A sensitive and robust plasmid analysis method by Capillary Electrophoresis-Laser Induced Fluorescence

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Cell and Gene Therapy USA Congress, Boston, MA

ABSTRACT

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 limitations on quantitation. 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 poster, 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 testing plasmid purity and monitoring plasmid degradation.

Plasmid Topology

Plasmid DNA can exist in different topological forms: covalently closed circular (CCC) or supercoiled (SC); open circular (OC) and linear (L) along with catenanes/multimers. During plasmid DNA degradation (Figure 1), the CCC form can become the OC form due to one single-stranded cleavage. When a double-stranded cleavage occurs, the plasmid DNA will become linearized.

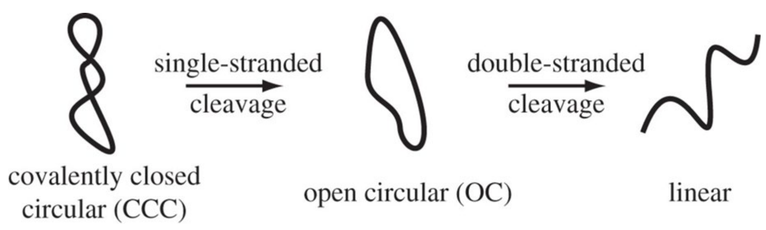


Figure 1. Plasmid Degradation.

Agarose Gel

Agarose gel is the standard method for analysis of plasmid topology. In Figure 2, 200 ng of a plasmid sample (Lane "P2") was run on a 1% TAE agarose gel with ethidium bromide (PN 161-0433, BioRad). About 835 ng of 1 kb plus DNA ladder (PN SM1331, Thermo) was loaded in Lane "M". The supercoiled (SC) and open circle (OC) 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 resolution and detection, providing better quantitative analysis.

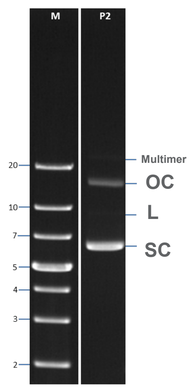


Figure 2. Plasmid analysis by agarose gel.

Capillary Electrophoresis

Capillary electrophoresis analysis for plasmid DNA was performed on a PA800 Plus Pharmaceutical Analysis System (SCIEX) equipped with an LIF detector. A dsDNA 1000 kit (PN 477410) consists of a seiving matrix in tris-borate-EDTA, pH 8.3, buffer (TBE), a coated capillary and Orange G marker was from SCIEX.

Vial Trays and Separation Method

Vial positions and separation timed program for the optimized plasmid analysis method are shown in Table 1 and Table 2. Cartridge temperature was set to 20°C. Sample storage temperature was set to 15°C.

Table 1. Vial positions for optimized plasmid method

Water		Gel Separation	Gel Rinse		
Water		Gel Separation	Gel Rinse		
Water		Gel Separation	Gel Rinse		
Water		Gel Separation	Gel Rinse		
Water		Gel Separation	Gel Rinse		
Water	Gel Rinse	Gel Rinse	Methanol Rinse		

BI (Inlet Buffer Tray)

Water	Gel	Gel Separation	Water Waste		
Water	Gel	Gel Separation	Water Waste		
Water	Gel	Gel Separation	Water Waste		
Water	Gel	Gel Separation	Water Waste		
Water	Gel	Gel Separation	Water waste		
Water	Water Waste	Gel Storage	Water Waste		

BO (Outlet Buffer Tray)

Table 2. Separation timed program for optimized plasmid method

Time (min)	Event	Value	Duration	Inlet	Outlet	Summary
	Rinse - Pressure	10.0 psi	20.00 min	BI:D2	BO:D2	Forward, In/Out vial inc 6
	Wait		0.00 min	BI:A2	BO:A2	In/Out vial inc 6
	Inject - Pressure	2.0 KV	10.0 sec	SI:A1	BO:B2	Override, forward
	Wait		0.00 min	BI:A2	BO:A2	In/Out vial inc 6
0.00	Separation - Voltage	7.8 KV	20.00 min	BI:C2	BO:C2	0.17 min ramp, reverse polarity, In/Out vial inc 6
1.00	Autozero					
20.00	End					

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 seive. Seiving 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 3A and 3B). The 10x dilution gel was used for further optimization experiments from this point on.

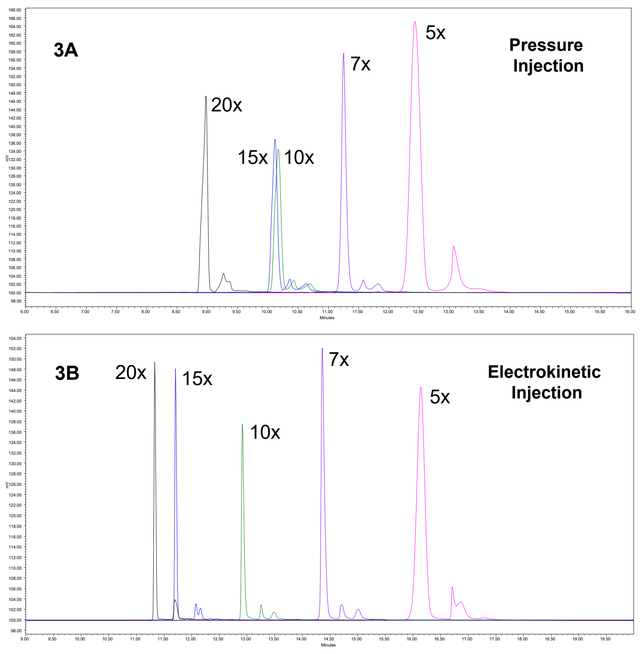


Figure 3. Varying gel dilution with pressure (0.2 psi for 4 sec; **Panel 3A**) or electrokinetic (2 kV for 5 sec, **Panel 3B**) injections. LIFluor EnhanCE stain (PN 477409, SCIEX) was used.

Since pressure injection may load more sample matrix than electrokinetic injection, an evaluation of pressure (Figure 4A) and electrokinetic injection (Figure 4B) was done with 10x dilution gel, LIFluor EnhanCE stain and 8 ng/µL plasmid DNA sample. Results in Figure 4A 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 4B). Among the conditions used with electrokinetic injection, 12 seconds injection time at 2.0 kV produced the best results.

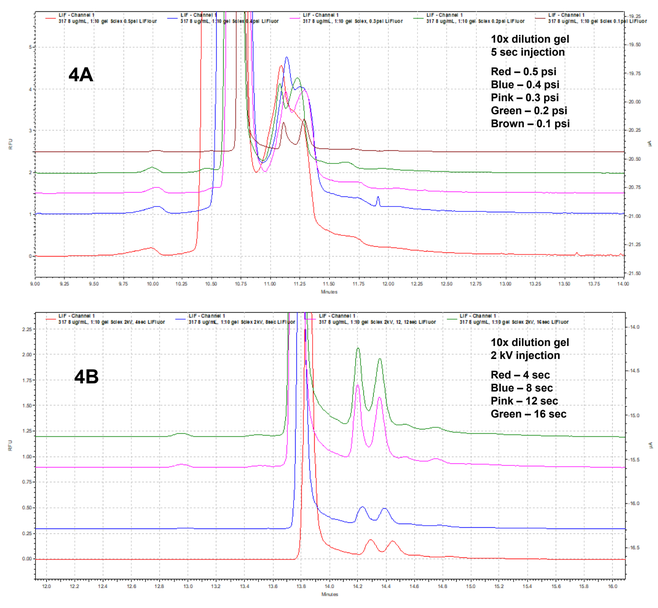


Figure 4. Effect of varying injection pressure (**Panel 4A**) and varying electrokinetic injection time (**Panel 4B**). LIFluor EnhanCE stain was used.

Different Fluorescent Dyes

LIFluor EnhanCE, Ethidium Bromide (EtBr), Sybr-gold (PN S11494, Thermo) and YOYO-1 were evaluated. Sybr-gold generated the best separation of supercoiled and open circle forms (Figure 5).

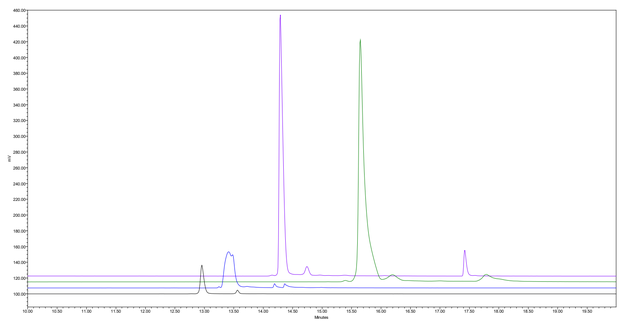
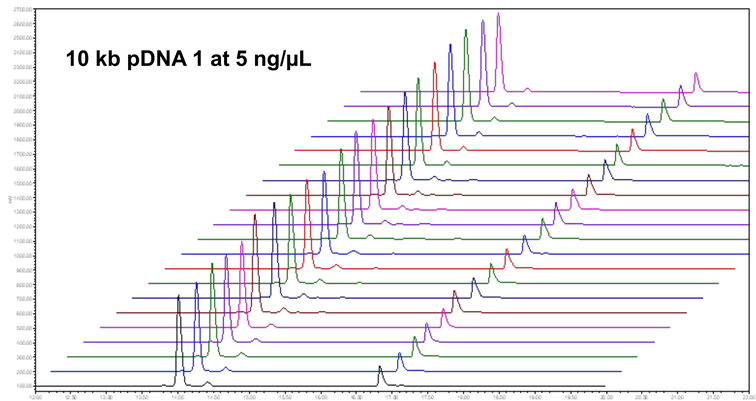


Figure 5. When LIFluor EnhanCE (black) and EtBr (blue) were used, sample concentration was at 8 ng/µL; LIF dynamic range was 100 RFU; injection was 2 kV for 5 sec. For Sybr-gold (purple), sample was 5 ng/µL; detection range was 1000 RFU; injection was 2 kV for 10 sec. For YOYO-1 (green), sample was 8 ng/µL; detection range was 1000 RFU; injection was 2 kV for 5 sec.

Robustness

Further optimization involved adding methanol rinse as the first step in conditioning. Methanol rinse improved separation profiles, extended the capillary life. Methanol rinse between injections or at specific intervals did not enhance resolution (data not shown). Robustness of optimized method was tested by running the same sample multiple times. Results in Figure 6 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 circle peaks were below 8%.



Time corrected area

Sample	Peak 1	Supercoil	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Open Circle	Peak 7	Peak 8
Average	*2100.88*	*201160.21*	*10201.20*	*1152.27*	*307.04*	*2758.39*	*585.70*	*38688.86*	*1895.49*	*554.78*
SD	*150.06*	*12772.81*	*656.03*	*117.90*	*58.51*	*232.26*	*139.01*	*2823.79*	*213.04*	*142.55*
%RSD	*7.14*	*6.35*	*6.43*	*10.23*	*19.06*	*8.42*	*23.73*	*7.30*	*11.24*	*25.69*

Figure 6. Twenty-one consecutive runs of a 10 kb plasmid using optimized method. Sybr-gold stain was used.

CE for Plasmid Stability Monitoring

pDNA 1 (10 kb) and pDNA 2 (7 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 Figure 7 and 8 indicate CGE can show topology changes. The supercoiled form decreased while open circle form increased over stress treatment (Figure 7). Results from CGE were consistent with those from AGE. The topoisoform percentages were not affected by the methanol rinse in CGE (Figure 8).

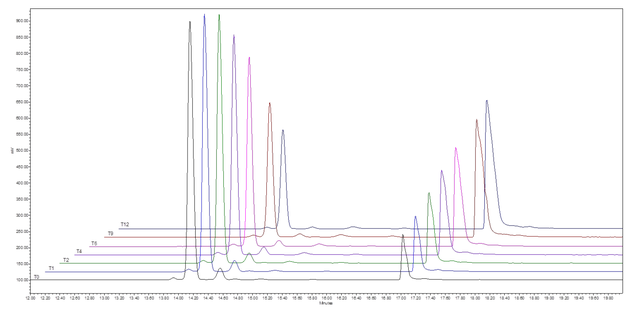


Figure 7. Robustness in monitoring degradation of pDNA 2 (7 kb) samples stressed at 40°C from zero (T0) to 12 (T12) weeks.

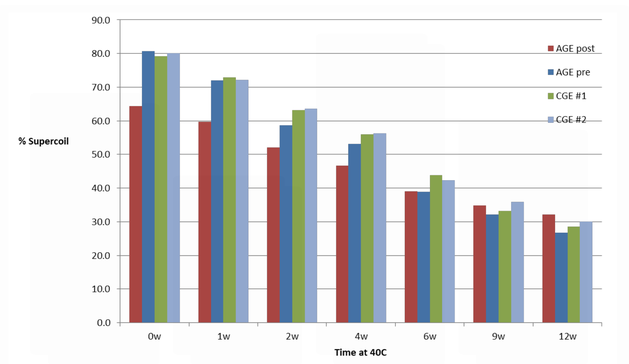


Figure 8. Robustness in monitoring degradation of pDNA 1 (10 kb) samples stressed at 40°C from zero (T0) to 12 (T12) weeks. CGE #1 is no methanol rinse and CGE #2 includes methanol rinse. "AGE post" was stained after running while "AGE pre" contained stain in the agarose gel.

CONCLUSIONS

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

Optimizing the gel dilution allowed plasmid isoforms to be baseline resolved and quantitated.

Electrokinetic injection performed better than pressure injection.

Intercatalation of different dyes have different impact on the topoisoform separation. Sybr-gold produced the best separation profile.

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.

Experiments with stressed plasmid samples showed CE-LIF can quantitate plasmid topoisoforms.

REFERENCE

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

Sciex Document Number: RUO-MKT-10-9327