

# Usefulness of microchip electrophoresis for reliable analyses of nonstandard DNA samples and subsequent on-chip enzymatic digestion

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The Hitachi SV1100 utilizes capillary electrophoresis on a microchip that is capable of rapidly sizing DNA fragments. Reproducibility of electrophoresis in different channels was shown by comparing the migration times of the internal controls, DNA fragments of 100 and 800 bp. The range of DNA sizing for this microchip is between 100 and 800 bp, and accuracy in sizing of a 322 bp DNA fragment of a pUC118 *PvuII* digest was observed, independent of DNA concentration. Although relatively good quantification of this fragment was observed with a DNA concentration of 1.83 ng·μL<sup>-1</sup>, error increased in a dose-dependent manner. Furthermore, the feasibility of sequential analysis with this microchip was shown by the reproducibility of successive electrophoreses of the internal

control in one channel. When the pUC118 *PvuII* digest was treated with endonuclease *KpnI* on the microchip for 10 min, sequential analysis showed that the 322 bp fragment completely disappeared and two peaks corresponding to the 130 and 192 bp fragments appeared. This analysis was performed within 4 min, and the peaks were estimated as 127 and 183 bp, respectively. These results indicate the potential of on-microchip endonuclease treatment of plasmid DNA with sequential analysis, offering high resolution in a short time.

**Keywords:** electrophoresis; microchip; plasmid; quantification; sizing.

Plasmid DNA is one of the most common genetic vectors used in molecular biological applications [1]. Common practice in plasmid analysis is to cut the plasmid DNA at one site with a restriction endonuclease. For DNA sizing and semiquantification of digested DNA fragments, agarose gel electrophoresis is performed with a linear DNA sizing marker followed by ethidium bromide staining. These methods are manual and time-consuming; each endonuclease treatment and run on an agarose gel requires about 1 h, and consumes microgram amounts of DNA fragments. Furthermore, after the electrophoresis, separate steps of imaging with densitometer scanning of the photograph or CCD imaging of the stained gel are necessary [2].

Miniaturization of analytical and biological instruments has developed rapidly in the past 10 years [3–8]. Microchip electrophoresis has recently attracted much attention in DNA analysis due to its high efficiency, high throughput, time-saving ability, easy operation, and low consumption of samples and reagents [9]. Some commercial instruments, such as the Agilent 2100 Bioanalyzer, Shimadzu MCE2010,

and Hitachi SV1100 and SV1210, have been developed, which has greatly promoted the further application of microchip electrophoresis. In microchip electrophoresis, nucleic acid fragments are separated by capillary electrophoresis in a chip with microfabricated channels, with automated detection as well as on-line data evaluation. Microfabricated devices are forecast to be fundamental to the postgenome era, especially in the field of genetics and medicine [10]. However, although there are many reports of the use of these instruments to evaluate standard DNA, DNA ladders, PCR products, and commercially available plasmid digests [11–20], little information is available about their use with biological materials [11]. It is therefore necessary to evaluate these microfabricated devices for DNA analysis of biological materials, for example plasmid DNA isolated from bacteria.

In the present study, we evaluated the ability of the Hitachi SV1100 to generate consistent results for the migration time of internal control DNA fragments of 100 and 800 bp on electrophoresis in different channels. Furthermore, we investigated the accuracy of sizing and quantification of endonuclease-digested plasmid DNA. The effects of DNA concentration on the accuracy of DNA sizing and quantification were also examined. Furthermore, we demonstrated the reproducibility of successive electrophoreses of the internal controls in one channel, and on-microchip endonuclease treatment of plasmid DNA and sequential analysis were performed to examine the feasibility of additional applications for DNA analysis.

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## Materials and methods

### Reagents and sample preparations

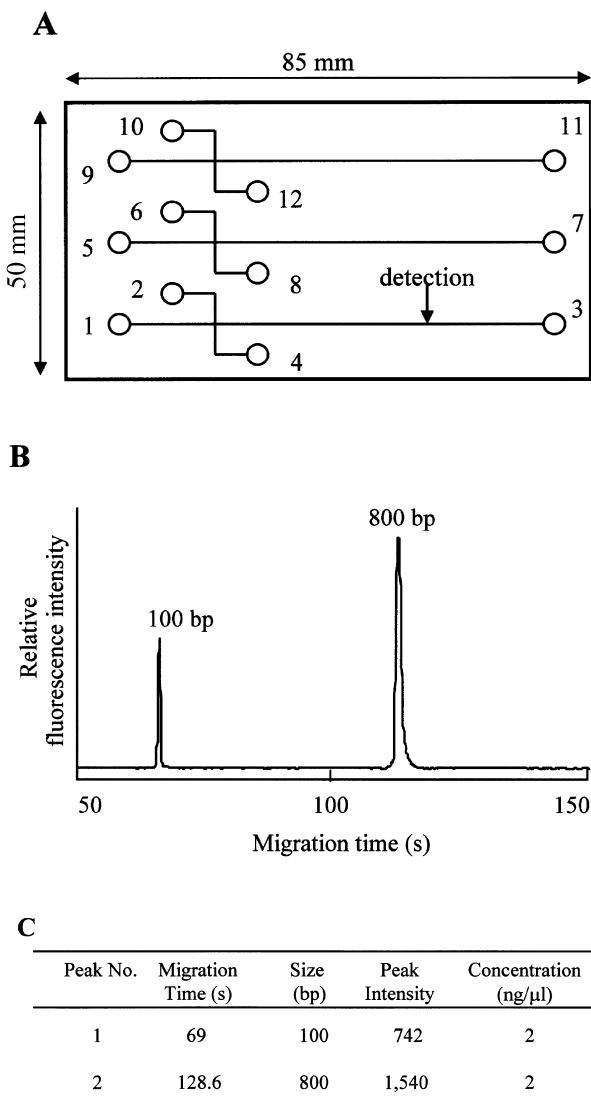
Restriction endonucleases *PvuII* and *KpnI* were purchased from TOYOBO (Tokyo, Japan). After digestion of pUC118 (3162 bp) with *PvuII*, digested DNA was purified by phenol/chloroform extraction, precipitated with two vols cold ethanol, centrifuged, washed once with 70% ethanol, allowed to dry under ambient conditions, and resuspended in TE buffer. The concentration of DNA was determined from absorbance at 260 nm by use of a Shimadzu UV160 spectrophotometer (Shimadzu, Kyoto, Japan). *PvuII*-digested pUC118 DNA fragments (2, 20, 40, 80, 160, and 320 ng· $\mu\text{L}^{-1}$ ) were subjected to electrophoresis on a microchip, and the variations of DNA sizing and concentrations were examined. After digestion of pUC118 with endonucleases, the size of the digested fragments was confirmed by gel electrophoresis on 1.5% or 3.0% agarose (Takara Shuzo, Kyoto, Japan) followed by ethidium bromide staining.

### Microchip preparation

Disposable *i*-chips (Hitachi Electronics Co., Tokyo, Japan), which are fabricated from polymethylmethacrylate and comprise an interconnected network of fluid reservoirs and microchannels, were used for all of the separation experiments (Fig. 1A). Three samples can be analysed on this chip. The loading gel containing ethidium bromide was infused from the buffer reservoir (wells 3, 7, and 11) into the microchannels of the *i*-chip by using a syringe, and wells 1, 2, 5, 6, 9, and 10 were filled with 10  $\mu\text{L}$  gel by using a pipette. Wells 4, 8, and 12 were the sample wells, and a pipette was used to fill each well with 1.0  $\mu\text{L}$  internal control, containing 2.0 ng· $\mu\text{L}^{-1}$  each 100 and 800 bp dsDNA fragments as markers for DNA sizing and quantification, and 9.0  $\mu\text{L}$  sample. Each sample can be analyzed in parallel within 4 min.

### Instrumentation

Experiments were performed on a Hitachi SV1100 microchip electrophoresis instrument (Hitachi Electronics 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 reader) that communicates with a personal computer. The sv1100b software includes data collection, presentation, and interpretation functions. Data is displayed as both a simulated gel image and electropherograms. Electropherograms of internal controls, 100 and 800 bp DNA fragments, are shown in Fig. 1B. Sizing and quantification of DNA fragments can also be presented in tabular form (Fig. 1C). The chip reader contains programmable high voltage power supplies, each of which is connected to a platinum electrode. These electrodes allow the instruments to perform multiple injections and other fluid manipulations from specific sample wells.



**Fig. 1. Design of the *i*-chip and the data output of the assay on a microchip.** (A) The chip performs capillary electrophoresis in each of three different channels, and three samples can be analysed on this chip. Wells 4, 8, and 12 are sample wells. The loading gel was infused from the buffer reservoir (wells 3, 7, and 11) into the microchannels using a syringe, and wells 1, 2, 5, 6, 9, and 10 were filled with gel by using a pipette. (B) Analysis of internal control, using the sv1100b software on 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 as 100 and 800 bp in DNA size, and 2 ng· $\mu\text{L}^{-1}$  in concentration, respectively.

### Microfluidic separation

All chips (*i*-chips), except for the analysis of DNA ladder consisting of 100–800 bp fragments (Hitachi Electronics Co.) and on-chip *KpnI* digestion of pUC118, were prepared according to the manufacturer's instructions with the supplied materials (gel, internal controls). To

examine variations of DNA sizing after sequential electrophoresis in different channels and in a single channel, 1.0  $\mu\text{L}$  internal control containing 100 and 800 bp DNA fragments and 9  $\mu\text{L}$  TE buffer instead of sample was added to the 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 analysis of DNA ladder consisting of 100–800 bp fragments, 8  $\mu\text{L}$  DNA ladder and 2  $\mu\text{L}$  TE buffer were added to the sample well and analysed. For analysis of on-chip *KpnI* treatment of *PvuII*-digested pUC118 fragments, 7  $\mu\text{L}$  40  $\text{ng}\cdot\mu\text{L}^{-1}$  *PvuII*-digested DNA, 1  $\mu\text{L}$  70 mM  $\text{MgCl}_2$ , and 1  $\mu\text{L}$  internal control were added to the sample well and analysed on the microchip. Then, 1  $\mu\text{L}$  *KpnI* (3–10  $\text{U}\cdot\mu\text{L}^{-1}$ ) was added to this well, and sequential analysis was performed after 5, 10, and 15 min incubation of the microchip on the Hitachi SV 1100.

## Results and discussion

### Reproducibility of the electrophoresis in different channels

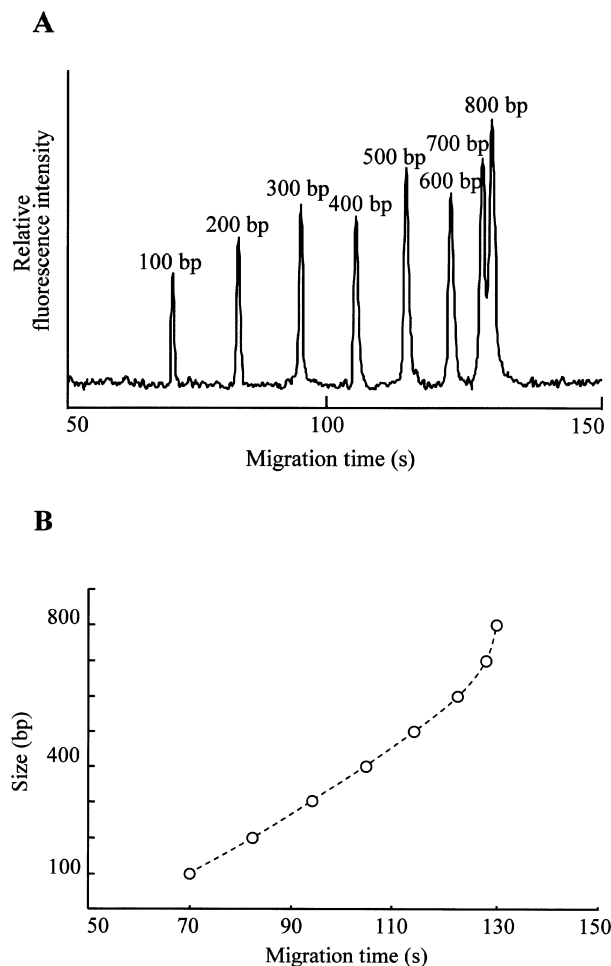
The Hitachi SV1100 performs capillary electrophoresis in each of three different channels, and three samples can be analysed on one chip. To evaluate the reproducibility of electrophoresis in the different channels, the migration times in each channel were examined with the internal controls, 100 and 800 bp DNA fragments (Table 1). The relative standard deviations in five different channels for the migration times of the 100 and 800 bp fragments were 2.67% and 2.98%, respectively, indicating the reproducibility of the electrophoresis even in different channels.

### Separation of the DNA ladder

An electropherogram of DNA ladder consisting of 100–800 bp fragments is shown in Fig. 2A. Eight peaks corresponding to the 100–800 bp fragments were separated clearly. A calibration curve was constructed by plotting each migration time against DNA size, and a linear relationship was obtained for fragment sizes of 100–600 bp (Fig. 2B).

**Table 1. Reproducibility of migration times of fragments in internal controls in five different channels with electrophoresis.** RSD, relative standard deviation.

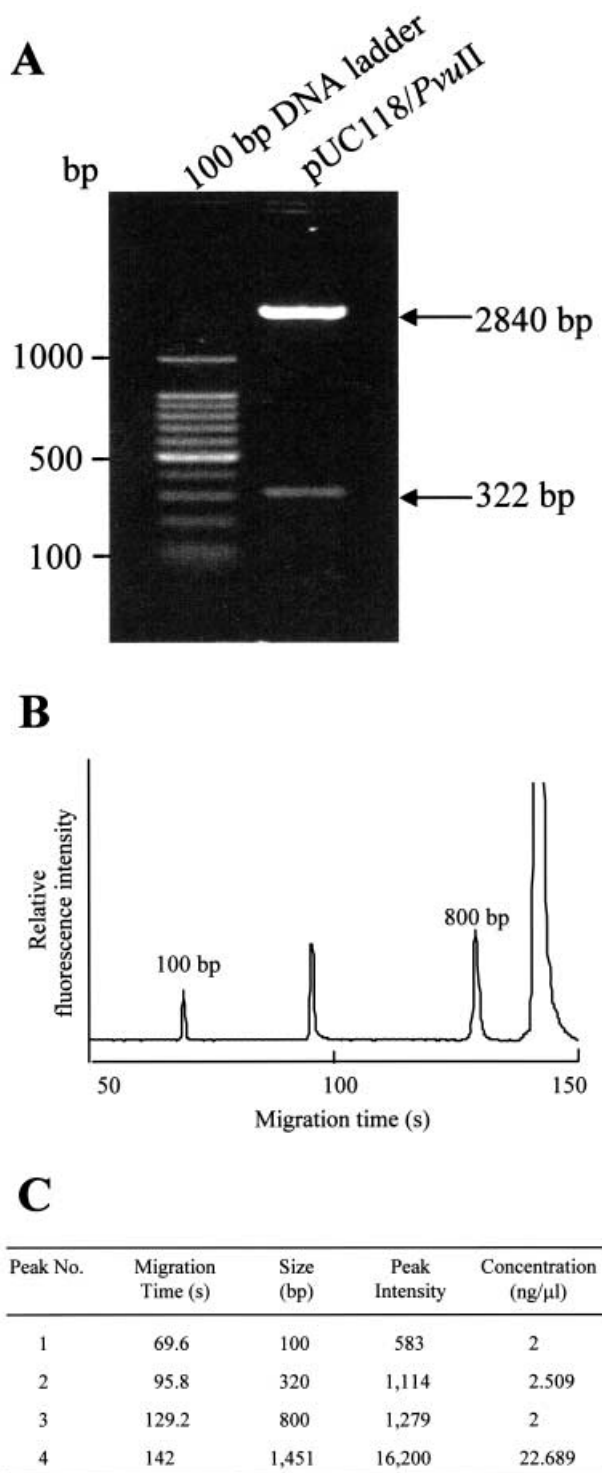
| Channel number | Migration time (s) |        |
|----------------|--------------------|--------|
|                | 100 bp             | 800 bp |
| 1              | 69.0               | 130.2  |
| 2              | 70.8               | 131.4  |
| 3              | 71.4               | 132.8  |
| 4              | 73.4               | 139.8  |
| 5              | 73.6               | 136.8  |
| Average        | 71.64              | 134.2  |
| RSD (%)        | 2.67               | 2.98   |



**Fig. 2. Relationship between base pair sizes and migration times of the DNA fragments.** (A) Electrophoretic separation of the 100 bp ladder (100–800 bp) using the sv1100b software on the Hitachi SV1100. (B) Relationship between the base pairs and migration time.

### DNA sizing and quantification of *Pvu II*-digested pUC118 fragments

*PvuII* digestion of pUC118 results in fragments of 322 and 2840 bp (Fig. 3A). These fragments were analysed to evaluate the ability of the Hitachi SV1100 to generate consistent results with respect to DNA sizing and quantification. The Hitachi SV1100 is capable of estimating DNA size between 100 and 800 bp. The concentration of the internal control DNA fragments measured automatically is 2.0  $\text{ng}\cdot\mu\text{L}^{-1}$ , and we used 20  $\text{ng}\cdot\mu\text{L}^{-1}$  *PvuII*-digested DNA for analysis so that the concentration of the 322 bp fragments would be 1.83  $\text{ng}\cdot\mu\text{L}^{-1}$ . Two peaks corresponding to the *PvuII*-digested pUC118 fragments were observed in electropherograms (Fig. 3B). As shown in Fig. 3C, the estimated DNA size differed from the predicted size of the 322 bp DNA fragment by only 2 bp (peak no. 2), whereas a large difference was observed in the prediction of the size of the 2840 bp DNA fragment (peak no. 4). The Hitachi SV1100 was able to give a more precise sizing of the 322 bp fragment compared with the rough estimate obtained from



**Fig. 3. Analysis of *PvuII*-digested pUC118 DNA fragments.** (A) Two bands corresponding to the 322 and 2840 bp DNA fragments are observed by conventional 1.5% agarose gel electrophoresis. (B) Two peaks other than the internal controls are shown in the electropherogram from the Hitachi SV1100. (C) Sizing and quantification results of each peak are presented in tabular form.

agarose gel electrophoresis. On the other hand, the estimated DNA concentrations of the 322 and 2842 bp fragments differed from the predicted concentrations by 37.0% and 40.4%, respectively. In the DNA size range 100–800 bp accuracy of DNA sizing was observed even with a digested fragment of plasmid DNA.

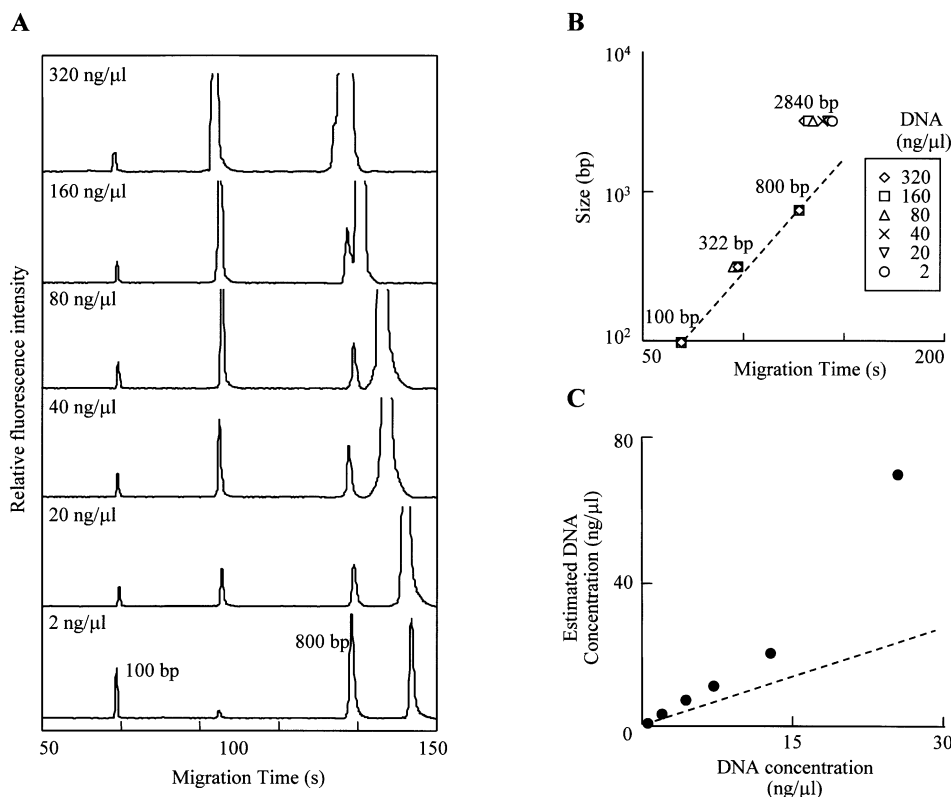
#### Effect of DNA concentration on the accuracy of DNA sizing and quantification

To examine the effect of DNA concentration on the accuracy of sizing and quantification of digested plasmid DNA fragments, samples containing 2, 20, 40, 80, 160, and 320  $\text{ng}\cdot\mu\text{L}^{-1}$  *PvuII*-digested pUC118 DNA were analysed on the Hitachi SV1100 (Fig. 4A). As shown in Fig. 4B, the estimated DNA size was very close to the predicted size of the 322 bp fragment for all concentrations, and DNA sizing results were found to be independent of DNA concentration. Similar results were observed with another commercial instrument [11]. On the other hand, large differences between estimates and predictions were observed for the 2842 bp DNA fragment, which is outside the range of DNA sizing with this chip; the DNA was estimated to be shorter than the predicted size in a dose-dependent manner. A similar phenomenon is also observed with conventional electrophoresis on agarose gels containing ethidium bromide. The accuracy of quantification of the 322 bp fragment is shown in Fig. 4C, indicating that the error apparently increased in a dose-dependent manner. The percentage errors for DNA quantification of the 322 bp fragment were +28.8%, +24.4%, +52.0%, +45.8%, +48.8% and +165.8% at the absolute concentrations of 0.18, 1.83, 3.66, 7.33, 14.66 and 29.31  $\text{ng}\cdot\mu\text{L}^{-1}$  DNA, respectively. These results indicate that the accuracy of quantification is relatively constant at  $< 1.83 \text{ ng}\cdot\mu\text{L}^{-1}$  DNA. It may be useful to use as an internal control 2.0  $\text{ng}\cdot\mu\text{L}^{-1}$  each of 100 and 800 bp DNA fragments, as markers for both DNA sizing and quantification.

#### Reliability in analysis of on-chip endonuclease digest of plasmid DNA

For microchip analysis, 10  $\mu\text{L}$  aliquots of sample solution are required to be loaded onto a microchip. However, only a small portion of loading sample is utilized for actual electrophoresis and analysis, and most of the applied sample remains in the sample well even after electrophoresis. Thus, successive electrophoresis were performed in one channel to examine the possibility of sequential analysis of remaining sample in the well just by pushing the start button after electrophoresis. Sequential electrophoresis was performed five times at 4 min intervals. Only slight variation of the migration times of the 100 and 800 bp fragments was observed, with relative standard deviations for each time of 0.25% and 0.28% (Table 2). These results demonstrated the possibility of using successive electrophoreses with at least a 20 min interval for the sequential analysis of a DNA sample in one channel.

We examined the feasibility of endonuclease digestion of plasmid DNA on a microchip and subsequent analysis. Mueller *et al.* [11] showed that signal intensity with the Agilent 2100 Bioanalyzer decreased at high salt



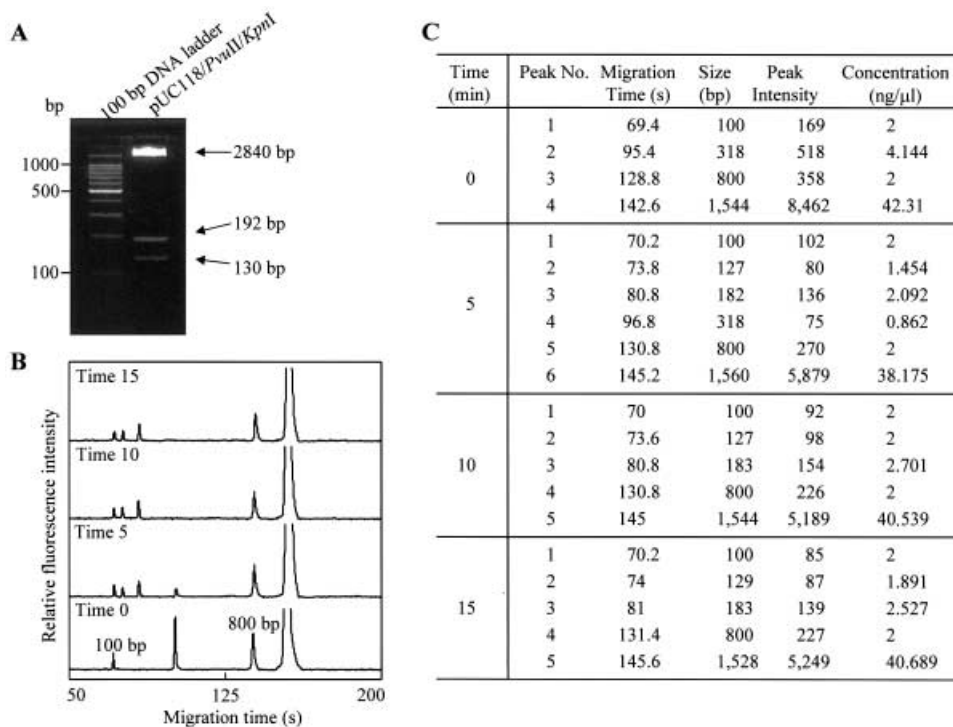
**Fig. 4.** Effects of DNA concentration on the accuracy of DNA sizing and quantification by the Hitachi SV1100. (A) Electropherograms from analysis of the pUC118 *PvuII* digest at different DNA concentrations. In all cases, the peak corresponding to the 322 bp DNA fragment appeared at a similar migration time, but the migration time for the 2842 bp DNA fragment decreased in a dose-dependent manner. (B) The estimated size of the 322 bp DNA fragment is very close to the predicted size for all concentrations, whereas large differences are observed in the prediction of the 2842 bp DNA fragments, which is out of the range of DNA sizing. (C) Plots of estimated DNA concentrations on the Hitachi SV1100. The filled circles indicate the estimated DNA concentrations of each sample, and the dashed line indicates the precise relationship between the estimated DNA concentrations and the applied sample concentrations. The error in DNA quantification increases in a dose-dependent manner.

**Table 2.** Reproducibility of migration times of fragments in internal controls in one channel with five successive electrophoreses. RSD, relative standard deviation.

| Electrophoresis number | Migration time (s) |        |
|------------------------|--------------------|--------|
|                        | 100 bp             | 800 bp |
| 1                      | 71.2               | 134.0  |
| 2                      | 70.8               | 133.4  |
| 3                      | 71.2               | 133.4  |
| 4                      | 71.2               | 133.2  |
| 5                      | 71.2               | 133.0  |
| Average                | 71.1               | 133.4  |
| RSD (%)                | 0.25               | 0.28   |

concentrations, and a similar decrease in signal intensity was observed in 100 mM NaCl with the Hitachi SV 1100, making it difficult to detect the peaks corresponding to each DNA fragment (data not shown). Endonuclease *KpnI* was used to treat *PvuII*-digested pUC118 DNA fragments on the microchip, because it works effectively under low-salts conditions (0 mM NaCl) and cleaves the 322 bp fragment into two fragments of 130 and 192 bp (Fig. 5A). For

on-chip *KpnI* digestion of pUC118 *PvuII* fragments, 1.0  $\mu$ L of 70 mM MgCl<sub>2</sub> per well was used instead of the original 10 $\times$  buffer because this ion is required for the endonuclease reaction [21]. Electropherograms of *PvuII*-digested pUC118 fragments before and after *KpnI* digestion are shown in Fig. 5B. Before *KpnI* treatment (time 0), the 322 bp fragment of *PvuII*-digested pUC118 was clearly observed between the internal controls. Partial digestion of the 322 bp fragment was already observed after 5 min incubation (time 5), and the peak corresponding to this fragment completely disappeared and two other peaks were observed by extension of incubation time for 5 min (time 10). The peak sizes were estimated to be 127 bp (peak 2) and 183 bp (peak 3), differing from the predicted fragment sizes of 130 and 192 bp by only 3 and 9 bp, respectively (Fig. 5C). The estimated DNA concentrations of the 130 and 192 bp fragments differed from the predicted concentrations by 40% and 37%, respectively. Fourteen minutes was required to obtain these results: 10 min for endonuclease treatment on a microchip, and 4 min for electrophoresis and analysis. Furthermore, most of the applied sample remained in the sample well because of the negligible sample consumption on a microchip. For analysis of endonuclease treatment of DNA by conventional methods, several hours are required,



**Fig. 5. Sequential analysis of *Kpn* I digestion of *Pvu*II-digested pUC118 DNA fragments.** (A) After *Kpn*I treatment of *Pvu*II-digested pUC118 DNA, two bands corresponding to the 130 and 192 bp DNA fragments are observed by conventional 3% agarose gel electrophoresis. (B) Electropherograms of pUC118 *Pvu*II digest show a 318 bp DNA fragment at time 0; after on-microchip digestion with *Kpn*I (time 15), two peaks corresponding to the 130 and 192 bp DNA fragments appeared instead of the 322 bp fragment. (C) Sizing and quantification results of each peak are presented in tabular form.

microgram levels of sample are consumed, and it is difficult to perform the real-time analysis of DNA digestion.

We believe that this method has considerable advantages, such as accuracy of evaluation of DNA size, high efficiency, easy operation, and low consumption of samples. As shown above, it is easy to examine the enzymatic reaction in a time-course experiment so this method will be useful for enzymatic kinetic analysis. In medical applications, this method will be a powerful tool in clinical diagnosis, for example for analysis of restriction fragment length polymorphisms. Furthermore, in recombinant DNA technology, this method may be suitable for partial digestion of large strands of DNA for the construction of gene banks.

Microchip electrophoresis is being developed predominantly for the sizing and quantification of DNA, RNA, proteins and metabolites. The present study showed the accuracy and reproducibility of DNA sizing of endonuclease-digested plasmid DNA fragments. Furthermore, we demonstrated the potential of on-microchip endonuclease digestion of plasmid DNA and sequential analysis. Although on-chip enzymatic assays were reported recently [22–25], as far as we know this is the first report of on-microchip endonuclease treatment of plasmid DNA and its sequential analysis using simple methods on a commercial instrument. Endonuclease treatment is one of the most basic and frequently used methods in molecular biology. This application, on-microchip endonuclease treatment and sequential analysis, will be useful for obtaining large

amounts of information in the postgenome era, especially fields such as genetics and medicine that require investigation of nucleic acids, because of its potential for rapid DNA analysis with small amounts of sample.

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