Optimization of a Robust and Sensitive Capillary Electrophoresis-Laser Induced Fluorescence Method for Monitoring Plasmid Stability and Purity

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Introduction

Plasmid DNA is an important part of the biopharma industry. It is used as a gene-delivery vehicle for DNA vaccination and as a key intermediate for processes like viral particle production for gene therapy and ex-vivo protein synthesis. Most plasmid DNA preparations contain several topological variants or isoforms including the supercoiled, open circular and linear forms of the molecule. Federal regulations require purity testing for manufactured injectable plasmid products and recommend establishing a release criterion of > 80% supercoiled content (Ref 1). Traditional agarose gel has sensitivity limitations in detecting low level of impurities. Capillary electrophoresis with Laser Induced Fluorescence detection (CE-LIF) provides a rapid, sensitive, reproducible and automated method for the quantitative analysis of plasmid DNA isoforms. In this technical note, we describe the development of a method for plasmid analysis by CE-LIF with the use of a common dye. Results obtained with two large plasmids at 7 to 10 kb demonstrate that this method is sensitive and robust, providing baseline resolution of supercoiled, open-circular and linear plasmid isoforms within 20 minutes. It is suitable for both testing plasmid purity and monitoring plasmid degradation.

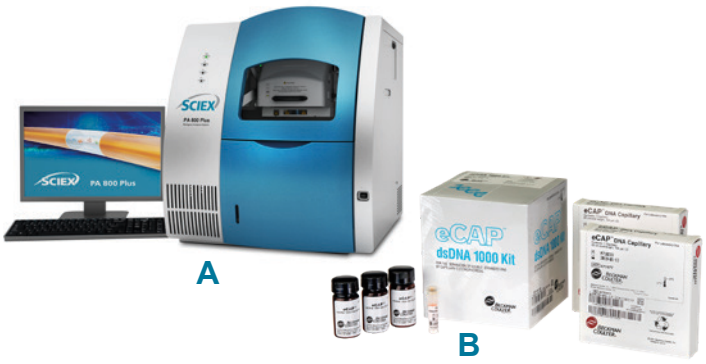

Figure 1. The PA800 Plus Pharmaceutical Analysis System (A) and the dsDNA 1000 Kit (B).

Key Features

- Robust Assay for plasmid purity and stability analysis.
- Superb resolution of different plasmid isoforms with a 3-minute window between supercoiled and open circular.
- Results obtained from plasmid degradation monitoring were comparable to those from traditional agarose gel analysis method.
- Automated sample separation and methanol rinse of coated capillary provide better reproducibility than agarose gel.
- Automated data processing speeds up data analysis time.

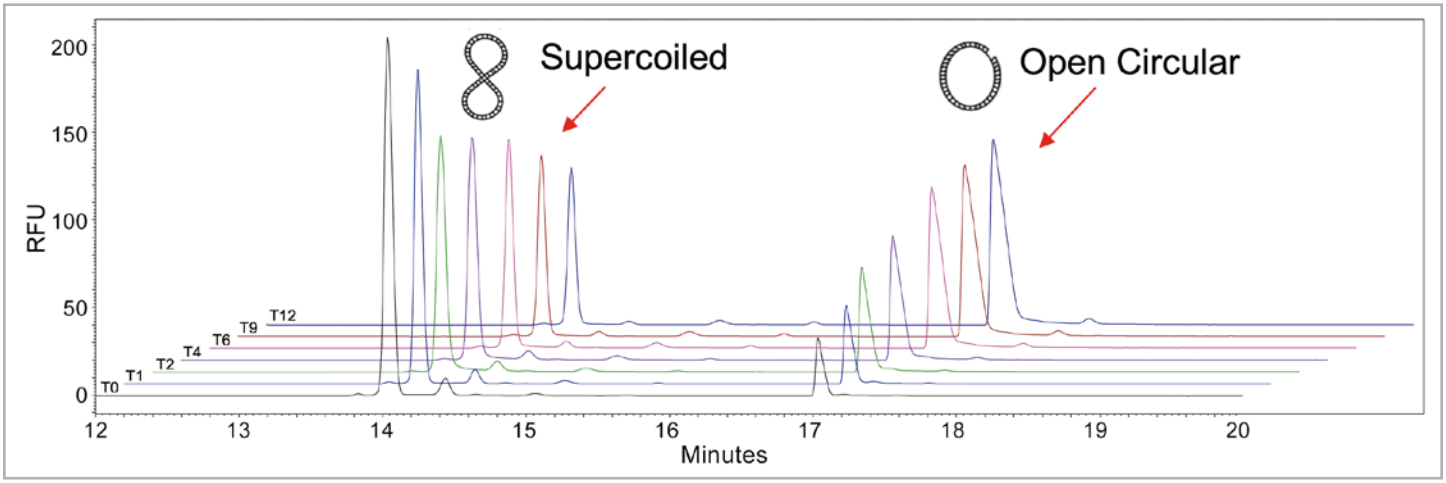

Figure 2. Monitoring the degradation of pDNA1 (7 kb) samples stressed at 40° C for zero (T0) to 12 weeks (T12) shows a good correlation between decrease in primary product (supercoiled) and increase in degraded product (open circular).

Materials and Instrument:

Materials: The dsDNA 1000 kit (PN 477410, Figure 1) was from SCIEX, Framingham, MA. A 10X concentrated stock solution of Tris Borate EDTA (10x TBE) was from Sigma Aldrich, St. Louis, MO (PN T4323). LIFluor EnhanCE fluorescent stain (PN 477409) was from SCIEX, Framingham, MA. SYBR Gold (PN S11494) was from Thermo, Carlsbad, CA. Plasmid DNAs were from an internal project at Pfizer, Chesterfield, MO. Methanol was from Fisher Scientific, Ontario, Canada (A4564).

Instrument and software: A PA 800 plus Pharmaceutical Analysis System (Figure 1) equipped with LIF detection and solid state laser with excitation wavelength at 488 nm and a 520 nm band pass emission filter was from SCIEX, Framingham, MA. Data acquisition and analysis was performed using 32 Karat software V10.2.

Methods

Gel Buffer reconstitution: To rehydrate the gel buffer, 20 ml of 0.2 µm filtered deionized water was added to the gel buffer vial. The mixture was gently stirred with a small stirring bar for up to 24 hours or till the dried gel was completely dissolved. Then, the gel solution was diluted in 1X TBE (90 mM Tris, 90 mM Borate, and 2 mM EDTA pH 8.3) at a dilution factor indicated in Results section. The diluted gel was filtered through a 0.45 µm filter. 1x TBE was prepared by a 10x dilution of TBE stock solution with deionized water followed by passing through a 0.2 µm filter. LIFluor EnhanCE stain was added to the gel at a concentration of 5 µl stain in 6 ml gel. Alternatively, SYBR Gold was added to gel at the amount of 1 µl in 10 ml gel.

Cartridge Assembly: DNA capillary was installed per instruction of kit insert (PN 726412) in the dsDNA 1000 kit. The total capillary length was 40.2 cm with 30 cm as the length to detection window.

Sample Preparation: Plasmid samples were diluted with 1x TBE buffer to either 8 ng/µl for analysis with LIFluor EnhanCE stain or 5 ng/µl for analysis with SYBR Gold. 95 µl of samples were then transferred to sample microvial for analysis on PA800 Plus instrument.

Capillary Initial Conditions Tab Settings: For analysis with SYBR Gold, the temperature for sample storage was set to 15º C. The cartridge temperature was set to 20º C. Maximum current was set to 300 µA. The LIF Dynamic Range was set to 1000 RFU with a data rate of 4 Hz. The Excitation was 488 nm and the emission was at 520 nm. The detector filter setting was set to normal and the filter peak width points were set to 16-25. For analysis with LIFluor EnhanCE stain, setting were the same as for SYBR Gold except the temperature for sample storage was set to 10º C; LIF Dynamic Range was set to 100 RFU.

Vial Positions: Figures 3 and 4 illustrated the vial positions used for capillary conditioning, plasmid separation and shut down methods with SYBR Gold. Figure 5 shows the vial positions for plasmid separation with LIFluor EnhanCE stain.

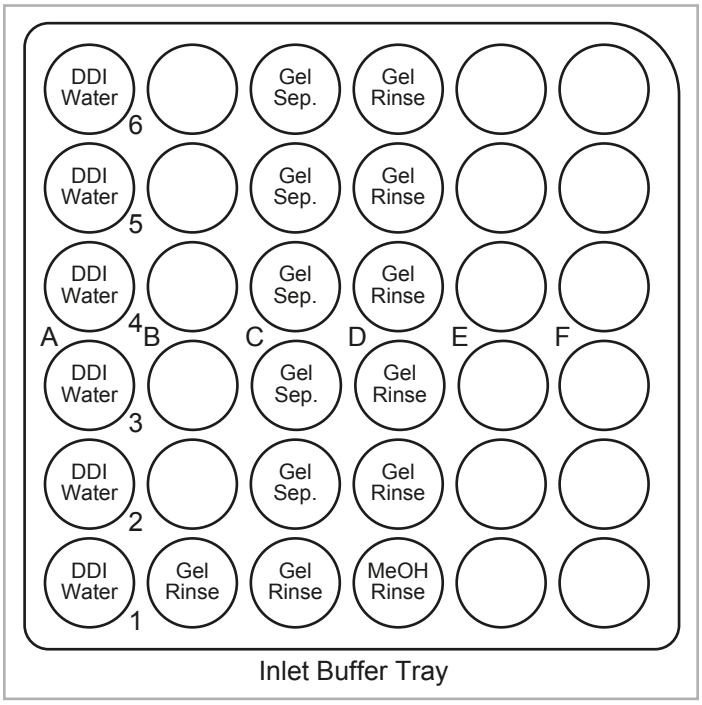

Figure 3. Vial configuration for BI (Inlet Buffer Tray)

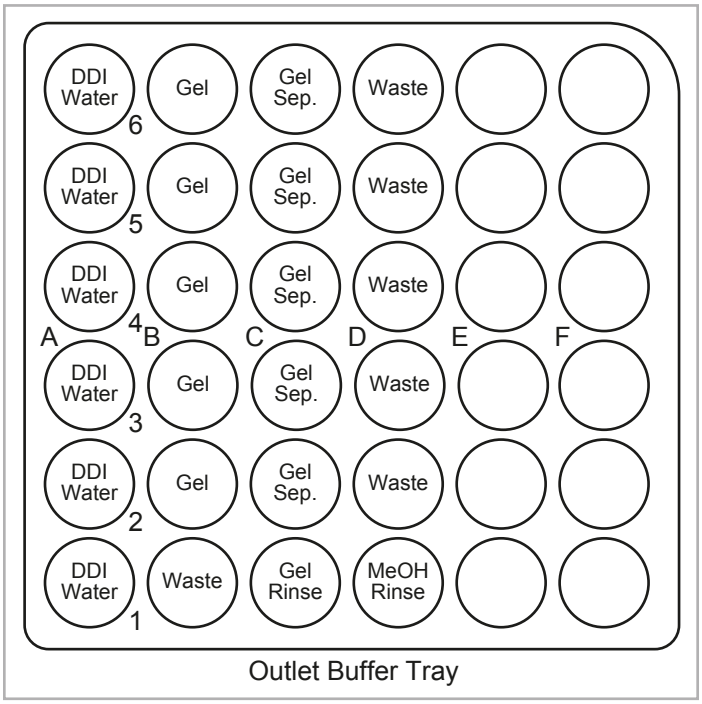

Figure 4. Vial configuration for BO (Outlet Buffer Tray)

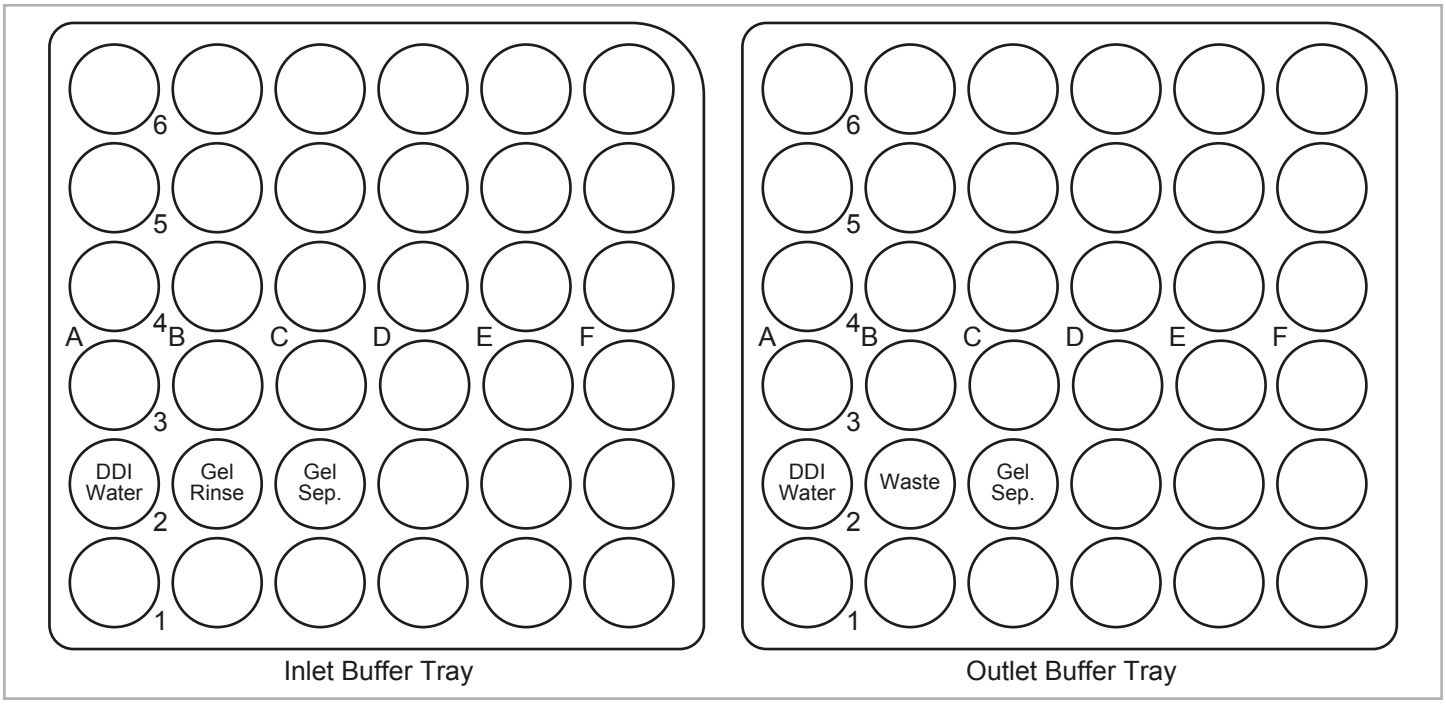

Figure 5. Vial positions for plasmid analysis with LIFluor EnhanCE Stain.

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	10.0 psi	10.00 min	BI:D2	BO:D2	forward, In / Out vial inc 6	Gel rinse to fill the capillary with dsDNA 1000 gel - Automatic increment every 6 runs
2		Wait		0.00 min	BI:A2	BO:A2	In / Out vial inc 6	Water dip to clean the capillary tip - Automatic increment every 6 runs
3		Inject - Voltage	2.0 KV	10.0 sec	SI:A1	BO:B2	Override, reverse polarity	Sample injection with gel in outlet vial
4		Wait		0.00 min	BI:A2	BO:A2	In / Out vial inc 6	Water dip to prevent sample carry-over - Automatic increment every 6 runs
5	0.00	Separate - Voltage	7.8 KV	20.00 min	BI:C2	BO:C2	0.17 Min ramp, reverse polarity, In / Out vial inc 6	Separation in dsDNA 1000 gel - Automatic increment every 6 runs
6	1.00	Autozero						
7	20.00	End						
8								

Figure 6. Time program for plasmid separation method with SYBR Gold.

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	2.00 min	BI:B2	BO:B2	forward	Gel rinse to fill the capillary with dsDNA 1000 gel
2		Wait		0.00 min	BI:A2	BO:A2		Water dip to clean the capillary tip
3		Inject - Pressure	0.2 psi	5.0 sec	SI:A1	BO:C2	Override, forward	Sample injection with gel in outlet vial
4		Wait		0.00 min	BI:A2	BO:A2		Water dip to prevent sample carry-over
5	0.00	Separate - Voltage	10.0 KV	25.00 min	BI:C2	BO:C2	0.17 Min ramp, reverse polarity	Separation in dsDNA 1000 gel
6	1.00	Autozero						
7	25.00	End						
8								

Figure 7. Time program for plasmid separation method with LIFluor EnhanCE stain and pressure injection.

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	2.00 min	BI:B2	BO:B2	forward	Gel rinse to fill the capillary with dsDNA 1000 gel
2		Wait		0.00 min	BI:A2	BO:A2		Water dip to clean the capillary tip
3		Inject - Voltage	2.0 KV	4.0 sec	SI:A1	BO:C2	Override, reverse polarity	Sample injection with gel in outlet vial
4		Wait		0.00 min	BI:A2	BO:A2		Water dip to prevent sample carry-over
5	0.00	Separate - Voltage	7.8 KV	25.00 min	BI:C2	BO:C2	0.17 Min ramp, reverse polarity	Separation in dsDNA 1000 gel
6	1.00	Autozero						
7	25.00	End						
8								

Figure 8. Time program for plasmid separation method with LIFluor EnhanCE stain and electrokinetic injection.

Capillary Condition Time Program: Capillary condition method was performed at the beginning of each sequence. Routine conditioning was done at 10 psi for 10 min with Gel/1x TBE/dye buffer. If a new capillary was used, conditioning was done by rinsing six to eight times at 10 psi for 20 minutes with Gel/1x TBE/dye buffer. With optimized method using SYBR Gold, a 10 min rinse with methanol was done before the regular gel rinse.

Shutdown Method Time Program: Shutdown method is performed at the end of each sequence. The capillary was rinsed with Gel/1x TBE/dye buffer at 10 psi for 20 minutes. The capillary was stored on the instrument at 20° C overnight or at 4° C for 2 to 3 days between runs. With optimized method using SYBR Gold, a 2 min rinse with methanol was done before the regular gel rinse.

Separation Method: Time programs for plasmid separation methods are listed in Figures 6, 7 and 8.

Agarose Gel Electrophoresis: A 1% agarose gel in TAE (Tris, Acetate EDTA, pH 8.3) that contained ethidium bromide at concentration of 0.5 µg/ml was used for analysis of plasmid samples. After the samples were loaded, electrophoresis was carried out at 80 to 150 Volts for 60 min.

Results and Discussion

Plasmid analysis by agarose gel: Agarose gel was used for analysis of plasmid topology. In Figure 9, 200 ng of a plasmid sample (Lane “P2”) was run on a 1% TAE agarose gel containing ethidium bromide (PN 161-0433, BioRad). About 835 ng of 1 kb plus DNA ladder (PN SM1331, Thermo) was loaded in Lane “M”. The super-coiled (SC) and open circular (OC) forms generated good, sharp bands. The linear (L) and multimer forms were barely detectable. Therefore, although the agarose gel method is easy to do, it has a high detection limit. A capillary electrophoretic method would improve sensitivity in detection, providing automated quantitative analysis.

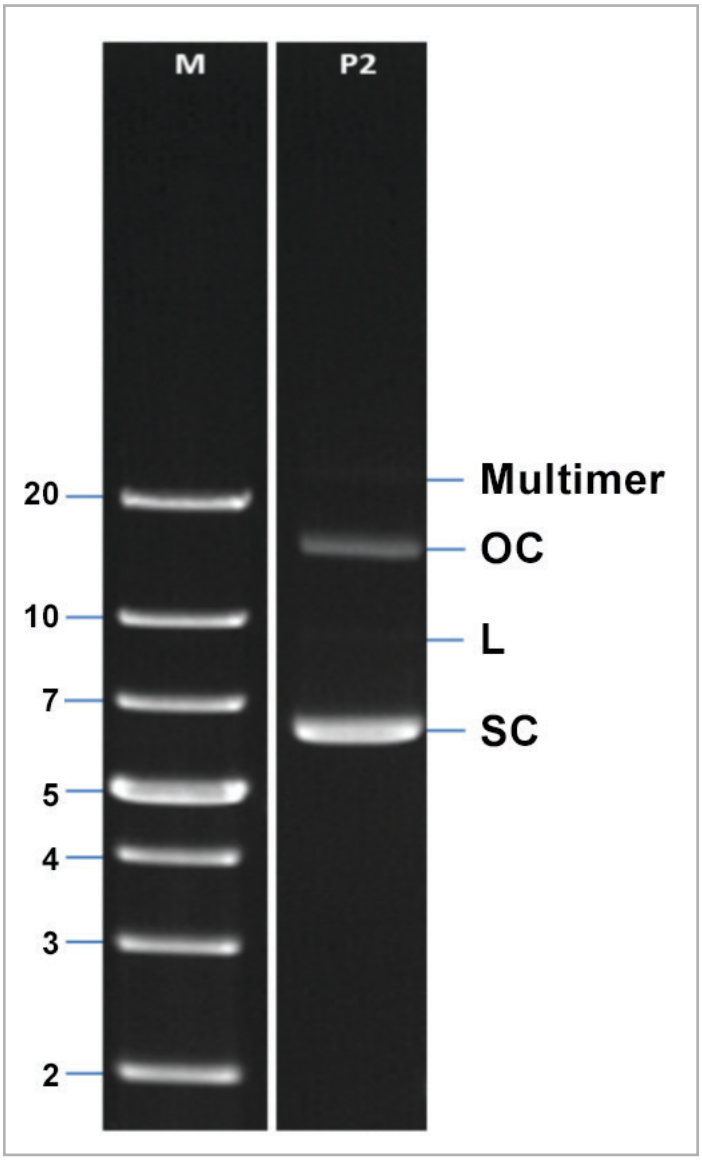

Figure 9. A 10 kb plasmid (pDNA2) was analyzed on 1% TAE agarose gel.

Optimization of Sample Injection and Separation Matrix Conditions: Although different plasmid DNA topoisoforms have similar molecular weights, their tertiary structures are different, allowing the gel buffer to sieve. Sieving was achieved by diluting the gel buffer to obtain the best resolution. During initial experiments, the gel buffer was diluted with 1xTBE at dilution factors of 5x, 7x, 10x, 15x and 20x. It was determined that gels diluted at 7x and 10x generated the best results (Figure 10A and 10B). The 10x dilution gel was used for further optimization experiments from this point on.

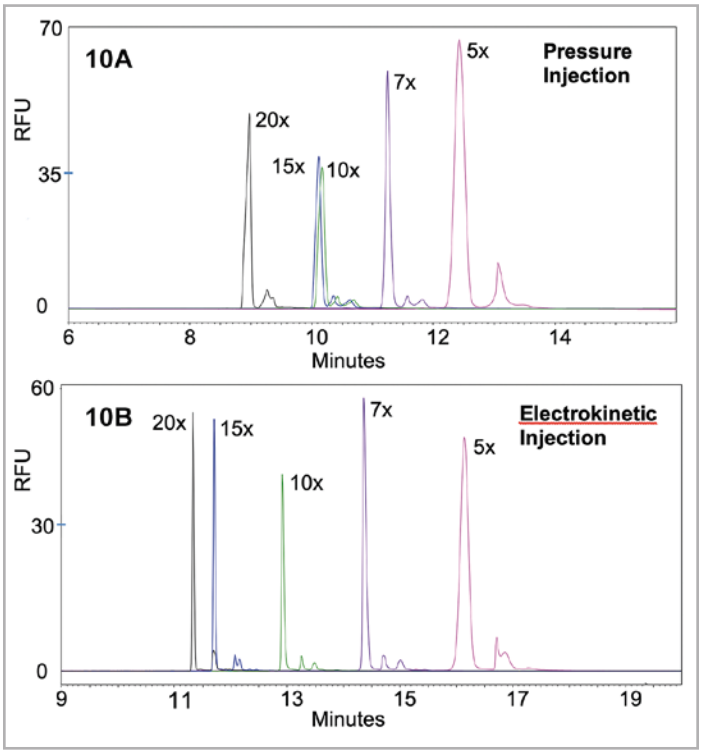

Figure 10. Varying gel dilution with pressure (0.2 psi for 4 sec; Panel 10A) or electrokinetic (2 kV for 5 sec, Panel 10B) injections. LIFluor EnhanCE stain and pDNA1 (7 kb) were used in this experiment.

Since pressure injection may load more sample matrix than electrokinetic injection, an evaluation of pressure (Figure 11A) and electrokinetic injection (Figure 11B) was done with 10x dilution gel, LIFluor EnhanCE stain and 8 ng/µL plasmid DNA sample. Results in Figure 11A showed that better resolution was obtained with lower injection pressure with best resolution at 0.1 psi for 5 seconds. Much better resolution was achieved when electrokinetic injection was used to introduce the samples (Figure 11B). Among the conditions used with electrokinetic injection, 12 seconds injection time at 2.0 kV produced the best results.

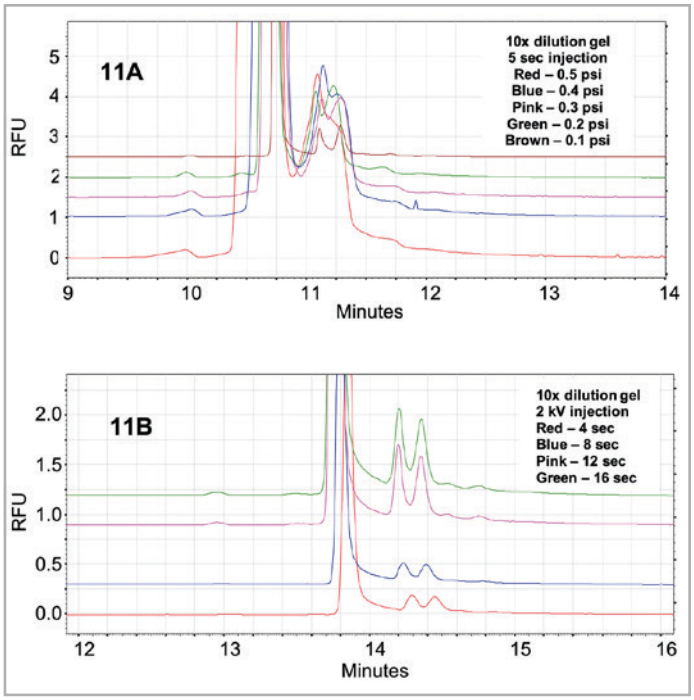

Figure 11. Effect of varying injection pressure (Panel 11A) and varying electrokinetic injection time (Panel 11B). LIFluor EnhanCE stain and pDNA2 (10 kb) were used in this experiment.

Different Fluorescent Dyes: Since LIFluor EnhanCE stain has a relatively low quantum yield (<0.01), SYBR Gold (PN S11494, Thermo) with a quantum yield of 0.7 was evaluated. Results in Figure 12 indicated that although good resolution and signal levels were obtained with both dyes, the signal level obtained with SYBR Gold was much higher than the signal level obtained with LIFluor EnhanCE stain. The resolution was also improved with SYBR Gold.

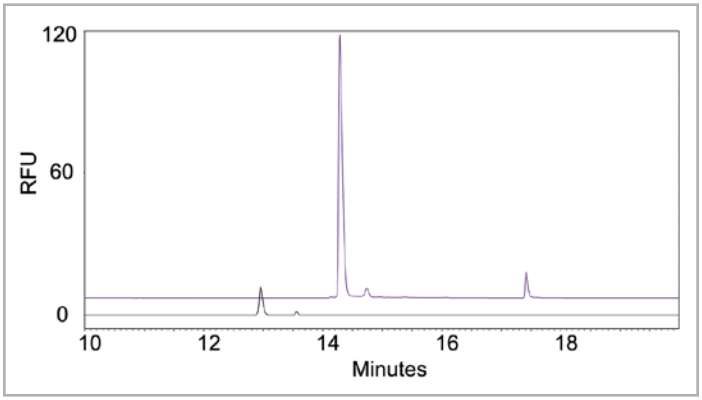

Figure 12. Comparison between LIFluor EnhanCE stain (black) and SYBR Gold (purple). pDNA1 (7 kb) was used in this experiment.

CE for Plasmid Stability Monitoring: pDNA1 (7 kb) and pDNA2 (10 kb) were stressed at 40° C to produce different isoforms. Samples were then analyzed by different topology methods: capillary gel electrophoresis (CGE) and agarose gel electrophoresis (AGE). Results in Figures 2 and 13 were obtained with pDNA1 (7 kb). Data in these two figures indicate CGE can show topology changes. The supercoiled form decreased while open circular form increased over stress treatment (Figure 2). Results from CGE were consistent with those from AGE (Figure 13). The topoisoform percentages were not affected by the methanol rinse in CGE (Figure 13). Similar results were also obtained with pDNA2 (10 kb) (Data not shown).

Robustness: Further optimization involved adding methanol rinse as the first step in conditioning. Methanol rinse improved consistency of separation profiles, extending the capillary life to over 100 runs from under 100 runs. Robustness of optimized method was tested by running the same sample multiple times. Results in Figure 14 demonstrates consistent peak profiles for 21 consecutive runs with different topological isoforms baseline resolved. Percent RSD values of time corrected area for the supercoiled and the open circular peaks were below 8%.

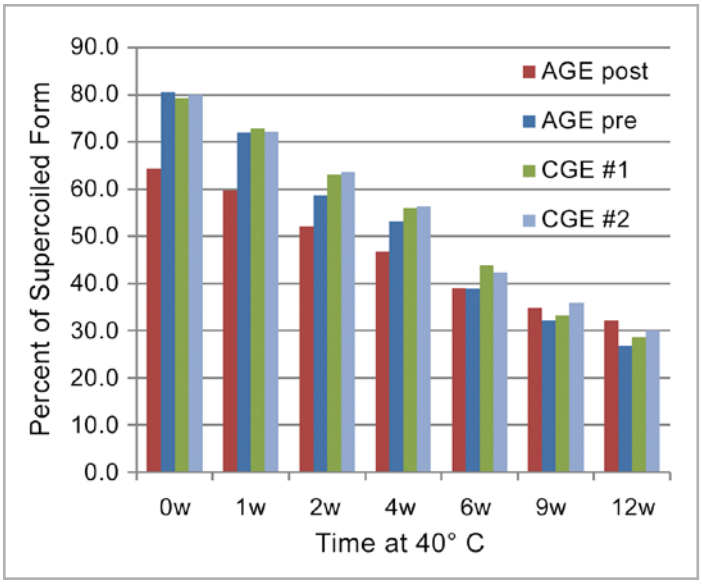

Figure 13. Robustness in monitoring degradation of pDNA1 (7 kb) samples stressed at 40° C for zero (T0) to 12 weeks (T12). CGE #1 is no methanol rinse and CGE #2 included methanol rinse. "AGE post" was stained after running while "AGE pre" contained stain in the agarose gel.

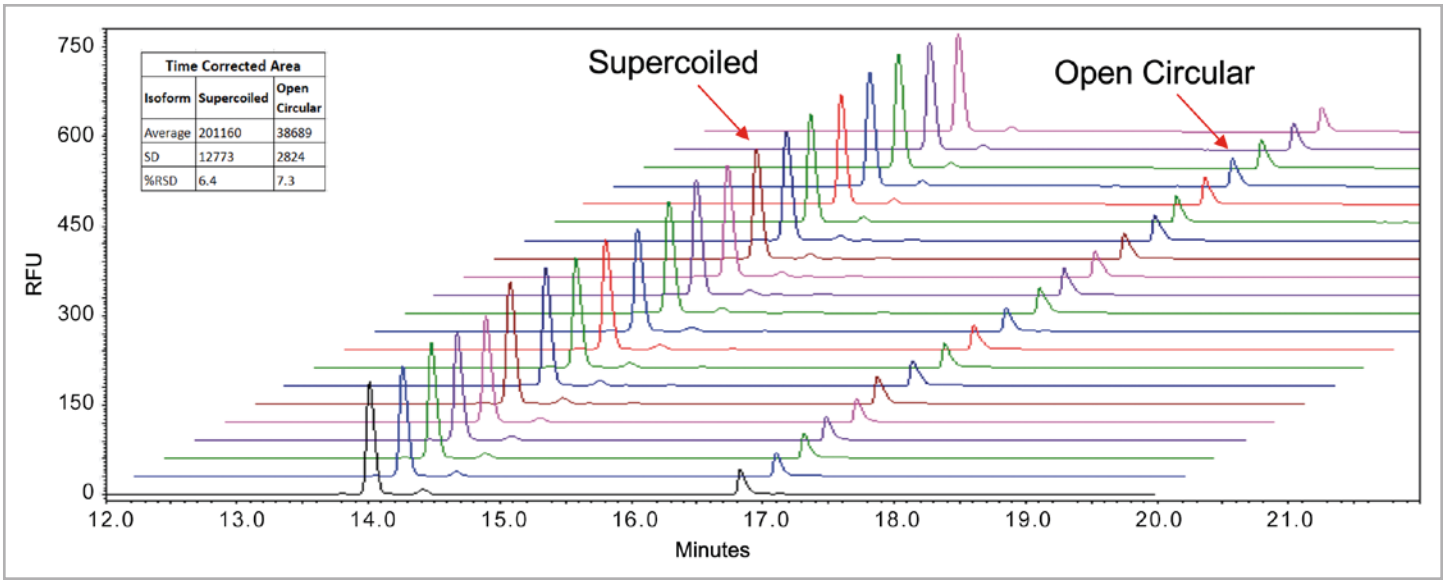

Figure 14. Method Robustness showing high repeatability: Twenty-one consecutive runs of a 10 kb plasmid using optimized method. SYBR Gold and pDNA2 (10 kb) were used.

Conclusions

1 A sensitive and robust CE-LIF method for plasmid analysis was developed.

2 Electrokinetic injection performed better than pressure injection due to more efficient injection of charged DNA analytes.

3 Optimized method using SYBR Gold produced higher sensitivity and better separation profile due to its high quantum yield and favorable impact on topoisoform separation through interaction with DNA as an intercalating dye.

4 Addition of a methanol rinse as the first step in conditioning extended number of injections per sequence and increased the capillary life time to over 100 injections. The topoisoform percents were not affected by the methanol rinse.

5 Experiments with stressed plasmid samples showed CE-LIF can quantitate plasmid topoisoforms and be used in monitoring plasmid degradation.

References

1. FDA. Points to consider on plasmid DNA vaccines for preventive infectious diseases. 1996. Docket no. 96N-0400.

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