Accurate Analysis of Double Stranded DNA over an Extended Size Range

Capillary Electrophoresis-Laser Induced Fluorescence Detection (CE-LIF)

Jane Luo, Mukesh Malik, Marcia Santos, Sahana Mollah and Handy Yowanto
SCIEX, Brea, California

Introduction

Analysis of double stranded DNA (ds DNA) is an integral part of manufacturing nucleic acids as therapeutics for gene and cell therapy as well as for DNA vaccines. The Food and Drug Administration (FDA) guidelines recommend manufacturers to include the gene map with relevant restriction sites for gene therapy vector constructs and vector diagrams identifying the gene insert, regulatory regions and pertinent restriction endonuclease sites when submitting applications for investigational new drugs (IND).[1-2] Analysis of restriction fragments from vectors of various sizes often requires accurate sizing over an extended size range. Traditional agarose gel method has limitation on long size coverage as well as poor fragment resolution, sizing accuracy and reproducibility. In this technical note, we describe CE-LIF based methods for accurate and robust analysis of ds DNA over an extended molecular size range from 100 base pairs (bp) to 15 kilo base pairs (kb).

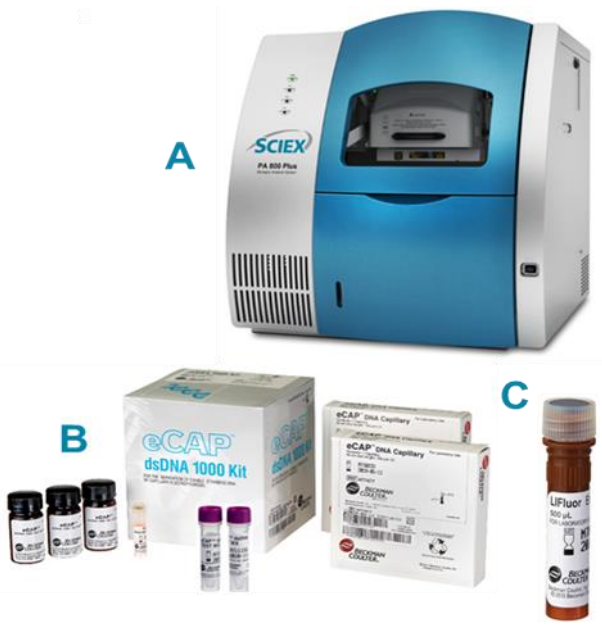


Figure 1. The PA 800 Plus Pharmaceutical Analysis System (A), the dsDNA 1000 Kit (B) and the LIFluor EnhanCE Stain (C).

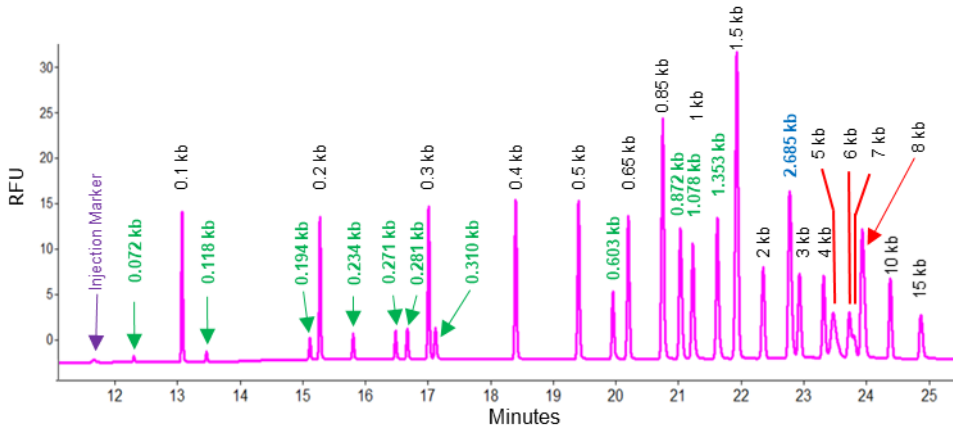


Figure 2. Example of successful separation and sizing of restriction fragments of virus and plasmid DNA using SCIEX dsDNA 1000 kit. Peaks with sizes labeled in green font are *Hae* III restriction fragments of ϕX 174 bacteriophage DNA. The linear pUC18dG plasmid DNA was labeled in blue font. Fragments in 1 kb plus DNA ladder are labeled in black font. The injection marker in purple font is LIF Performance Test Mix. Separation was performed on a 30 cm effective length DNA capillary at 7.8 kv voltage for 30 minutes.

Key Features

- Complete set of reagents for dsDNA fragment analysis with better kit shelf life than microchip based reagent kits
- Excellent resolution over an extended fragment size range with the same gel matrix
- Accurate size determination by standard curve
- Good Sensitivity (LOD of 0.79 ng/ml) and dynamic range (2.7 log)
- Good repeatability with % RSD of less than 0.5% for migration time and less than 2.5% for corrected peak area
- Ideal for restriction enzyme digestion analysis of DNA vectors

Materials and Instrument

Materials:

The eCAP dsDNA 1000 kit (PN 477410, Figure 1B), the LIFluor EnhanCE fluorescent stain (PN 477409, Figure 1C), dsDNA 1000 Test Mix (PN: 477414, Figure 1B), linearized pUC18dG (PN 608008) and LIF Performance Test Mix (PN: 726022) were from SCIEX, Framingham, MA. The 1 kb plus (PN 10787018) and 1 kb (PN 15615-016) DNA ladders were from Thermo, Carlsbad, CA. HPLC grade water (W5-4) was from Fisher Chemicals, New Lawn, NJ.

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

Methods

Gel buffer reconstitution: This step must be done one day before running samples. To rehydrate the gel buffer, 20 ml of 0.2 µm filtered deionized water was added to the gel buffer vial. After the gel buffer vial was securely capped, the vial was placed on a rotator or the gel mixture was gently stirred with a small stirring bar for up to 24 hours or till the dried gel was completely dissolved. The hydrated gel should be good for one month if stored at 4°C.

Cartridge Assembly: DNA capillary (PN 477477) was installed per instructions on kit insert (PN 726412) in the dsDNA 1000 kit. The total capillary length was 40.2 cm with 30 cm as the length to the detection window. For analysis of large sized DNA fragments, a 50.2 cm total length capillary was also used with the length to the detection window as 40 cm. Since the inner wall of the DNA capillary is coated, the cartridge assembly was carried out within 5 to 10 minutes. The capillary ends were immersed in liquid (water or buffer) as soon as the cartridge assembly was complete to prevent the coating from drying out.

Sample Preparation: All samples were diluted with filtered deionized water to 2 to 20 ng/µl for analysis on the PA 800 Plus instrument.

LIF Calibration: To ensure consistent response of LIF detector throughout this study, the LIF detector was calibrated using LIF Calibration Wizard and Performance Test Mix (PN: 726022) following the instructions in LIFluor EnhanCE user's guide (PN 725824). The target RFU value was set as 62. This calibration was done whenever capillary or LIF detector or the laser was changed. After the calibration was complete and the CCF was accepted, vials of water were placed back to the "home" positions (BI:A1 and BO:A1) so that the capillary ends were placed in water to keep them wet.

Instrument Performance Test: Instrument and gel buffer performance may be tested using dsDNA 1000 Test Mix (PN 477414) and LIFluor EnhanCE stain (PN 477409). Test Mix can be diluted by adding 1 mL of filtered deionized water (> 18MΩ) to the test mix vial. 5 µl LIFluor EnhanCE stain may be added to 6 mL of 1X rehydrated dsDNA 1000 gel buffer. Separation may be carried out following instructions in LIFluor EnhanCE user's guide (PN 725824).

Preparation of Buffer Trays and Sample Trays: Vial positions for buffer trays are indicated in Figure 3. Each "DDI Water" vials were filled with 1.5 ml double deionized (DDI) water. Waste vials were filled with 1 ml DDI water. "Gel Fill" vials and "Gel Sep." vials were filled with 1.5 ml gel. Vial "A6" on the outlet tray was filled with 1 ml gel. Two sets of water dip vials were used during sample separation for minimal sample carryover and better repeatability. Vial increment was done for every 8 injections. Injecting samples against a half-filled vial of gel buffer (BO:A6) facilitated improved precision in peak area.

Each ds DNA sample was transferred to a Microvial (PN 144709) at 50 to 100 µl. Sample vials were loaded onto the sample inlet tray.

Instrument Set up:

The "Initial Conditions" and "LIF Detector Initial Conditions" were set up as indicated in Figure 4 and Figure 5 respectively. Same set up was used for all three methods: Capillary Conditioning, Separation and Shut Down.

The time program for Capillary Conditioning was illustrated in Figure 6. Buffer vials used for conditioning were purposely positioned in row #6 to avoid moving the capillary tips over buffer vials used for sample separation.

Figures 7 and 8 show time programs for separation and shut down methods for running the dsDNA 1000 test mix. For DNA fragments with sizes larger than 2 kb, the separation time was extended to longer time as indicated in figure legends.

When the 40 cm effective length DNA capillary was used, injection condition was 0.5 psi for 10 sec. The separation time was 25 minutes at 12 kv, 35 minutes at 10 kv, 45 minutes at 8 kv, 65 minutes at 6 kv and 75 minutes at 5 kv.

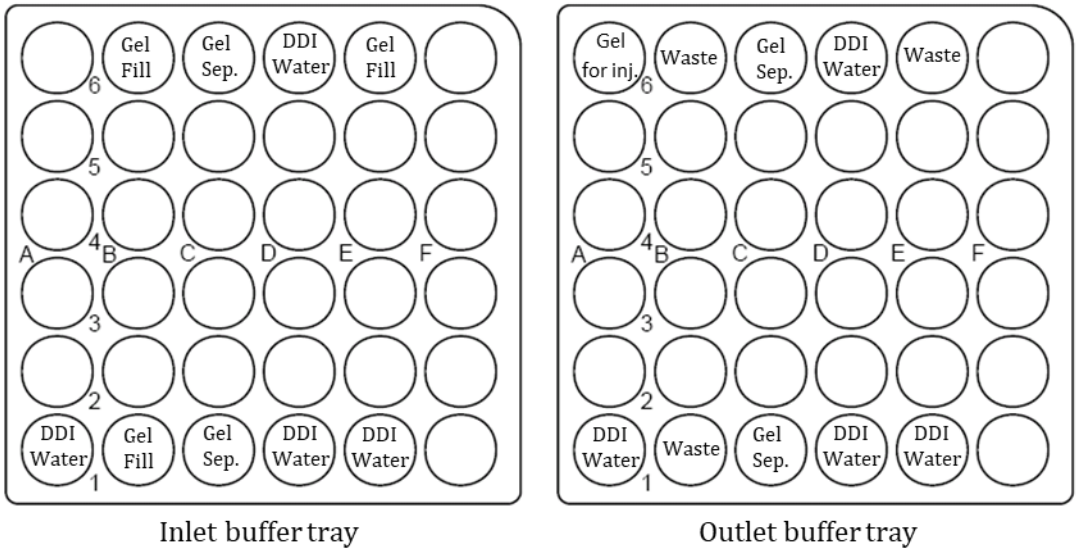


Figure 3. Schematic set up for Inlet (left) and Outlet (right) buffer trays for up to 8 sample injections.

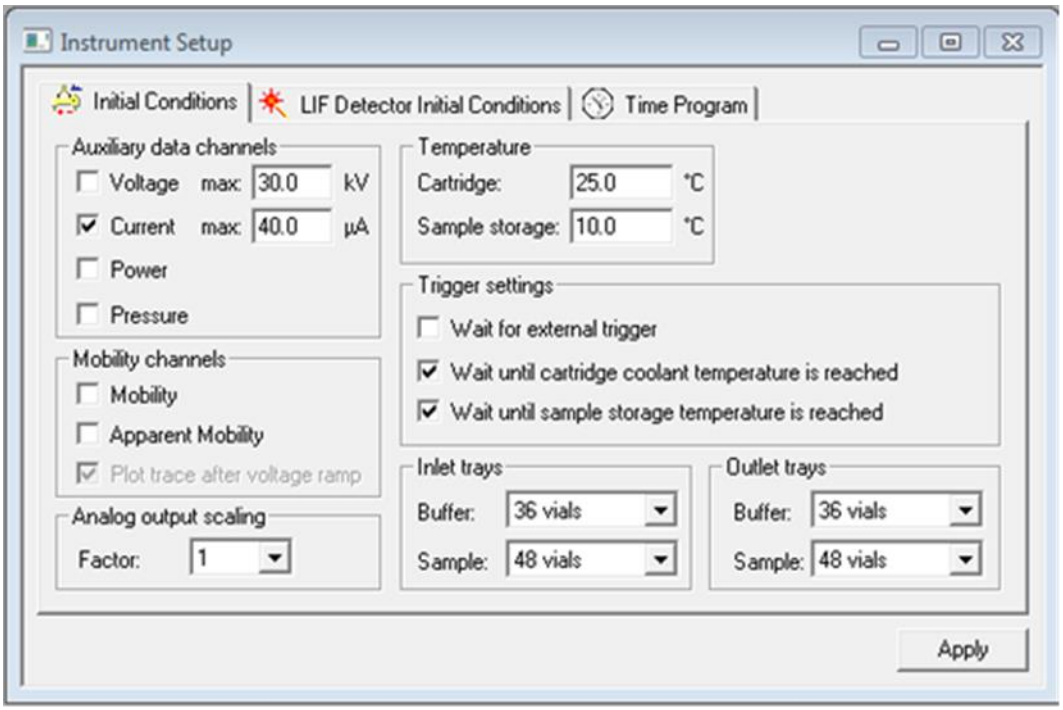


Figure 4. Settings for “Initial Conditions” tab.

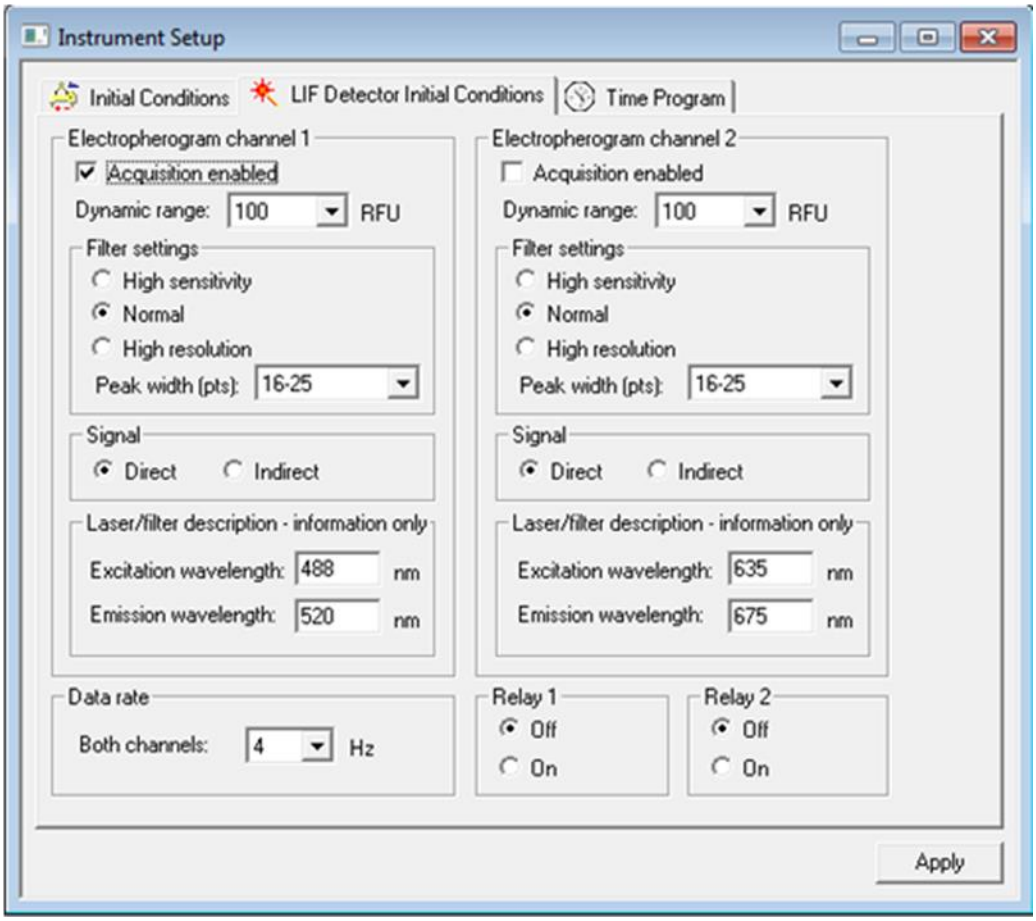
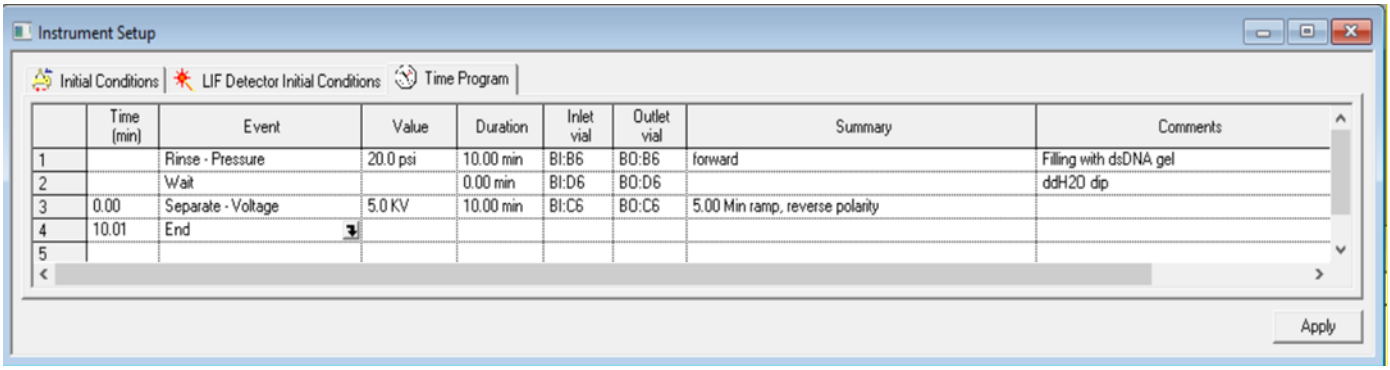
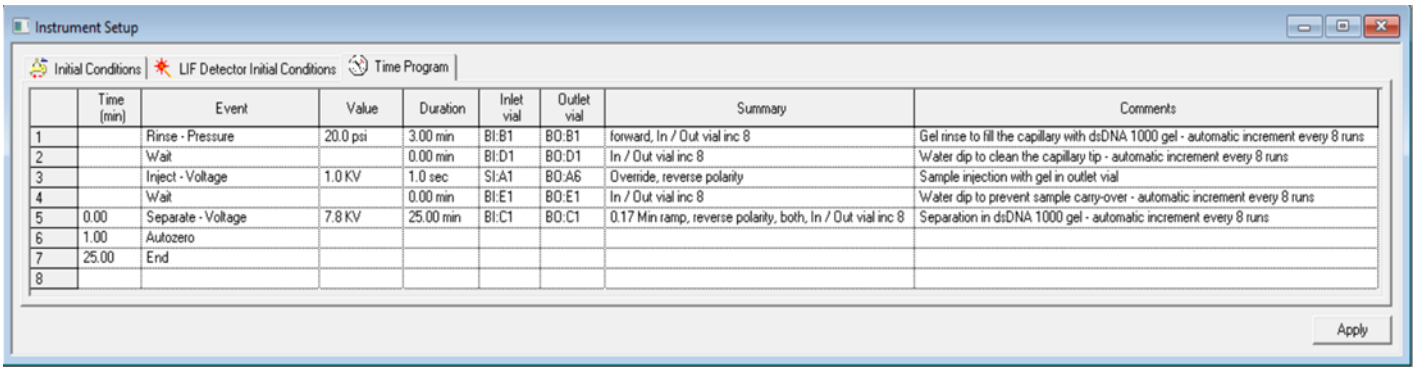


Figure 5. Settings for “LIF Detector Initial Conditions” tab.



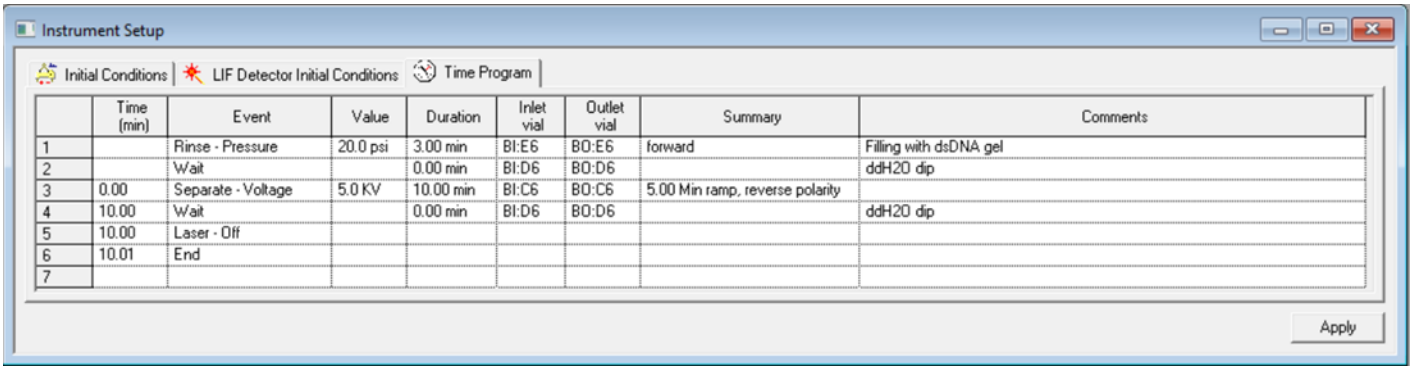
	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	10.00 min	BI:B6	BO:B6	forward	Filling with dsDNA gel
2		Wait		0.00 min	BI:D6	BO:D6		ddH20 dip
3	0.00	Separate - Voltage	5.0 KV	10.00 min	BI:C6	BO:C6	5.00 Min ramp, reverse polarity	
4	10.01	End						
5								

Figure 6. Time Program Settings for the Conditioning Method.



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	3.00 min	BI:B1	BO:B1	forward, In / Out vial inc 8	Gel rinse to fill the capillary with dsDNA 1000 gel - automatic increment every 8 runs
2		Wait		0.00 min	BI:D1	BO:D1	In / Out vial inc 8	Water dip to clean the capillary tip - automatic increment every 8 runs
3		Inject - Voltage	1.0 KV	1.0 sec	SI:A1	BO:A6	Override, reverse polarity	Sample injection with gel in outlet vial
4		Wait		0.00 min	BI:E1	BO:E1	In / Out vial inc 8	Water dip to prevent sample carry-over - automatic increment every 8 runs
5	0.00	Separate - Voltage	7.8 KV	25.00 min	BI:C1	BO:C1	0.17 Min ramp, reverse polarity, both, In / Out vial inc 8	Separation in dsDNA 1000 gel - automatic increment every 8 runs
6	1.00	Autozero						
7	25.00	End						
8								

Figure 7. Time Program Settings for the Separation Method of the dsDNA 1000 Test Mix.



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	3.00 min	BI:E6	BO:E6	forward	Filling with dsDNA gel
2		Wait		0.00 min	BI:D6	BO:D6		ddH20 dip
3	0.00	Separate - Voltage	5.0 KV	10.00 min	BI:C6	BO:C6	5.00 Min ramp, reverse polarity	
4	10.00	Wait		0.00 min	BI:D6	BO:D6		ddH20 dip
5	10.00	Laser - Off						
6	10.01	End						
7								

Figure 8. Time Program Settings for the Shut Down Method.

Results and Discussion

Analysis of the *Hae* III restriction fragments of ϕX174

The ϕX174 is a small, single-stranded DNA virus that infects *Escherichia coli*. It is also called a bacteriophage. It was the first genome to be sequenced. [3] Its replicative form (RF) is a double stranded circular DNA molecule of 5386 base pairs (bp). When it is digested by a restriction enzyme *Hae* III, 11 DNA fragments are generated.[4] These fragments have been used as popular DNA standards for size determination of DNA fragments in the past 40 years. It is available from SCIEX as dsDNA 1000 Test Mix (PN 477414). Figure 9a shows the restriction map of ϕX174 with *Hae* III sites, the origin of replication, and location of 11 genes indicated. Figure 9b is a representative electropherogram obtained by separation of the *Hae* III fragments of ϕX174 on PA800 Plus using dsDNA 1000 gel, LIFluor EnhanCE dye and coated DNA capillary at 40.2 cm total length. Instrument set up and separation conditions were described in the Methods section. All 11 fragments including two fragments differing by 10 bps were baseline resolved within a 25 minutes run. As shown in Figure 10, a calibration curve was generated with results from Figure 9b. The best curve fitting was obtained with the quartic (4th degree polynomial) model with excellent R square values: 0.9992 at the linear scale (panel A) and 0.9997 at log scale (panel B).

Further experiments were carried out to determine the limit of detection (LOD) and limit of quantitation (LOQ) as well as the linearity of detector response to concentration of the DNA sample in this method. The *Hae* III fragments of ϕX174 were serially diluted by 2 fold each time and separated. Figure 11A shows the electropherograms obtained with some of the diluted samples. The concentration of the 872 bp fragment is indicated next to each electropherogram trace. The signal to noise ratio for the 872 bp peak was 5.4 at the concentration of 0.79 ng/ml, and

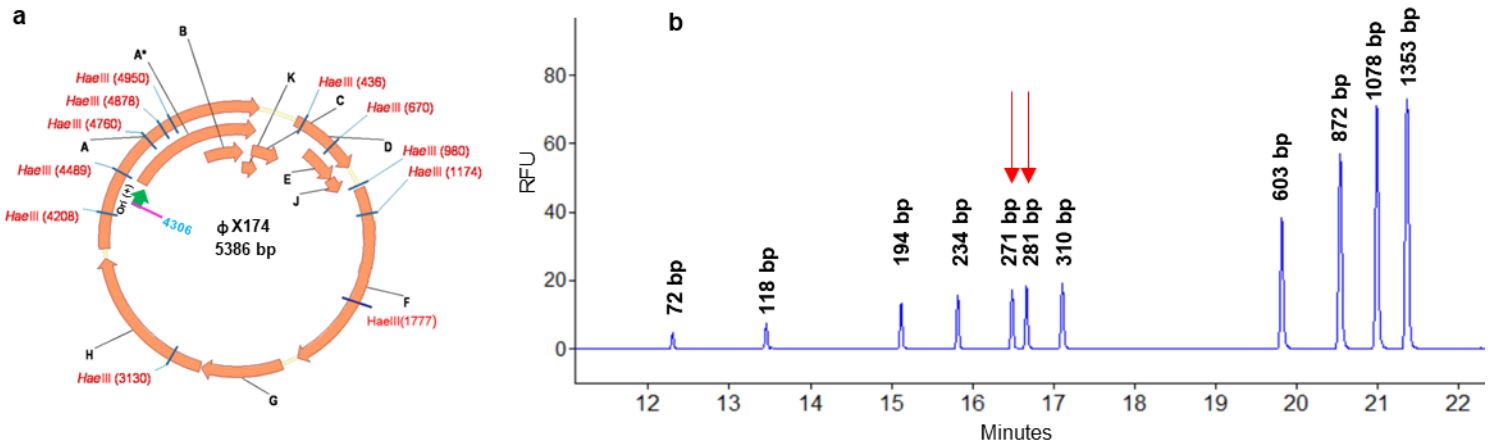


Figure 9. Baseline resolution of 11 *Hae* III restriction fragments of ϕX174. Panel "a" shows the map of ϕX174 genome with *Hae* III sites indicated. The ϕX174 genome contains 11 genes (A, A*, B-H, J, K) represented by thick arrows in orange color. Location of the origin of replication is indicated by the green arrow. Panel "b" shows an electropherogram of the 11 *Hae* III fragments with their sizes labeled in base pairs (bp). Separation was performed with a 30 cm effective length DNA capillary (40.2 cm in total length), dsDNA 1000 gel and the LIFluor EnhanCE stain. The two red arrows indicate that the two fragments with 10 bp size difference were baseline resolved.

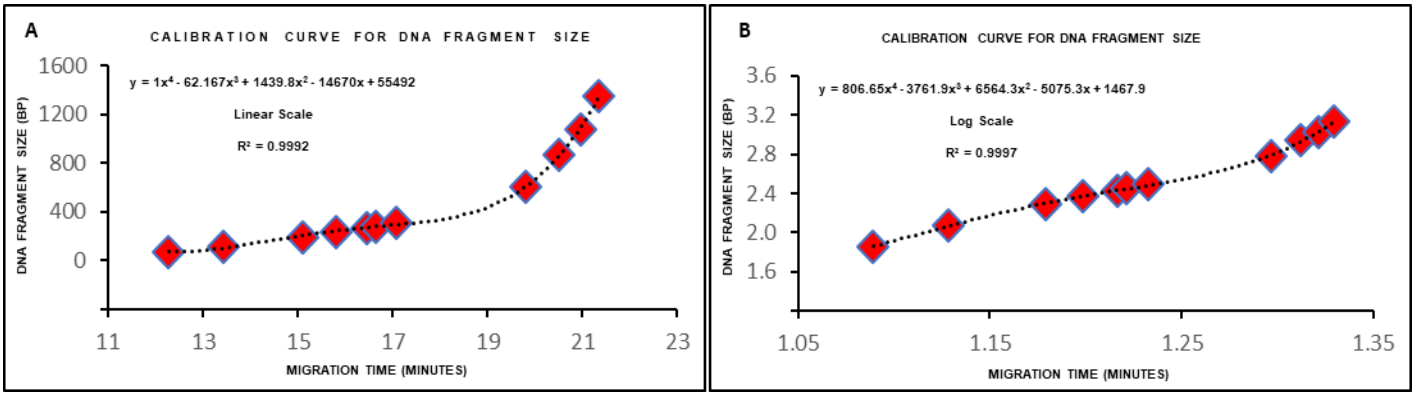


Figure 10. Calibration curve for DNA fragment size determination using dsDNA 1000 test mix (ϕX174 *Hae* III restriction fragments) as standards. DNA fragment sizes in bp were plotted against migration time in minutes. The best curve fitting model was quartic (4th degree polynomial). The R square value was 0.9992 for the linear scale (Panel A) and 0.9997 for the log scale (Panel B).

16.1 at 1.6 ng/ml. Therefore, the LOD is 0.79 ng/ml and the LOQ is around 1 ng/ml. Figure 11B shows that good linearity was obtained when plotting the corrected peak area of the 872 bp fragment against its concentration from 0.79 ng/ml to 404 ng/ml. The R square value was 0.995. The dynamic range was 2.7 log.

Analysis of DNA fragments over extended size range

Many of the vectors used in producing nucleic acid therapeutics for gene and cell therapy as well as for DNA vaccination have sizes well over 10 kb. Restriction enzyme digestion of these large vectors often generates some fragments that are larger than 1 kb as well as fragments that are smaller than 500 bps. Analysis of these fragments over an extended size range requires the use of DNA size standards that provide a long size range coverage. Several DNA standards with broader range of size coverage were tested. Among them, the 1 kb DNA ladder and 1 kb plus DNA ladder from Thermo were selected.

Analysis with 1 kb DNA ladder

The 1 kb DNA ladder includes 12 large fragments created by repeating a 1018 bp fragment for 1 to 12 times. It also contains additional 11 fragments with sizes from 75 bp to 1636 bp that are

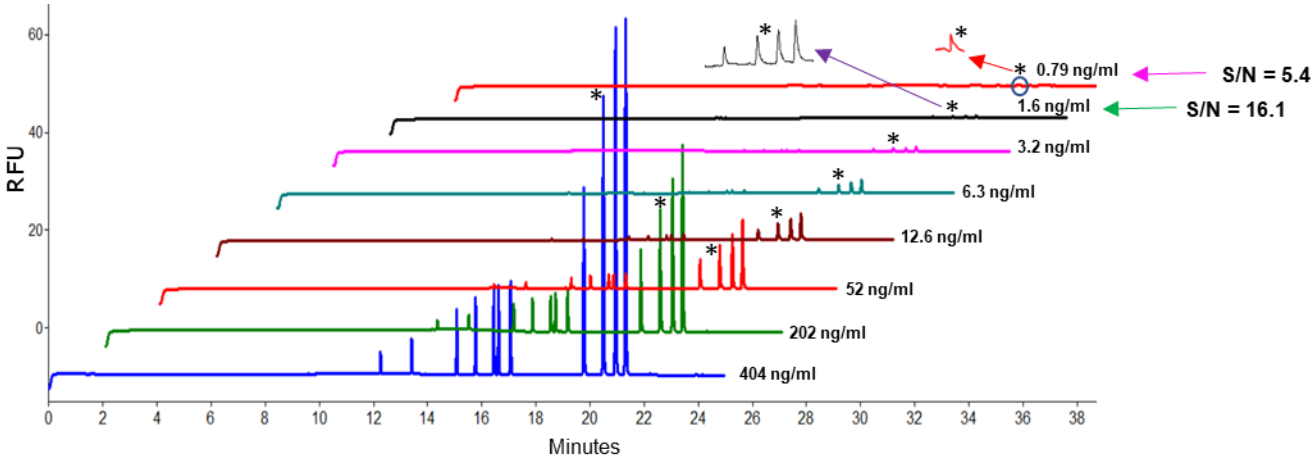


Figure 11A. Good detector response to different concentrations of the sample. The dsDNA 1000 Test Mix was serially diluted before analysis. The 872 bp fragment used for quantitative analysis was labeled with "*". Its concentration in each sample is labeled**.**

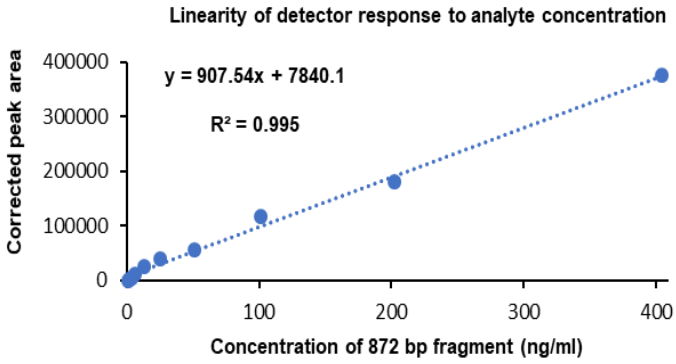


Figure 11B. Good linearity between detector response to analyte concentration. Corrected peak area of the 872 bp fragment was plotted against its concentration in ng/ml.

generated by *Hinf* I digestion of a plasmid vector pBR322. For the 1 kb DNA ladder, the best separation was obtained with the separation voltage at 6 kv and a 40 cm effective length DNA capillary. As shown in Figure 12, an experiment was done to test method repeatability. The 1 kb DNA ladder at the concentration of 20 ng/ul was injected at 0.5 psi for 10 sec for 8 runs and separated using dsDNA 1000 gel and LIFluor EnhanCE dye. Each separation was performed at a voltage of 6 kv for 65 minutes with 20 psi pressure at both capillary ends. Results in Figure 12 showed that the peak patterns were consistent between the 8 different runs. Fragments under 500 bp with size differences of 20 to 50 bp were well separated. The picture inset in Figure 12 shows a zoomed-in view of the area where large DNA fragments with sizes of 1.6 kb to 12 kb were baseline separated as well. Good repeatability was demonstrated by the RSD% values for the 1636 bp peak: 0.43% for migration time and 2.19% for corrected peak area.

Analysis with 1 kb plus DNA ladder

The 1 kb plus DNA ladder contains 18 fragments in the range of 100 bp to 15,000 bp. Two different methods were evaluated with this DNA ladder. The first one included using the DNA capillary at 30 cm effective length and electrokinetic sample injection. An experiment was done to simulate analysis of fragments produced by restriction enzyme digestion of a plasmid and a virus. The 1 kb plus DNA ladder (at 6.25 ng/µl), linearized plasmid pUC18dG (at 3 ng/µl) and *Hae* III fragments of ϕX174 (at 2.5 ng/µl) were injected at 1 kv for 2 seconds. Separation was performed at 7.8 kv for 30 minutes. Results in Figure 2 demonstrated that excellent resolution was achieved with baseline resolution of fragments differing by 6-10 bp around 200-300 bp region, 30-50 bp around 800 bp region and 150 to 315 bp around 1 to 3 kb area. A calibration curve was created by plotting the size of DNA fragments in the 1 kb plus ladder against migration time using the quantitative analysis feature of the 32 Karat software. The R square value was 0.9982 at the log scale using the quartic model (data not shown). This calibration curve was used to deduce predicted sizes for the linearized pUC18dG and 10 of the *Hae* III

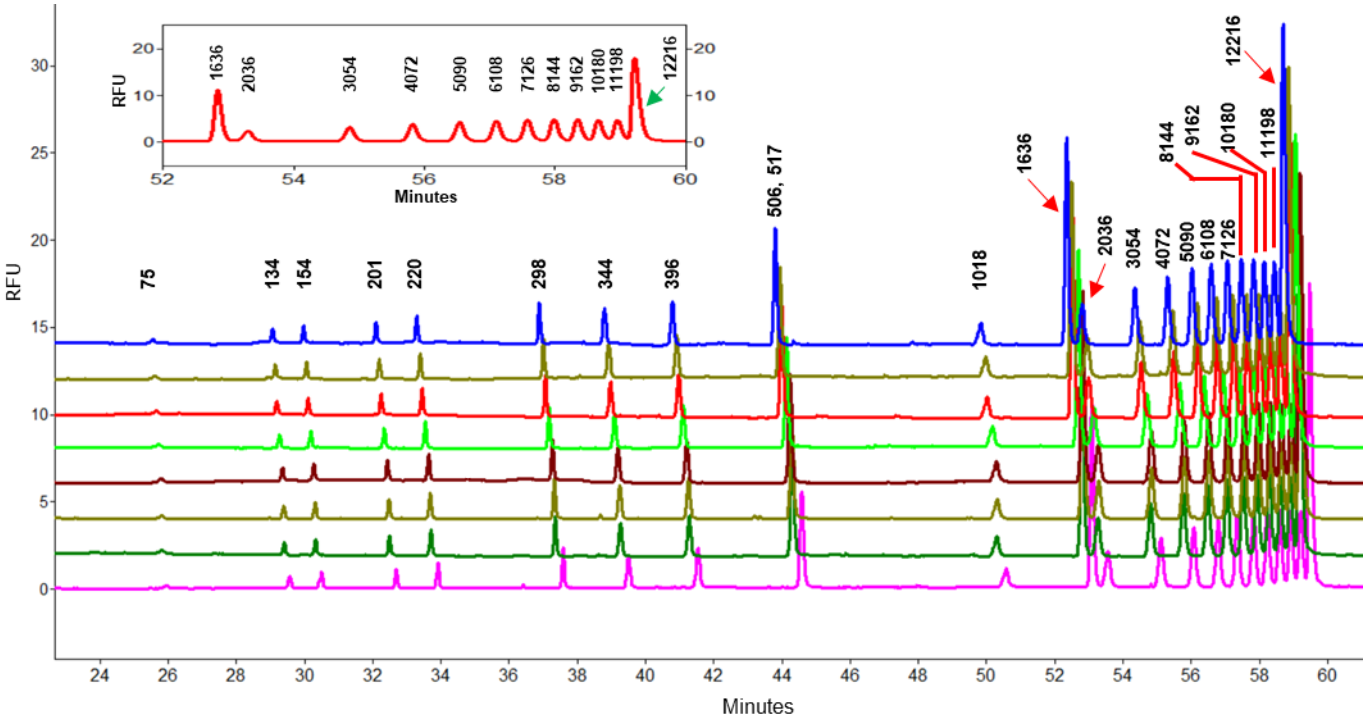


Figure 12. Good repeatability in separation of DNA fragments in the 1 kb DNA ladder. The 1 kb DNA ladder at the concentration of 20 ng/ul was injected 8 times and separated on PA800 Plus using dsDNA 1000 gel and LIFluor EnhanCE dye. Each separation was performed at a voltage of 6 kv for 65 minutes with 20 psi pressure at both capillary ends. The RSD% for the 1636 bp fragment was 0.43% for migration time and 2.19% for corrected peak area. The picture insert shows a zoomed-in view of the area where large DNA fragments with sizes of 1.6 kb to 12 kb were baseline resolved. The effective length of the DNA capillary used was 40 cm.

fragments of ϕX174. The predicted sizes were compared to their corresponding theoretical sizes (Table 1). The differences between predicted sizes and theoretical sizes were no more than 5 to 7% of the theoretical sizes for all fragments, demonstrating the accuracy of size determination for restriction fragments by this method. In addition, this method can be potentially useful for analysis of host cell DNA in cell culture-produced vaccines in which over 80% of residual DNA was under 1000 bp with the major peak around 150 bp and some minor peaks in the range of 1000 bp to 10 kb. [5-6]

The second method with the 1 kb plus DNA ladder involves using the DNA capillary at 40 cm effective length and pressure injection for sample loading. Figure 13 shows results obtained by injecting a mixture of the 1 kb plus DNA ladder and the 1 kb DNA ladder at 0.5 psi for 10 seconds. Separation was carried out at 10 kv for 35 minutes. Large DNA fragments were separated with a resolution of 1 kb. In the region between 850 to 2000 bp, the resolution was 136 to 150 bp. Fragments smaller than 500 bp were well resolved when the size differences between them were 20 bp or larger. Interestingly, a 298 bp fragment was separated

Table 1. Comparison of theoretical sizes with predicted sizes for 11 fragments.

Theoretical Size	118	194	234	271	281	310	603	872	1078	1353	2685
Predicted Size	120	204	236	265	270	295	609	931	1054	1331	2804
Δ Size	2	10	2	-6	-11	-15	6	59	-24	-22	119
% Δ Size / TS	2	5	1	-2	-4	-5	1	7	-2	-2	4

Notes: "Δ Size" is the difference between theoretical size and the size predicted using 1 kb plus DNA ladder. "% Δ Size / TS" stands for percent size difference relative to the theoretical size.

from a 300 bp fragment while a 396 bp fragment was not resolved from a 400 bp fragment. These differences may be due to differences in sequence composition or differences in interaction with the intercalating dye. Resolution achieved with this method was similar to that obtained with the first method.

Users can choose which method to use based on their samples.

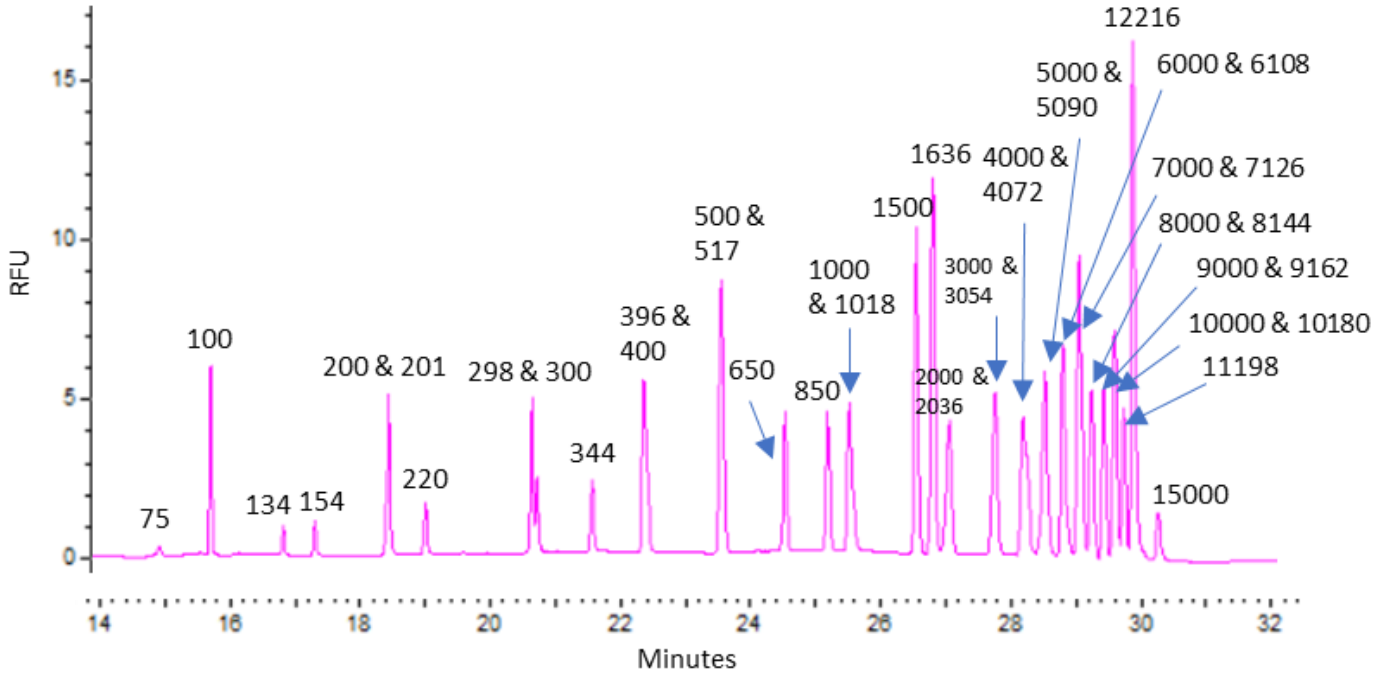


Figure 13. Good resolution was achieved in separating fragments from a mixture of the 1 kb DNA ladder and the 1 kb plus DNA ladder. Samples containing 20 ng/µl of both DNA ladders were injected at 0.5 psi for 10 seconds. Separation was performed with a 40 cm effective length DNA capillary at 10 kv for 35 minutes.

Conclusions

This technical note demonstrated the following advantages of using SCIEX dsDNA 1000 gel, coated DNA capillary and LIFluor EnhanCE dye over the traditional agarose gel and microchip based methods in analysis of dsDNA fragments:

1. Accurate size determination is achieved over a longer size range (72 bp to 15 kb) with the same dsDNA 1000 gel matrix and within 35 minutes
2. Finer resolution for more accurate size determination
    a. 6 to 10 bp around 200-300 bp
    b. 30 to 50 bp around 800 bp to 1kb
    c. 136 to 315 bp around 1.5 kb to 3 kb
    d. 0.5 kb to 1 kb around 4 kb to 12 kb
3. Better sensitivity and assay repeatability for more consistent results with LOD of 0.79 ng/ml and %RSD of less than 0.5% for migration time and less than 2.5% for corrected peak area
4. Wider dynamic range (2.7 log) allows detection of both abundant fragments and rare fragments
5. Longer kit shelf life (15 months vs 4 to 6 months) provides cost-effectiveness and efficient inventory management

In addition, co-injection of sample and DNA standards provides higher accuracy in size determination than traditional agarose gel electrophoresis.

Furthermore, users can choose different capillary length, DNA standards, sample injection and separation conditions to suit their analysis needs of different size range.

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