

Analysis of plasmid samples on a microchip

Li Ding,* Kathi Williams,¹ Walter Ausserer, Luc Bousse,² and Robert Dubrow³

Caliper Technologies Corp., 605 Fairchild Drive, Mountain View, CA 94043-2234, USA

Received 28 October 2002

Abstract

We have developed a LabChip-based plasmid assay that runs on the Agilent 2100 Bioanalyzer. The assay determines the sizes and relative concentrations of the multiple forms of plasmid samples. Twelve samples can be analyzed on each chip in an automated run lasting approximately 30 min. By using a supercoiled DNA sizing standard of 2–16 kb, the size of the analyzed plasmid can be determined. The resulting MW has a relative standard deviation (CV) <5% and error <5%. Plasmids from 2–8 kb can be separated with resolution better than 1 kb. Topological isoforms in a plasmid sample can also be separated. However, due to differential staining, the heterogeneity of plasmid samples can only be measured if the signal of each isomer peak can be calibrated with pure standards for every isomer form. For a typical plasmid preparation which predominately is in the supercoiled form, the normalized corrected peak area for the supercoiled form correlates with the plasmid concentration in a broad range of 1–100 ng/μl. The measurement is semiquantitative with a CV lower than 20%. A number of applications of this assay on a Labchip will be shown. © 2003 Elsevier Science (USA). All rights reserved.

Keywords: Plasmid; Microchip; Supercoiled DNA; Sizing; Quantification; Electrophoresis; Separation

Plasmid DNA is one of the most common genetic vectors used in molecular biology applications. Preparation of plasmids needs to be routinely analyzed for size, concentration, and homogeneity. Plasmids are extrachromosomal DNA molecules that vary in size from 1 kb to more than 200 kb. Most plasmids are double-stranded, covalently closed circular molecules that may exist in several forms differing in topology [1]. Prominent topological isomers of DNA are the covalently closed circular (ccc)⁴ form, the open circular (oc) form, and the linear form [2]. These three isomers correspond to the traditional nomenclature of isomer form I, II, and

III. They can be separated by electrophoresis in agarose gels [2–4]. In most bacterial plasmid preparations, the majority of plasmids exist in the covalently closed circular form [1,5]. In the ccc form, both DNA strands are covalently closed and the DNA is supercoiled. When one strand is nicked, the coiling is lost, which results in the relaxed oc form (also called nicked form). Linear forms are produced when both strands are cleaved once at the same position. Because of the widespread use of plasmids, a fast and quantitative plasmid assay method has become increasingly desirable.

Currently the primary method for plasmid analysis is agarose gel electrophoresis followed by ethidium bromide staining [1]. This method is manual and time consuming. A typical run requires about an hour and consumes microgram levels of plasmid sample. After the electrophoresis, separate steps of imaging and data acquisition are necessary. Commonly used imaging techniques include densitometer scanning of the photograph of the stained agarose gel [6] or CCD imaging of the stained gel itself [7]. Due to the narrow linearity of most imaging techniques and the lack of internal standards, the estimated plasmid size and isoform quantitation are at best semiquantitative. Because of the complexity of

* Corresponding author. Present address: Arcturus Engineering Inc., 400 Logue Ave., Mountain View, CA 94043-4019. Fax: 1-650-962-3039.

E-mail address: lding@arctur.com (L. Ding).

¹ Current address. Transgenomic Inc., 2032 Concourse Dr., San Jose, CA 95131.

² Current address. Biospect Inc., 951 Gateway Blvd., South San Francisco, CA 94080.

³ Current address. Nanosys Inc., 2625 Hanover St., Palo Alto, CA 94304.

⁴ Abbreviations used: ccc, covalently closed circular; oc, open circular; DMSO, dimethyl sulfoxide; Taps, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; PCR, polymerase chain reaction.

current imaging techniques, most routine plasmid is performed qualitatively by visual estimation. Even the qualitative analysis is sometimes difficult because of uncertainties in assigning the bands to their corresponding forms. The electrophoretic mobility of plasmids of different shapes changes with the electrophoresis operating conditions [4]. Therefore, a common practice for plasmid analysis is to first cut the plasmid at one site with a restriction enzyme. The linear form is analyzed on an agarose gel with a linear DNA sizing standard. This additional step makes the assay even more time consuming and the information on the sample topological isomer composition is also lost. A more automated alternative method for plasmid analysis is available based on capillary gel electrophoresis [2,8–11]. Though the method offers high resolution and sensitivity, it requires a highly specialized instrument and an analysis of a single plasmid sample requires about an hour.

In this work, we present a method for plasmid analysis based on a microfabricated and miniaturized analytical device on a glass chip that can be automatically and quantitatively analyzed on an Agilent 2100 Bioanalyzer. Previously assays for double-stranded DNA fragments [12,13] and proteins [14] have been developed on the same platform utilizing electrophoresis on a microchip. This instrument uses epifluorescent detection with a 10 mW semiconductor laser that emits at 630 nm. It also contains 16 individually programmable high-voltage power supplies. When an electrical field is applied on the chip, the injected plasmids may be separated according to their mass and three-dimensional compactness in the sieving polymer-filled separation channel. After binding to the fluorescent plasmid dye, the migrating plasmid peaks are detected at the end of the separating channel by the laser-induced fluorescence detector. The migration time and area of each peak are automatically recorded. Since a short separation channel is employed and high electrical field strength is applied, the speed of analysis is dramatically increased compared to either agarose gel electrophoresis or capillary gel electrophoresis [13]. This high-speed analysis combined with the multiplexing chip design results in the automated analysis of one plasmid sample in 1 min, and a set of 12 samples on one microchip in approximately 30 min.

The resulting assay can separate plasmid topological isomers. The relative amount of a particular form of a plasmid in different samples is a straightforward comparison between individual analyses. The absolute quantification of any form of a particular plasmid is possible when a calibration standard is available. For the supercoiled form, the assay can detect as little as 1 ng of plasmid sample in 1 μ l solution. In a broad range of plasmid concentrations, there is a linear correlation between the corrected areas of the supercoiled peak to the plasmid concentration. The plasmid size can be determined with accuracy better than 5%. The application of

the microchip-based assay may include high-throughput plasmid sample sizing, and automation of assays based on the determination of plasmid isoform distributions.

Materials and methods

Reagents

Supercoiled DNA ladder was purchased from Invitrogen Corp. (Carlsbad, CA). The DNA in the ladder was precipitated with a standard ethanol precipitation procedure and the pellet was resuspended in TE buffer in a volume equivalent to that of the initial sample. The prepared ladder sample was stored at 4 °C and used within a month. Plasmid pSP72, pGEM-3Zf (+), pGL3 control, pSV- β -galactosidase control, and pBR322 were purchased from Promega Corp. (Madison, WI). Plasmid p β gal-control was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). DMSO (dimethyl sulfoxide >99.9%) was obtained from Sigma-Aldrich (St. Louis, MO). The gel matrix dilution buffer was 200 mM Taps (*N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid), 2 mM EDTA, pH 8.0. Both chemicals are from Sigma-Aldrich. Plasmid samples were diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Microchip fabrication

The chips were made from soda-lime glass, in which channels were photolithographically defined, and etched using a hydrofluoric acid-based wet etching solution. The etch depth in all chips used in this work was 13 μ m, and the channel width on the mask was 10 μ m, which after the etching resulted in channels 36 μ m wide. The channels were then completed by thermally bonding another soda-lime glass plate with 2-mm-diameter holes drilled in it to the lower plate. After dicing, the size of each chip is about 17.5 mm square. More details of chip fabrication can be found in Refs. [13–15]. Fig. 1A shows the chip design with which all the data presented here was obtained.

Microchip preparation

For all the separation experiments on the chip, every channel in the glass chip was filled by loading sieving matrix into well C4 and applying pressure for 30 s. The sieving matrix used was a polymer based on polydimethylacrylamide [16] at 0.2% in the running buffer (200 mM Taps, 2 mM EDTA, pH 8.0), containing 1:400 (v/v) Agilent dye for the staining for plasmid DNA (Agilent Technologies, Palo Alto, CA; No. 5065-4475, excitation/emission 642/660 nm, chemical name not available). Wells A4 and B4 were also filled with this solution. The other wells on the chip (A1–A3, B1–B3, C1–C3, D1–D4) were then filled with 5 μ l marker buffer

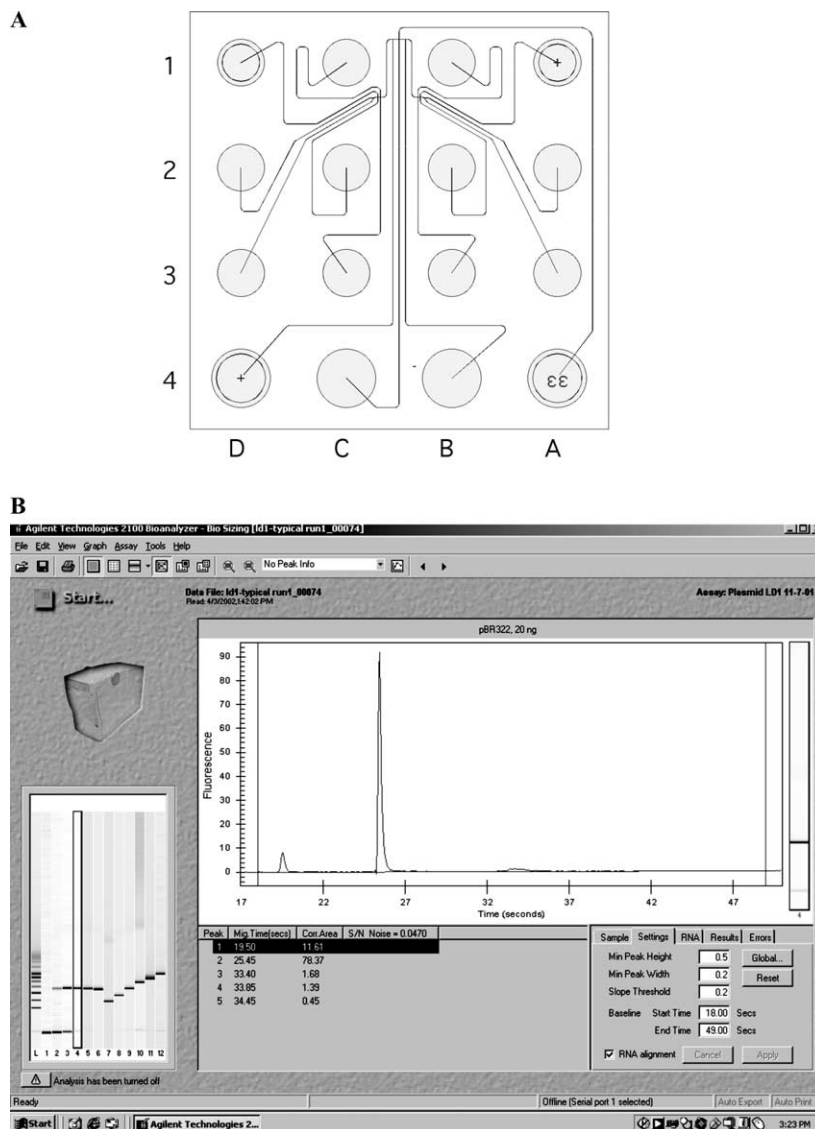


Fig. 1. The chip design and the data output of the plasmid assay on a microchip. The chip is designed for the analysis of 12 samples plus one ladder sample from one single run. (A) Chip design. The location of the wells is shown in light gray. Wells A4 and C4 are the separation buffer and waste wells. D4 is first used as the ladder sample well. Then D4 and B4 are used as load wells. All other wells contain samples. (B) Screenshot of the output data on analysis of 12 plasmid samples using Bio Sizing software on the Bioanalyzer 2100. Each analysis is represented as a lane in the gel-like image. A selected analysis is presented in the form of an electropherogram. The corresponding analytical results are tabulated below the electropherogram. The plasmid samples shown on each lane of the gel-like image are as follows: L, supercoiled DNA ladder; 1, blank; 2, 0.8 ng pBR322; 3, 4 ng pBR322; 4, 20 ng pBR322; 5, 100 ng pBR322; 6, 500 ng pBR322; 7, 100 ng pSP72; 8, 100 ng pGEM-3Z(+); 9, 100 ng pBR322; 10, 100 ng pGL3-control; 11, 100 ng pSV β gal-control; 12, 100 ng p β gal-control.

(200 mM Taps, 0.4 mM EDTA, 4 mM Tris, pH 8.0, containing 4.5 μ g/ml of a 50-bp dsDNA fragment as the front marker). Usually well D4 was loaded with 1 μ l of the supercoiled DNA standard sample, and the well was then called the ladder well. Each of the sample wells A1–A3, B1–B3, C1–C3, and D1–D3 was then loaded with 1 μ l of one of the 12 plasmid samples in TE buffer.

Measurement instrument

Separation and detection were performed on an Agilent 2100 Bioanalyzer instrument (Agilent Technolo-

gies; G2940BA), which was controlled by dedicated software running on a PC.

Cloning of different size PCR products on the pCR II vector

The kit for TOPO TA cloning on to pCR II-TOPO vector from Invitrogen was used for the cloning of a series of DNA fragments made from PCR. DNA fragments of 500, 1000, and 2000 bp were purchased from BioVentures (Murfreesboro, TN). A DNA fragment of 750 bp was made with components from the TOPO TA cloning kits

according to the procedure provided by the manufacturer. The cloning of each of the DNA fragments was carried out according to the procedure provided by the manufacturer. After the corresponding positive clones were selected, plasmid DNA was prepared from each positive clone with the QIAprep miniprep kit from Qiagen (Valencia, CA). The size of the insert DNA fragment and the vector of each clone were analyzed by restriction digestion with *EcoRI*. The DNA sizing of the *EcoRI* digest was done with the Agilent DNA 7500 kit (Agilent Technologies). The result of the analysis confirms that the selected clones contain the target sequences. The control pCRII DNA was prepared from a negative clone (a dark blue colony).

Topoisomerase treatment of plasmid pBR322

DNA topoisomerase I (Calf thymus) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The conditions for the enzymatic reaction with plasmid pBR322 were determined empirically based on the manufacturer's recommendations. Briefly, in a 10- μ l reaction mixture, 1 μ g pBR322 in 35 mM Tris-HCl, pH 8.0, 72 mM KCl, 5 mM MgCl₂, 5 mM DTT, 5 mM spermidine, and 0.01% BSA was mixed with different amounts of topoisomerase as specified under Results. The reaction mixture was then incubated 30 min at 37 °C and then 1 μ l of the reaction mixture was loaded in the sample well for the chip-based plasmid assay.

DNA topoisomerase II from *Drosophila melanogaster* was purchased from USB (Cleveland, OH). The reaction mixture (10 μ l) contains 1 μ g plasmid pBR322 mixed with different amounts of the enzyme as indicated in each experiment in a buffer of 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 15 μ g/ml BSA, 1 mM ATP. The reaction was carried out at 37 °C for 30 min. One microliter of the reaction mixture was then analyzed on the plasmid assay chip.

For the complete conversion of the plasmid BR322 into its oc form, 10 units of topoisomerase I was used in the 10- μ l reaction mixture as described above. For the inhibition of topoisomerase I and II reactions, 1 μ l of either 10 mM (*S*)-(+)-camptothecin or 10 mM etoposide (both chemicals are from Sigma-Aldrich, St. Louis, MO) was added to the corresponding reaction mixture to make the final concentration of the inhibitors to 1 mM. The concentration of the topoisomerase I in the inhibition assay is 0.01 unit/ μ l. The topoisomerase II concentration in the inhibition assay is 0.2 unit/ μ l.

UV treatment of plasmid pBR322

Ten microliter of plasmid pBR322 at 0.1 μ g/ μ l in TE buffer was placed in a 1.5-ml microcentrifuge tube. The sample was irradiated for different time periods (0 to

60 min) on an UV transilluminator (UVP Model White/2UV LMW 20, Upland, CA) at 302 nm.

Results and discussion

An automated plasmid analysis method for multiple samples

The method presented in this paper is a “lab-on-a-chip” automated assay for plasmid samples that integrates the staining, separation, and detection steps on a microchip. Shown in Fig. 1A is the design of the chip. After the chip is prepared as described under Materials and methods, it is placed in a Bioanalyzer. The designated software controls the program of power supplies connected to each well to achieve the loading, injection, and separation of a plasmid sample on the chip. The plasmid sample is stained by the Agilent intercalating dye contained in the buffer in the channels as it is electrophoretically driven through the chip to the injection intersection. A plug of the sample is injected into the separation channel [17], and is then separated by electrophoresis in the separation channel. The separated plasmid-dye complex is detected by laser-induced fluorescence after a separation length of 1.4 cm. Data are displayed in a real-time manner as either an electropherogram or a lane in a gel-like image. Fig. 1B is an example of the analysis of 12 samples representing a plasmid at different concentrations (lanes 1–6) and plasmids of different sizes (lanes 7–12). For each selected peak in an electropherogram, migration time and corrected area are presented in the digital form. The analysis of the output data for different parameters of the plasmid samples is discussed in the following sections

Separation of different topological forms of the same plasmid

Plasmids of identical sequences may exist in different topological isomers [1,2]. Plasmid samples prepared from bacteria are commonly a mixture of the covalently closed circular form, the open circular form, and the linear form. Because the different forms differ in compactness of their 3D structure, they migrate differently in the gel-matrix-filled chip during electrophoresis. The electropherogram of a typical preparation of plasmid pBR322 is presented in Fig. 2A. Peak 1 on the electropherogram corresponds to the maker peak generated by the 50-bp dsDNA in the sample buffer. It is utilized by the data analysis software for the alignment of each individual sample on the same chip. The ccc form has the most compact structure with the highest electrophoretic mobility (peak 2); it appears earlier than the oc form (peak 4). The identification of the ccc and the oc form in the plasmid preparation mixture was facilitated

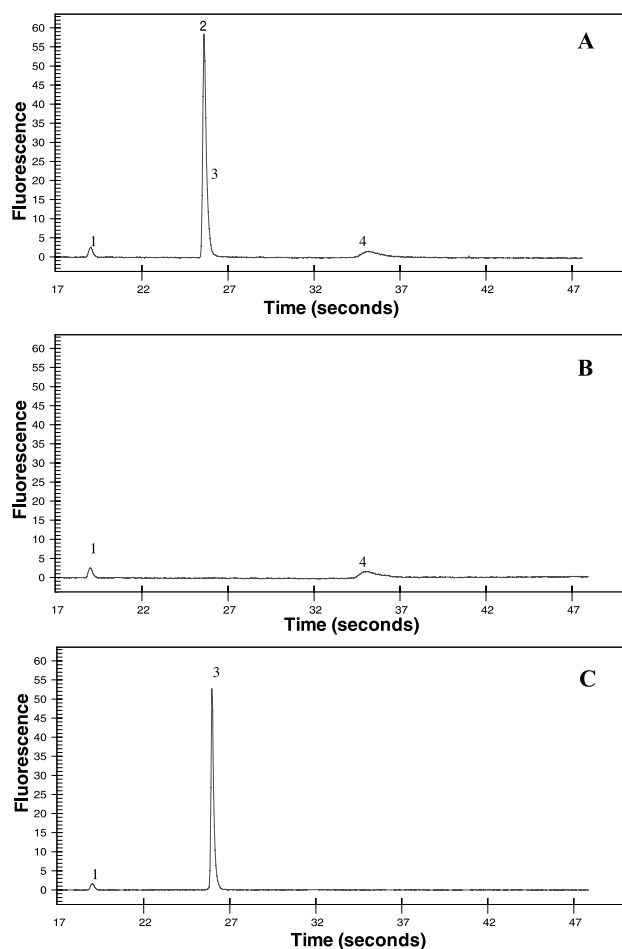


Fig. 2. Separation and identification of topological isoforms of plasmid pBR322. One hundred nanograms of predominantly one form of pBR322 plasmid is analyzed on one microchip. The migration time is aligned with the front marker. The fluorescence scale is the same for all the electropherograms. (A) Electropherogram of 100 ng of a freshly prepared 100 ng pBR322. (B) Electropherogram of 100 ng pBR322 that is completely relaxed by topoisomerase I (predominantly in the open circle form). (C) Electropherogram of 100 ng of a complete digestion of 100 ng pBR322 by *EcoRI* (dominantly in the linear form).

by the analysis of the same plasmid sample treated with Topoisomerase I (Fig. 2B). Because the enzyme catalyzes the conversion of the ccc form into the oc form [18], the only product peak (peak 4) must correspond to the oc form. When compared to Fig. 2A, Fig. 2B misses the major peak (peak 2) in Fig. 2A. This disappeared peak must correspond to the ccc form. Peak 2 on Fig. 2A has a small shoulder (peak 3) with longer migration time than the main peak. The shoulder can be fully resolved from the major peak if a lower concentration of polymer is used as the electrophoresis matrix (data not shown). This minor component is identified as the linear form of the plasmid pBR322 because it has an identical migration time (26.05 s) with the linear plasmid peak (peak 3) on Fig. 2C. The linear plasmid form analyzed on Fig. 2C was generated by restriction digest of the same plasmid with a single cutter enzyme *EcoRI*.

The linearized plasmid form migrates between the ccc form (25.70 s) and the oc form (35.30 s) with a migration time of 26.05 s. This order of migration of the ccc form, the linear form, and then the oc form holds true for all the plasmids tested under the defined assay conditions.

Separation of supercoiled plasmids of different sizes

As described under Materials and methods, the supercoiled DNA ladder is a mixture of 11 supercoiled circular plasmids of different lengths (approximately 2 to 16 kb). Smaller plasmids of 2 to 8 kb are different by 1 kb in molecular weight while the larger plasmids are different by 2 kb. Fig. 3 shows that these 11 plasmids can be fully separated on the chip with the smaller plasmid having shorter migration times. Across the chip, between runs, the peaks of the plasmids in the ladders superimpose perfectly when aligned with the front marker with a CV of 0.1 to 0.2%.

Sizing of plasmids

As we have discussed in the introduction, the conventional method of plasmid sizing involves multiple steps including converting the plasmid to its linearized form. Since the ccc form is the predominant form in most plasmid samples and it generates sharp peaks in the microchip-based assay, we decided to determine the molecular weight of the analyzed plasmid by comparing its ccc migration time to the point-to-point standard curve obtained on the same chip using the supercoiled DNA ladder. Examples of the gel-style displays of six plasmids are shown in lanes 7–12 of Fig. 1B. One microliter of each plasmid was loaded on the chip at a concentration of 100 ng/ μ l determined by the sample OD₂₆₀ absorbance. After the front marker on each analysis is aligned by the software with that in the ladder sample, the molecular weight for each selected peak can be calculated by the software (Bio Sizing A02.10). For each plasmid, the first major peak on the electropherogram is selected and is assumed to be its supercoiled form. The calculated molecular weight of the six plasmids ranging from approximately 2.5 to 7.6 kb is summarized on Table 1. Within this range, the determined MW is within 5% of the expected MW. The relative standard deviations (CV) for all the measurements are below 5%. Compared to the conventional gel electrophoresis method of plasmid size determination, the method described here is much faster, quantitative, and consumes less plasmid sample.

The determination of the relative sizes of plasmids is enough for a large number of applications. One such application is the screening for clones that contain target insert(s) on a cloning vector by comparing the resulting clone size to that of the cloning

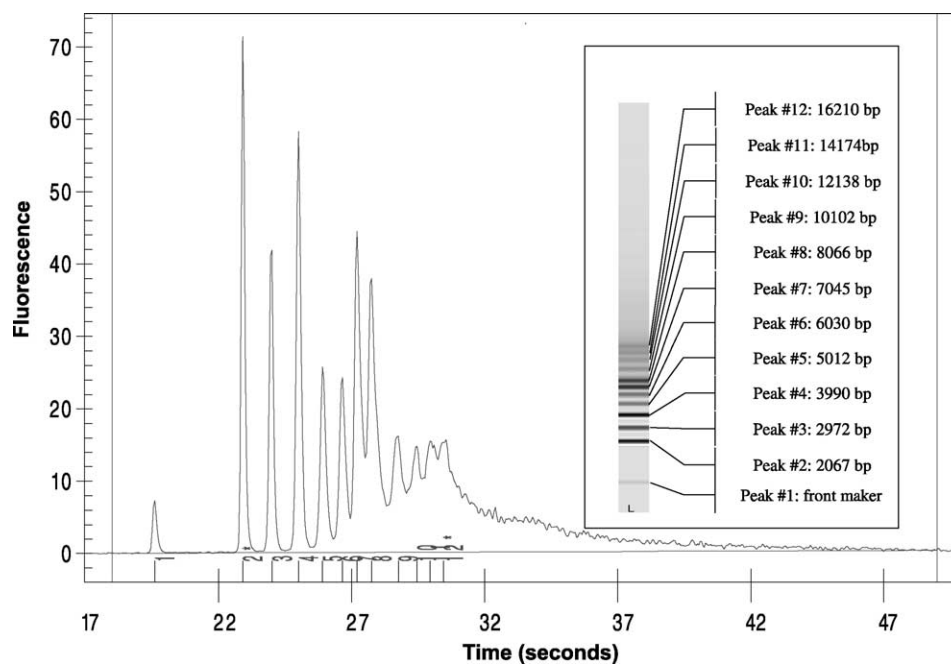


Fig. 3. Electropherogram of a mixture of 11 supercoiled DNA plasmids with known molecular weights (supercoiled DNA ladder from Invitrogen). The gel image of the same analysis is shown on the inset. Each individual peak (1–12) is marked on the electropherogram. The first peak is the front marker in the sample buffer, and the other peaks corresponding to the 11 plasmid standards in the ladder. The molecular weight of each standard peak is listed on the inset. *The lower and upper peaks from the supercoiled DNA.

Table 1

Determination of the plasmid molecular weight (MW) based on its supercoiled form migration time

Plasmid	Expected MW (bp)	Measured average MW (bp)	Standard deviation (\pm bp)	CV (%)	Error (%)
pSP72	2462	2468	75	3.0	0.3
pGEM-3Z(+)	3197	3263	96	2.9	2.1
pBR322	4363	4360	192	4.4	0.1
pGL3-control	5256	5369	117	2.2	2.1
pSV- β gal	6820	6492	193	3.0	4.8
p β gal-control	7931	7598	214	2.8	4.2

The expected MW is based on the plasmid sequences from manufacturers. The measured average MW is from six individual measurements of plasmid samples at 100 ng/ μ l in TE buffer.

vector. For example, Fig. 4 shows the superimposed electropherograms of plasmid preparations from four positive clones in comparison to that of the vector PCRII. The vector PCRII has a MW of 3.9 kb. The positive clones have inserts of 500-, 750-, 1000-, and 2000-bp PCR fragments cloned into the vector. The result shows that positive clones with only a 500-bp insert can be distinguished from the vector. In other words, it can be identified as the positive clone. For this set of plasmids, differences in size as small as 250 bp can be resolved (compare the 500-bp insert plasmid vs 750-bp insert plasmid, or the 750-bp insert plasmid vs 1000-bp insert plasmid). A baseline resolution is obtained between a tested clone with 750-bp insert and the vector. The corresponding migration time difference between the vector with or without the 750-bp insert is about 0.5 s. This example illustrates that this assay can be adopted for direct screening of

plasmid preparations that differ in their sizes without a restriction digestion.

Relative, semiquantitative determination of the supercoiled plasmid concentration

If the supercoiled form of the plasmid is the dominant component (greater than 90% in most fresh plasmid preparations), the total plasmid concentration can be estimated by measuring the concentration of the supercoiled form. We have tested the correlation of the corrected area of the ccc peak to the total plasmid concentration of plasmid pBR322. To make the corrected areas comparable between samples from different analyses, we normalized each corrected area of the ccc peak by the corresponding corrected area of the 50-bp dsDNA front marker. The resulting area ratio is then used for the quantification of the supercoiled plasmid.

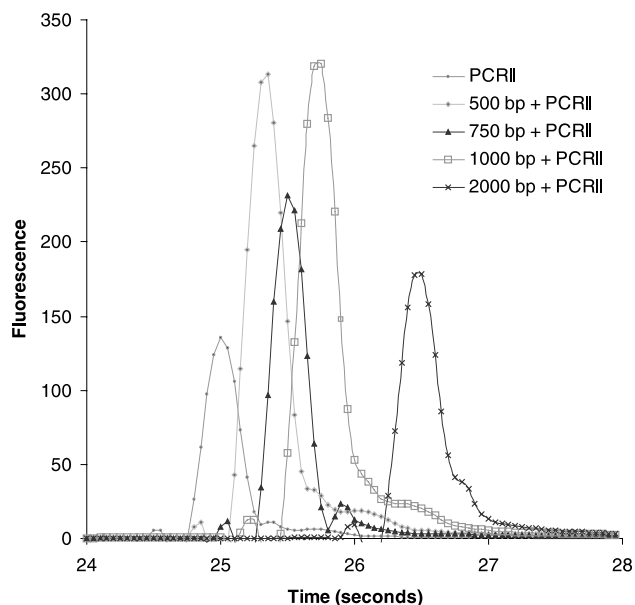


Fig. 4. Comparison of the migration times of the supercoiled form of the plasmids with different size inserts after cloning on the same vector. PCR product of 500, 750, 1000, and 2000 bp was cloned into PCR II vector by TA-TOPO cloning method. Plasmid preparations from each positive clone were analyzed by the microchip plasmid assay and the electropherogram of each analysis is superimposed on that of the vector after they are aligned on the front marker. Shown here is the superimposed electropherograms of the supercoiled peaks of the five plasmid samples as indicated by the figure legend.

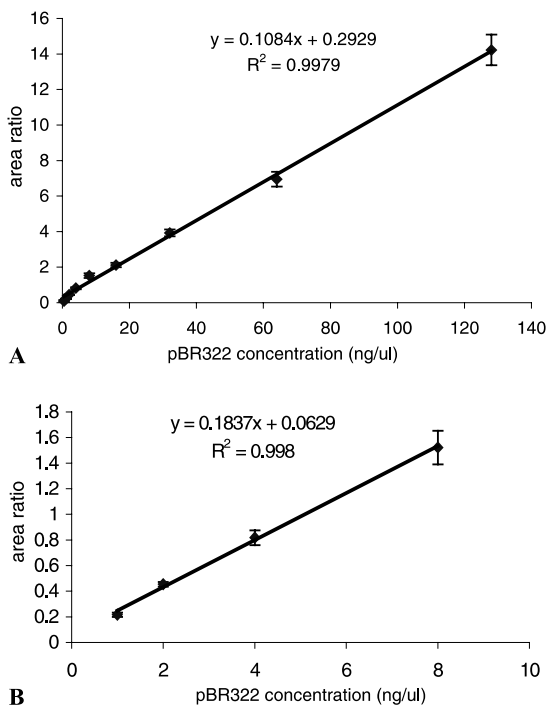


Fig. 5. Linear correlation of the plasmid concentration to the ratio of the corrected area under the supercoiled peak to the corrected area under the front marker peak. (A) The linear correlation at the range of plasmid concentrations of 1 to 128 ng/μl. (B) The linear correlation at the plasmid concentration range 1–8 ng/μl. The error bars on the figure represent the standard deviations from 12 individual experiments.

Fig. 5A is the linear fitted plot of the corrected area ratio to the total concentration of plasmid pBR322 dilutions ranging from 1 to 128 ng/μl. Within this range, the regression coefficient was 0.9979. It shows that the area ratios correlate with the total plasmid concentration linearly. At a narrow range of lower concentrations of plasmid (Fig. 5B), the linearity of the assay remains ($R^2 = 0.998$) though the slope of the fitted curve changed. To accommodate the changes in the slope of the standard curves at different concentration ranges, a point-to-point standard curve is used by the software for the determination of the concentration of a plasmid sample. Under the given conditions, the assay is only semiquantitative because the CVs for all of the data points in the defined concentration range were between 5 and 20%. At concentrations lower than 1 ng/μl, the assay can still detect the supercoiled peak of the plasmid pBR322 above the baseline but the assay was no longer quantitative because its CV was greater than 30%. On the other hand, if the concentration of the plasmid sample is too high (higher than 200 ng/μl), it may generate an electropherogram with peaks corresponding to different forms of plasmids but the corrected area of the supercoiled peak is no longer linear to the plasmid concentration (data not shown). Even worse, chip failure is often associated with chips containing samples at concentrations above the working range (data not shown).

The corrected area of the supercoiled form for different plasmids at the same concentration can vary up to 300% (data not shown). This is believed to be due to differential dye staining of different plasmids. To determine the concentration of the supercoiled plasmid form, it is necessary to compare the assayed sample to a standard curve generated with the same plasmid supercoiled form. Without such standard curve, relative concentrations of different preparations of the same plasmid samples can be obtained by comparing the corresponding corrected areas.

Comparison of freshly prepared versus stored plasmid pBR322

Freshly prepared plasmid from bacteria has the supercoiled DNA as its predominant form. Purified supercoiled plasmid samples are degraded to other forms during storage in aqueous solution [19]. Fig. 6 shows the electropherograms of two plasmid pBR322 samples. Both samples are at the concentration of 1 μg/μl in TE buffer. Compared to the sample freshly assayed after removal from -20°C (Fig. 6A), the sample that has been stored at 4°C for 12 months (Fig. 6B) has about 1/10 of the supercoiled form in the same total amount of the plasmid sample. Though harder to quantify, the area between the ccc form and the oc form has increased significantly in the stored sample. More significantly, the corrected area ratio between the supercoiled form and

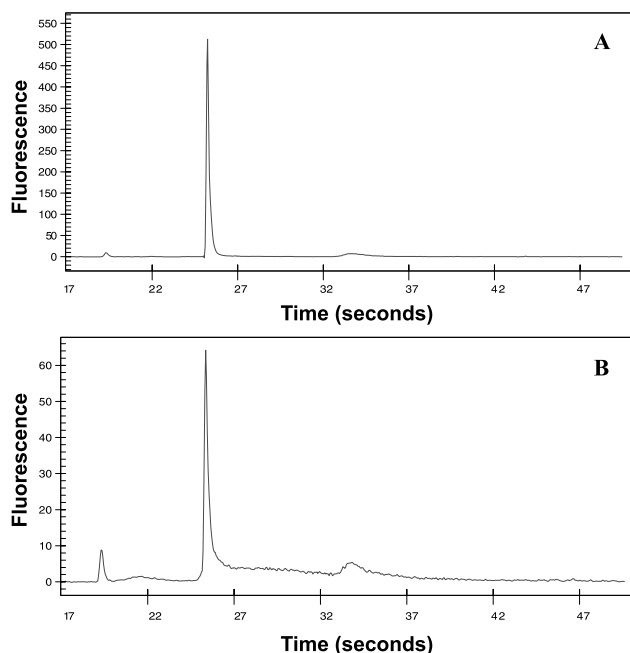


Fig. 6. Comparison of a preparation of supercoiled plasmid pBR322 sample stored in -20°C to a similar sample preparation stored in 4°C for 1 year. (A) Electropherogram of 100 ng of supercoiled pBR322 sample stored in -20°C after its preparation. (B) Electropherogram of 100 ng of supercoiled pBR322 sample stored in 4°C for 1 year after its preparation.

the oc form decreased dramatically from 15.2 ± 1.68 to 3.35 ± 0.20 (average value of five independent analyses similar to the one shown in Fig. 6). The result correlates with the previous known phenomenon that during the storage of plasmids, some environmental elements induce cleavage of the circular DNA, resulting in the relaxation of the supercoiled DNA.

Comparison of plasmid pBR322 samples exposed to UV light

One of the environmental elements leading to DNA single-stranded breaks is radiation [20]. During the course of a 60-min exposure of the plasmid pBR322 to short UV light, the peak corresponding to the supercoiled form decreased, while the peak under the oc form increased (Fig. 7). This result indicated that plasmid pBR322 is converted continuously from the ccc form to the oc form. In addition to reducing the peak height of the supercoiled form, exposure to UV light correlates with the broadening of the peak that may be an indication of the heterogeneity of the supercoiled form induced by the DNA single-stranded breaks. A minor new peak is also found after exposures longer than 30 min (the shoulder migrating after the oc form). This unidentified peak is likely to be the dimer form of the plasmid, which is another product of UV radiation [2]. Fig. 7 shows that after a 60-min UV exposure, no de-

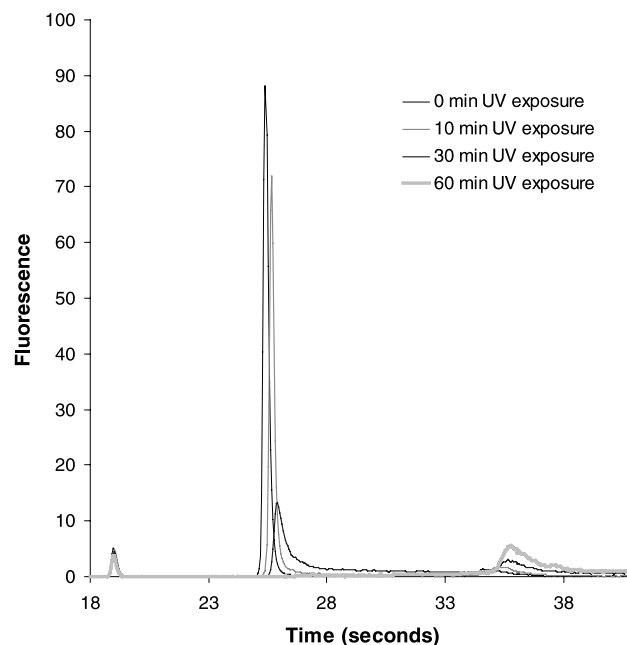


Fig. 7. Analysis of plasmid pBR322 samples treated for different times with UV-light exposure. Superimposed electropherograms of 100 ng of plasmid pBR322 exposed to short-wavelength UV for different times (0, 10, 30, and 60 min).

tectable level of ccc form was left in the plasmid sample. Most plasmid was in the oc form while the amount of the dimer form seems to be increased compared to the sample at shorter UV exposure.

Detection of DNA topoisomerase activities using pBR322 plasmid as the substrate

Both DNA topoisomerases I and II cleave supercoiled DNA and produce the relaxed form of the DNA [18]. The measurement of the enzyme activity typically involves radioactively labeled plasmid as the substrate to provide the sensitivity necessary for the measurement of the product. Our microchip-based plasmid assay provides a substitute for such assays. To illustrate the assay sensitivity, different amounts of either DNA topoisomerase I or II were incubated with $1\ \mu\text{g}$ pBR322 in corresponding reaction buffers in a final volume of $10\ \mu\text{l}$. After a 30-min incubation at 37°C , $1\ \mu\text{l}$ of the reaction mixture was analyzed by the chip-based plasmid assay. Fig. 8A is an electronically generated gel image of the analysis of 12 reaction mixtures. As shown in both lane 1 and lane 7, the substrate pBR322 preparation is predominantly in the supercoiled form after incubation in the absent of DNA topoisomerase. With increasing concentration of either topoisomerase II (lanes 2 to 6) or topoisomerase I (lanes 8 to 12), the band corresponding to the supercoiled form decreases while the band corresponding to the relaxed form increases. The enzyme activity can be easily detected at $0.02\ \text{unit}/\mu\text{l}$ for topo-

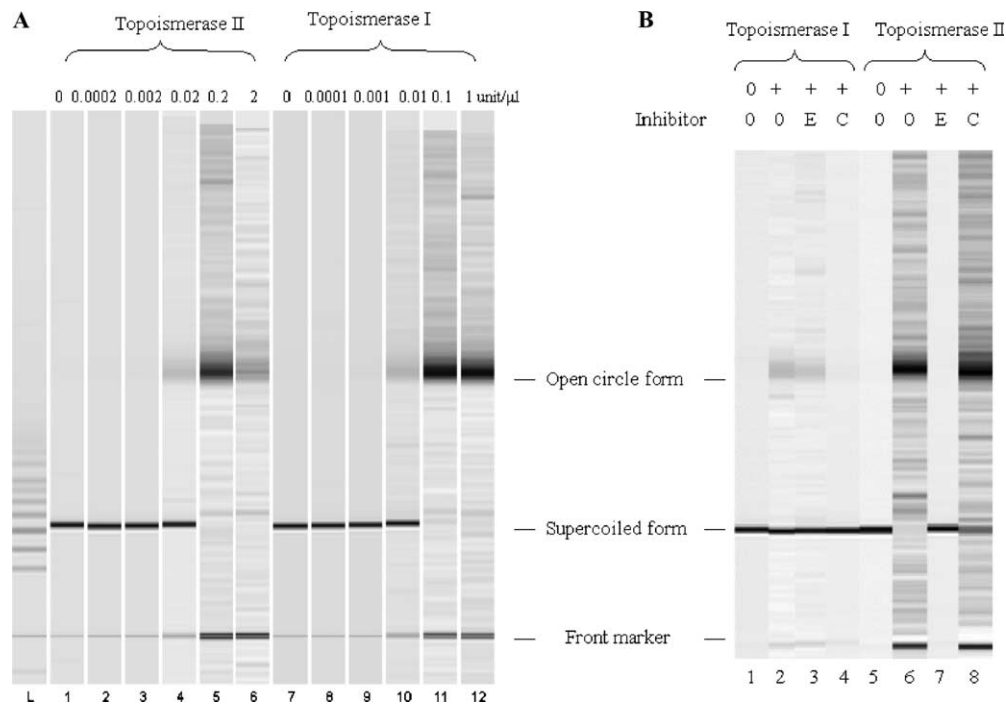


Fig. 8. Assay of topoisomerase I and topoisomerase II activity by measuring the relaxation of supercoiled plasmid pBR322. Plasmid pBR322 100 ng was mixed with different amount topoisomerase preparations or other reagents in corresponding reaction buffers as indicated on top of each sample. Each reaction mixture was then analyzed on the plasmid analysis chip. (A) Plasmid pBR322 is relaxed by increasing amounts of topoisomerase I or topoisomerase II. The final concentrations of the enzymes are indicated on the top of each lane on the gel image. The lane number of each sample is labeled on the bottom of the image. L is the ladder well loaded with the supercoiled DNA standard (the same as in Fig. 3). The identities of the bands in the sample wells are indicated on the side of the gel image. (B) The relaxation of the supercoiled pBR322 by topoisomerase I or II is inhibited by different inhibitors. One hundred nanograms of supercoiled pBR322 was incubated in 37°C for 30 min in the absence (lane 1) or the presence (lane 2) of 0.011 unit topoisomerase I. Lane 3 and lane 4 are the analysis of similar reaction mixtures with addition of 1 mM of either etoposide (E) or camptothecin (C). Lanes 4–8 are reaction mixtures in topoisomerase II reaction buffer. Lane 5 is the supercoiled plasmid incubated in the absent of the enzyme; lanes 6, 7, and 8 are the plasmid incubated with 0.2 unit topoisomerase II. Lane 7 is the product from the enzymatic reaction in the presence of 1 mM etoposide (E). Lane 8 is the product from the enzymatic reaction in the presence of 1 mM camptothecin (C). The image is compiled from analysis on a different chip by aligning the front marker band on each gel image electropherogram.

isomerase II and 0.01 unit/ μ l for topoisomerase I. Detection of the enzymes at even lower concentrations is possible if the electropherograms instead of the gel-style image are used for the data analysis (data not shown). To demonstrate the utility of the Topoisomerase assay based on the plasmid assay, we have conducted the topoisomerase assay in the presence of either topoisomerase I inhibitor camptothecin [21] or topoisomerase II inhibitor etoposide [22]. Lane 2 on Fig. 8B shows the activity of 0.01 unit/ μ l topoisomerase I on plasmid pBR322 by the production of the oc form (compare to lane 1 which is the reaction mixture without any enzyme). When 1 mM camptothecin is present during the enzymatic reaction, the formation of the relaxed oc form is inhibited (lane 4). On the other hand etoposide at 1 mM does not have any observed effect on the enzyme activity (lane 3). Similarly, when topoisomerase II was assayed at 0.2 unit/ μ l, its activity is readily observed as the shifting of the plasmid band from the ccc form into the oc form (compare lane 6 to lane 5). In contrast to

topoisomerase I, inhibition by 1 mM etoposide but not 1 mM camptothecin on topoisomerase II was shown in lane 7 and lane 8. The specificity of the inhibitors on the topoisomerases in this test is consistent with the previous reports [21,22].

The absolute quantification of different forms of a plasmid is limited

We have shown that different forms of plasmids can be detected by the microchip assay (Fig. 2). However, to do so it requires the sample loaded on the chip to be at relative high concentration (50 to 100 ng/ μ l for a typical plasmid sample). Partially it is because the oc form is a minor fraction of most plasmid samples. But another major reason for the low fluorescent signal of the oc form is due to the fact that it is differentially stained by the fluorescent dye used for the assay. Compare the electropherogram of pBR322 plasmid before and after a 60-min UV treatment in Fig. 7. The

supercoiled plasmid has been completely shifted to the oc form. The corrected area under the supercoiled peak in the untreated sample is 87.45 and the oc peak in the same sample is 4.95. After the treatment, the supercoiled form is not observed while the oc peak has an increased corrected area of 12.95 (from 4.95 to 17.90). The difference of the supercoiled corrected area 87.45 versus the corrected area of the oc form 12.95 is an indication of the differential staining of the different forms. Therefore the quantification of a particular plasmid form by this assay requires a standard of known quantity of the same form. Without such standards, only a comparison of the relative amount of different forms in plasmid preparations is feasible with the current assay.

Other limitations of the assay

The plasmid assay described here is based on the detection of on-chip-stained plasmid DNAs fractionated by electrophoresis in the separation channel on the microchip. A number of limitations are determined by the staining properties of the Agilent dye. For example the samples that can be analyzed by this assay are limited to relatively pure plasmid preparations because the dye is not specific to only plasmid DNA. Both double-stranded DNA and RNA are sufficiently stained by the same dye. Therefore both DNA and RNA contaminants in plasmid samples at comparable concentrations could interfere with the assaying of the plasmid. Most plasmid preparations from procedures that include the removal of RNA and genomic DNA are compatible with the microchip assay.

Conclusion

We have developed a plasmid assay on a microchip. Analyzed on the Agilent 2100 bioanalyzer with the dedicated software, the assay provides a tool for assessing a number of characteristics of multiple plasmid samples in one rapid automated process. The use of the internal front marker allows analysis of multiple samples through a single separation channel with very high reproducibility and reliability. With the use of an external supercoiled DNA ladder, the plasmid sample can be sized with high precision. For each purified plasmid sample, the major topological isoforms can be separated. The migration order for all plasmids tested was the supercoiled ccc molecules appearing first, followed by the linearized plasmid, and then the open circle form. The relative amount of the plasmid isoforms in different samples of the same plasmid can be compared. If the standard of a supercoiled plasmid is available, then the concentration of the supercoiled form in the plasmid preparation can be estimated in a

broad range of sample concentrations of 1 to 100 ng/ μ l. Compared to the conventional agarose gel electrophoresis analysis, the microchip-based plasmid assay consumes less sample, provides results in digital form, and completes the analysis of 12 samples in approximately 30 min.

Acknowledgments

We thank Odilo Mueller, Monika Dittmann, and Tanja Neumann from Agilent Technologies for their assistance.

References

- [1] J. Sambrook, D.W. Russell, *Molecular Cloning: A Laboratory Manual*, third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
- [2] T. Schmidt, K. Friehs, M. Schleef, C. Voss, E. Flaschel, Quantitative analysis of plasmid forms by agarose and capillary gel electrophoresis, *Anal. Biochem.* 274 (1999) 235–240.
- [3] H.V. Thorne, Electrophoretic characterization and fractionation of polyoma virus DNA, *J. Mol. Biol.* 24 (1967) 203–211.
- [4] P.H. Johnson, L.I. Grossman, Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-stranded DNAs, *Biochemistry* 16 (1977) 4217–4225.
- [5] R. Martin, *Gel Electrophoresis: Nucleic Acids*, Bios Scientific, London, 1996.
- [6] C. Sundfors, Y. Collan, Basic of quantitative polymerase chain reaction. 2. Electrophoresis and quantitation of polymerase chain reaction products, *Electrophoresis* 17 (1996) 44–48.
- [7] J.C. Sutherland, D.C. Monteleone, J.G. Trunk, P.V. Bennett, B. Sutherland, Quantifying DNA damage by gel electrophoresis, electronic imaging and number-average length analysis, *Electrophoresis* 22 (2001) 843–854.
- [8] O. De Carmejane, J.J. Schwinefus, S.C. Wang, M.D. Morris, Electrophoretic separation of linear and supercoiled DNA in uncoated capillaries, *J. Chromatogr. A* 849 (1999) 267–276.
- [9] S.A. Nevins, B.A. Siles, Z.E. Nackerdien, Analysis of gamma radiation-induced damage to plasmid DNA using dynamic size-sieving capillary electrophoresis, *J. Chromatogr. B. Biomed. Sci. Appl.* 741 (2000) 243–255.
- [10] G. Rancci, C.A. Maggi, D. Parente, Capillary electrophoresis of supercoiled DNA molecules: parameters governing the resolution of topoisomers and their separation from open forms, *Anal. Chem.* 72 (2000) 821–826.
- [11] D.T. Mao, R.M. Lautamo, Separation of supercoiled DNA using capillary electrophoresis, *Methods Mol. Biol.* 162 (2001) 333–344.
- [12] O. Mueller, K. Hahnenberger, M. Dittmann, H. Yee, R. Dubrow, R. Nagle, D. Ilsley, A microfluidic system for high-speed reproducible DNA sizing and quantitation, *Electrophoresis* 21 (2000) 128–134.
- [13] L. Bousse, C. Cohen, T. Nikiforov, A. Chow, A.R. Kopf-Sill, R. Dubrow, J.W. Parce, Electrokinetically controlled microfluidic analysis systems, *Annu. Rev. Biophys. Biomol. Struct.* 29 (2000) 155–181.
- [14] L. Bousse, S. Mouradian, A. Minalla, H. Yee, K. Williams, R. Dubrow, Protein sizing on a microchip, *Anal. Chem.* 73 (2001) 1207–1212.

- [15] L. Bousse, R. Dubrow, K. Ulfelder, in: D.J. Harrison, A. van den Berg (Eds.), *Micro Total Analysis Systems'98*, Kluwer Academic, Dordrecht, 1998., pp. 271–275.
- [16] R.S. Dubrow, U.S. Patent 6042710 (1999) Methods and compositions for performing molecular separations.
- [17] S.C. Jacobson, R. Hergenroder, L.B. Koutny, R.J. Warmack, J.M. Ramsey, Effects of injection schemes and column geometry on the performance of microchip electrophoresis devices, *Anal. Chem.* 66 (1994) 1107–1113.
- [18] J.J. Champoux, DNA topoisomerases: structure, function, and mechanism, *Annu. Rev. Biochem.* 70 (2001) 369–413.
- [19] R.K. Evans, Z. Xu, K.E. Bohannon, B. Wang, M.W. Bruner, D.B. Volkin, Evaluation of degradation pathways for plasmid DNA in pharmaceutical formulations via accelerated stability studies, *J. Pharm. Sci.* 89 (2000) 76–87.
- [20] H. Sies, W.A. Schulz, S. Steenken, Adjacent guanines as preferred sites for strand breaks in plasmid DNA irradiated with 193 nm and 248 nm UV laser light, *J. Photochem. Photobiol.* 32 (1996) 97–102.
- [21] C.F. Stewart, Topoisomerase I interactive agents, *Cancer Chemother. Biol. Response Modif.* 19 (2001) 85–128.
- [22] T.R. Toonen, K.R. Hande, Topoisomerase II inhibitors, *Cancer Chemother. Biol. Response Modif.* 19 (2001) 129–147.