kb. Regarding the ligation of pUC19/*Eco* RI, for peak 2, which corresponded to self-ligated pUC19, the estimated DNA size was 1.2 kb. This estimated DNA size differing from the predicted size of 2686 bp pUC19 must be due to the conformation of the DNA, nicked circular. The relative mobilities of superhelical circular, nicked circular, and linear DNAs depend primarily on the concentration and type of gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the superhelical circular DNA [11]. Although peaks 3 and 4 may correspond to polymeric circle formation of pUC19, the amounts of DNA were apparently smaller than that of monomeric circles. Fifty nanograms of pUC19/*Eco* RI were subjected to microchip electrophoresis (Fig. 4A and C). While the fluorescence intensity corresponding to pUC19/*Eco* RI (peak 1, Fig. 4B) apparently decreased through consumption due to self-ligation, no significant decrease in the fluorescence intensity for ligation of pUC19/*Eco* RI/CIP (peak 1, Fig. 4D) was observed. CIP was used to prevent self-ligation of DNA

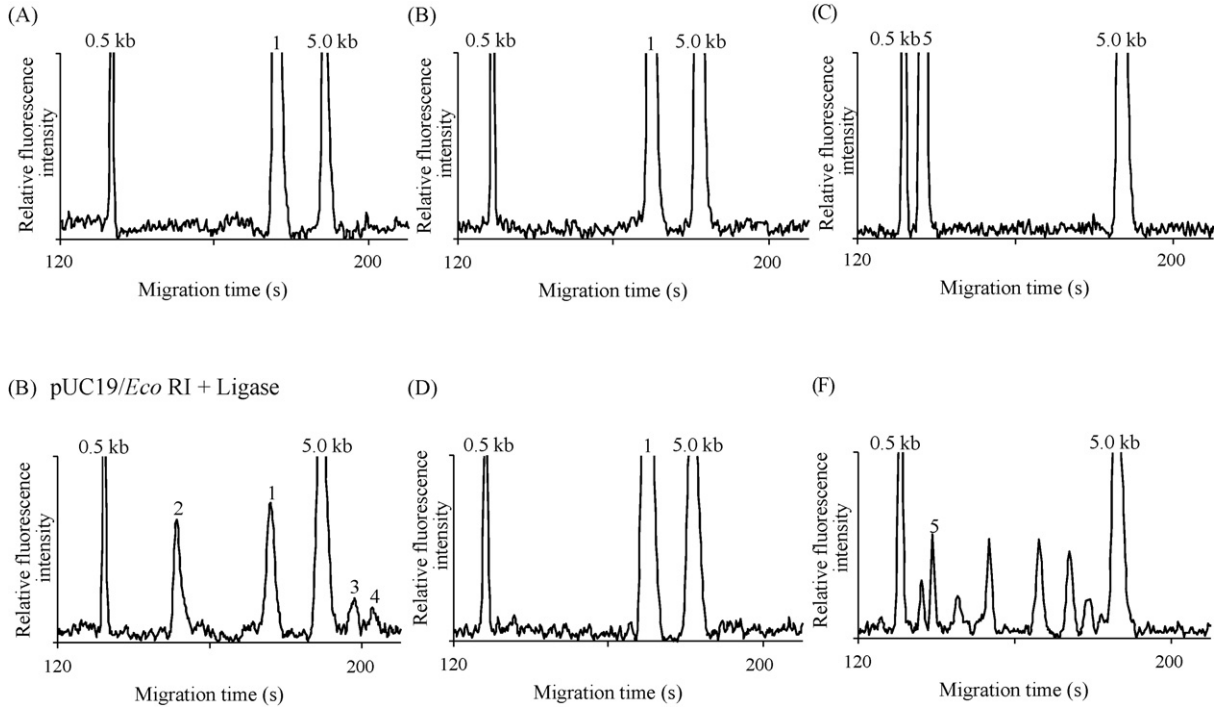


Fig. 4. Electropherograms of pUC19/*Eco* RI (A), pUC19/*Eco* RI after ligation (B), pUC19/*Eco* RI/CIP (C), pUC19/*Eco* RI/CIP after ligation (D), the 667 bp *Eco* RI-digested DNA segment (E), and the 667 bp *Eco* RI-digested DNA segment after ligation (F).

because alkaline-treated DNA fragments lack the 5′-phosphoryl termini required by DNA ligase. These results indicate that the 5′-phosphate group of pUC19/*Eco* RI fragments was sufficiently removed. The DNA size of the *Eco* RI-digested DNA (667 bp) was estimated to be 0.7 kb (peak 5, Fig. 4E). While the fluorescence intensity corresponding to *Eco* RI-digested DNA (migration time, 137.8 s) apparently decreased, several peaks appeared (Fig. 4F). These peaks must correspond to the formation of monomeric and polymeric circles of insert DNA during ligation.

Electropherograms of ligation mixtures of the 667 bp *Eco* RI DNA fragment and pUC19/*Eco* RI with and without CIP treatment are shown in Fig. 5. Without ligase, similar electropherograms were obtained with (Fig. 5C) and without (Fig. 5A) CIP, two peaks being observed between the internal controls in each case. The DNA size of the peak 1 material corresponding to the 667 bp *Eco* RI fragment was estimated to be 0.6 kb, and that of the peak 2 material corresponding to pUC19/*Eco* RI to be 2.5 kb (Fig. 5A and C). In the electropherogram of the ligation mixture without CIP, there were several peaks other than those of the insert DNA (peak 1), vector (peak 2), and internal controls (Fig. 5B). DNA fragments linearized with a restriction endonuclease without CIP become easily circularized through ligation. As shown in Fig. 4B, self-ligation of pUC19 seldom yielded polymeric circles, so most peaks observed in the electropherogram corresponded to the formation of monomeric pUC19 and/or monomeric and polymeric insert DNA. In the electropherogram of the ligation mixture with CIP treatment, several peaks were also observed (Fig. 5D). Interestingly, the fluorescence intensity corresponding to the insert DNA (peak 1, Fig. 5D) apparently decreased compared to without ligase (Fig. 5C), but the decrease was not as great as that observed for pUC19/*Eco* RI/CIP (peak 2). CIP treatment of pUC19/*Eco* RI prevents self-ligation, a small portion of the vector being consumed for ligation with insert DNA. In both electropherograms (Fig. 5B and D), a large DNA molecule that migrated more slowly than pUC19 was observed (peak 3), its estimated DNA size being 3.3 and 3.1 kb, respectively. A similar phenomenon of slower migration than the T vector was observed for pYU2, of which the predicted DNA size was 3524 bp (Fig. 3F, peak 3). This peak may correspond to pYU1 (predicted DNA size, 3353 bp), because of the similar DNA sizes and sequences of pYU1 and pYU2. A significant increase in the fluorescence intensity of peak 3 with CIP treatment (Fig. 5D) was observed compared to without CIP treatment (Fig. 5B). This indicates the effective construction of recombinant DNA with CIP treatment. After the transformation of each ligation mixture, blue-white color selection was performed. Approximately 32.5% of the colonies on an X-gal plate were white for the ligation mixture without CIP, and approximately 97.3% for the ligation mixture with CIP (data not shown). Twenty-three white colonies were randomly selected from each X-gal plate, and it was confirmed that all of them harbored the correct insert DNA.

DNA ligation is one of the most frequently used methods in molecular biology. Analysis of a ligation mixture and estimation of the size of cloned DNA by agarose gel electrophoresis are relatively difficult because of the low resolution on DNA sizing. To determine whether the ligation was successful or not, transformation is essential, followed by plasmid isolation. Transformation is time-, sample-, and reagent-consuming. In the present study, we showed the potential of microchip electrophoresis for the analysis of a

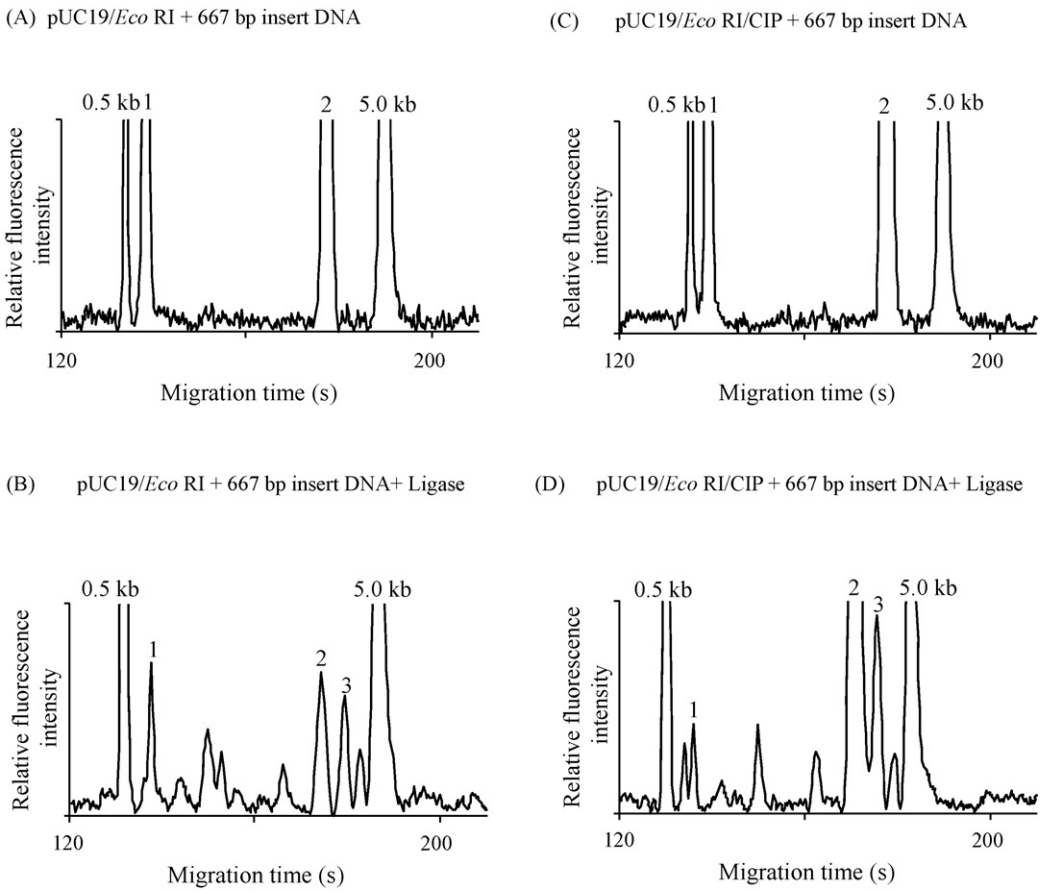


Fig. 5. Electropherograms of pUC19/*Eco* RI and the 667 bp *Eco* RI-digested DNA segment (A), pUC19/*Eco* RI and the 667 bp *Eco* RI-digested DNA segment after ligation (B), pUC19/*Eco* RI/CIP and the 667 bp *Eco* RI-digested DNA segment (C), and pUC19/*Eco* RI/CIP and the 667 bp *Eco* RI-digested DNA segment after ligation (D). Peaks 1 and 2 correspond to the 667 bp *Eco* RI-digested DNA segment and pUC19/*Eco* RI with and without CIP treatment, respectively. pYU1 is represented by peak 3.

ligation mixture, it being easy to determine whether the construction of cloned DNA was successful or not before transformation. On microchip electrophoresis, we can recognize recombinant plasmid DNA. Gel electrophoresis has been used for kinetic studies on a DNA-modifying enzymes [12]. Good quantification of the pUC19/*Eco* RI fragment was possible with a DNA concentration of <8.0 ng/μl, so microchip electrophoresis must also be useful for enzymatic kinetic analysis, i.e., that involving a phosphatase and DNA ligase.

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