Unlock the full potential of the DNA 20 kb Plasmid and Linear kit for comprehensive plasmid topology and linear DNA sizing analysis

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This technical note demonstrates rapid, high-resolution analysis of plasmid topological isoforms and accurate sizing of linear DNA fragments across a wide size range using the DNA 20 kb Plasmid and Linear kit from SCIEX. In nucleic acid therapeutics—such as cell and gene therapy and RNA vaccines—this kit helps with quickly characterizing new DNA templates, verifying the purity of supercoiled (SC) plasmids and determining the sizes of linearized plasmid DNA fragments. It overcomes the limitations of traditional methods such as agarose gel electrophoresis and ion exchange chromatography (IEX) by providing fast, automated and high-resolution separation of topological isoforms and accurate sizing analysis of linearized plasmids up to 20 kb on the BioPhase 8800 system. Excellent assay repeatability and reproducibility were achieved with relative standard deviations (RSDs) of <1% for migration time (MT) and corrected peak area percentages (CPA%) of the SC isoform for multiple plasmids in multiple injections.

Key plasmid and linear DNA analyses features of the DNA 20 kb Plasmid and Linear kit

- **High-resolution separation of plasmid topological isoforms and impurities across a wide size range (2.7-18.9 kb):** enables thorough characterization of plasmid isoforms and efficient analysis of plasmid purity and stability
- **Excellent assay repeatability and reproducibility:** achieves RSDs of <1% for MT and CPA% of the SC isoform for 5 plasmids in 12 injections (Figure 1)
- **Fast time to answer:** analyzes plasmid isoforms in 2 minutes per sample and 3.4 hours for a full plate of 96 samples on the BioPhase 8800 system
- **High-resolution analysis of linear DNA fragments over an extended size range (0.1-20 kb):** provides size determination of linearized plasmid with accuracy within 2% and 10% for the 7.9 kb and 18.9 kb plasmid, respectively
- **Ready-to-use kit together with a pre-assembled bare-fused silica (BFS) cartridge for topology and linear sizing analysis:** streamlines the operations, ensuring consistent results and saving cost and time.

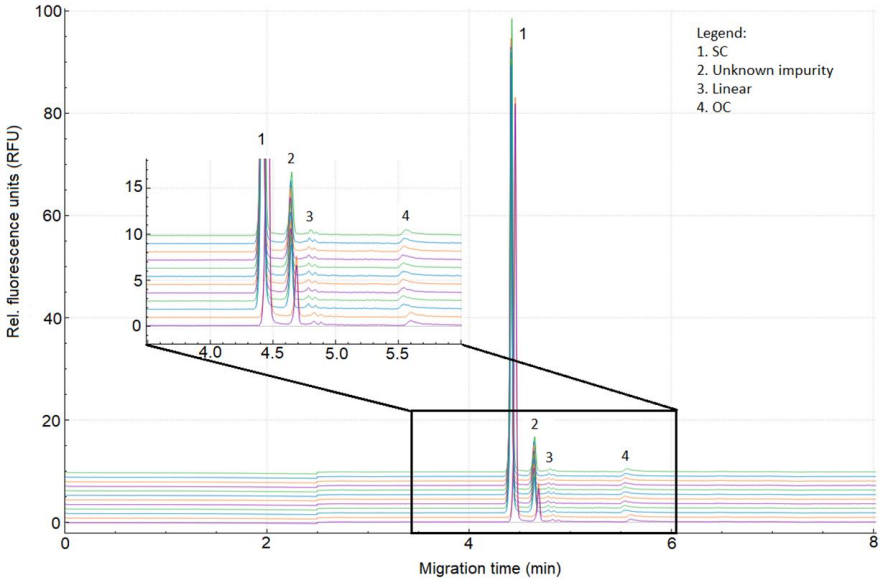

Figure 1. Excellent assay repeatability in analyzing topological isoforms of a 7.9 kb plasmid. The plasmid sample was injected from the same well for 12 consecutive injections and analyzed on the BioPhase 8800 system with a BioPhase BFS capillary cartridge - 8 x 30 cm using the DNA 20 kb Plasmid and Linear kit.

Introduction

Plasmids are small, extrachromosomal circular DNA molecules capable of replicating autonomously, separate from nuclear DNA and independent of cell division. They have become a critical starting material for manufacturing nucleic acid therapeutics.

Plasmid DNA is the backbone of viral vector development in cell and gene therapy and serves as a template for generating messenger RNA (mRNA) vaccines. The global demand for plasmid DNA has surged in recent years, driven by advances in cell and gene therapy and nucleic acid vaccines. As a result, demand has also increased for analytical tools for characterization and purity testing of plasmid samples.

Traditional agarose gel electrophoresis has limitations in resolution and automated quantitation. The IEX method faces challenges separating topological isoforms of large-sized plasmids and managing carry-over.[1] Currently, multiple capillary electrophoresis (CE)-based separation matrices are needed for automated analysis of plasmid topological isoforms and sizing analysis of linearized plasmid fragments. A more streamlined solution is needed.

This technical note highlights a solution for the rapid, automated, high-resolution separation of plasmid topological isoforms for characterization and purity analysis. It also showcases accurate sizing analysis of linearized plasmid fragments using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system. This innovative kit is effective for analyzing plasmids and linear DNA commonly used in nucleic acid therapeutics, establishing it as a valuable analytical tool in developing advanced cell and gene therapeutics and nucleic acid vaccines.

Methods

Materials: The DNA 20 kb Plasmid and Linear kit (P/N: 5311708) was from SCIEX (Framingham, MA) and contained DNA 20 kb Plasmid and Linear gel, DNA 20 kb Plasmid and Linear sample buffer, DNA 20 kb Plasmid test mix, SYBR™ Gold Nucleic Acid gel stain*, DNA 20 kb Plasmid and Linear conditioning solution, Acid wash/regenerating solution, and CE Grade water. The BioPhase BFS capillary cartridge - 8 x 30 cm (P/N: 5080121), the BioPhase BFS capillary cartridge - 8 x 50 cm (P/N: 5080123) and BioPhase sample and reagent plates (4,4,8) (P/N: 5080311) were from SCIEX. Rainin LTS filter tips were from Mettler Toledo (Oakland, CA). Nuclease-free water (NFW) (P/N: AM9932) and 1 Kb Plus DNA Ladder (P/N: 10787018) were obtained from Thermo Fisher Scientific (Waltham, MA).

The 2.7 kb pUC19 Vector plasmid DNA (P/N: N3041S) at 1,000 µg/mL was from New England Biolabs (NEB) (Boston, MA). The 4.4 kb pBR322 plasmid DNA (P/N: SD0041) at 0.5 µg/µL and the 7.9 kb plasmid pCMV·SPORT-βgal (P/N: 10586014) was from Thermo Fisher Scientific. The 5.8 kb gWiz-GFP plasmid DNA (P/N: 5006) and the 18.9 kb pALD-X80 AAV Helper plasmid (P/N: 5017-10) were from Aldevron (South Fargo, ND). The BamHI-HF (P/N: R3136S) and NotI-HF (P/N: R3189S) restriction enzyme kits, each containing its corresponding 10x reaction buffer, were from NEB.

Preparation of the gel buffer: The gel buffer was prepared by diluting the SYBR™ Gold Nucleic Acid gel stain 250-fold in the DNA 20 kb Plasmid and Linear gel (the separation gel). To analyze a full plate of 96 samples, 20 mL of the gel was removed from the bottle pre-warmed to room temperature and transferred to a 50 mL conical tube. Then, 80 µL of SYBR™ Gold Nucleic Acid gel stain (pre-thawed) was added. The tube was capped tightly and gently inverted 20 times to mix well, avoiding air bubbles. Finally, the tube was wrapped in aluminum foil to prevent photobleaching before use.

Sample preparation for analysis using the DNA 20 kb Plasmid and Linear kit: The plasmid DNA sample was thawed on ice for about 20 minutes and diluted in the DNA 20 kb Plasmid and Linear sample buffer to a final concentration of 1 ng/µL. The diluted plasmid sample was then transferred at 100 µL per well to the sample plate for analysis on the BioPhase 8800 system.

Restriction enzyme digestion of plasmids: For digestion of the 7.9 kb plasmid pCMV·SPORT-βgal, a 50 µL reaction was set up by adding 42 µL of NFW, 5 µL 10x reaction buffer, 2 µL of pCMV·SPORT-βgal and 1 µL of BamHI-HF. The reaction components were thoroughly mixed by pipetting up and down, followed by a quick spin. After incubation at 37°C for 1 hour, the reaction mixture was cooled to room temperature for about 5 minutes and stored at -20°C. Right before analysis on the BioPhase 8800 system, the sample was diluted in the DNA 20 kb Plasmid and Linear sample buffer to a final concentration of 0.1 ng/µL. For digestion of the pALD-X80 AAV Helper plasmid with

NotI-HF, a 50 µL reaction was set up by adding 43 µL of NFW, 5 µL 10x reaction buffer, 1 µL of pALD-X80 AAV Helper plasmid and 1 µL of NotI-HF. After mixing and a quick spin, the reaction mixture was incubated at 37°C for 1.5 hours. The NotI-HF was heat-inactivated by incubation at 65°C for 20 minutes, then cooled to room temperature for 10 minutes. The digested pALD-X80 AAV Helper plasmid sample was stored at -20°C and diluted to 0.1 ng/µL before analysis on the BioPhase 8800 system.

Instrument and software: The BioPhase 8800 system with UV/LIF (P/N: 5089278)—equipped with laser-induced fluorescence (LIF) detection utilizing an excitation wavelength of 488 nm and an emission wavelength of 520 nm—was from SCIEX. Data acquisition and analysis were performed using BioPhase software 1.2 from SCIEX.

Instrument setup: Buffer plates and sample plates were prepared based on the experimental design and plate map generated by the BioPhase software, as described in the DNA 20 kb Plasmid and Linear kit application guide.[2] The methods used on the BioPhase 8800 system were provided in the same application guide.

Data processing: Results were analyzed using BioPhase software. The Optimizer feature on the Integration tab was enabled to automatically select a best-fit analysis based on a minimum signal-to-noise (S/N) ratio of 10. Minor adjustments were made for peak integration.

Results and discussion

High-resolution separation of plasmid topological isoforms across a wide size range (2.7–18.9 kb): Plasmid DNA can exist in various topological isoforms, including SC, open circular (OC), linear and concatenated/multimer arrangements. DNA degradation can occur during plasmid extraction, downstream purification and storage, causing the SC isoform to convert to the OC and linear isoforms. The SC isoform has significantly higher transfection efficiency than the OC, while the linear isoform has the lowest transfection efficiency. Regulatory agencies recommend manufacturers establish a specification of >80% SC content to bulk-release plasmid DNA vaccines.[3,4] Therefore, separating SC from other isoforms and accurately quantifying SC is essential for assessing product quality, monitoring stability, and ensuring consistency between batches. Figure 2 shows an overlay of 5 electropherograms obtained with 5 different plasmid samples. The SC and OC isoforms were well-resolved for each plasmid sample within 6 minutes of separation time. In addition, the linear isoform and some unknown impurities were detected, indicating this method is sensitive enough to detect potential plasmid degradants. The percentage of SC (SC%) was calculated as CPA% using BioPhase software. The SC% for pUC19 (2.7 kb), pBR322 (4.4 kb), pCMV·SPORT-βgal (7.9 kb) and pALD-X80 (18.9 kb) was 94.88%, 96.63%, 88.48% and 93.83%, respectively, indicating high plasmid quality. The SC% for gWiz-GFP (5.8 kb) was 82.53%, with OC% at 12.47%, indicating lower quality and consistent with sample information from the vendor. These results demonstrate that topological analysis using the DNA 20 kb Plasmid and Linear kit can accurately evaluate samples with varying quality levels across a wide size range (2.7–18.9 kb) for purity and potential degradation.

Determination of the optimal plasmid concentration for topological isoform analysis: The plasmid concentration needs to be optimized to achieve optimal results for topological analysis. A simple titration experiment achieves this optimization. For example, the gWiz-GFP (5.8 kb) plasmid was serially diluted from 2.50 ng/µL to 0.13 ng/µL with the DNA 20 kb Plasmid and Linear sample buffer. Samples at all dilution points were separated using the DNA 20 kb Plasmid and Linear gel on the BioPhase 8800 system. Results were analyzed using the BioPhase software with the minimum S/N set at 10. Figure 3A shows the overlay of the electropherograms obtained at different plasmid concentrations. The signal level for all peaks grew with increasing plasmid concentration while the peak profile remained consistent. The CPAs of the unknown impurity peak and OC isoform were plotted against the plasmid concentration in ng/µL (Figure 3B). The plot shows that the CPA of the unknown impurity has a good linear relationship with the plasmid concentration in the range of 0.13–2.50 ng/µL with an R^2 value of 0.9974 (blue dotted line). In the same plasmid concentration range, the R^2 value for the OC isoform was 0.9378, indicating a suboptimal linear relationship between CPA and the plasmid concentration (orange dotted line). However, in the plasmid concentration range of 0.13–1.00 ng/µL, the R^2 value for the OC isoform is 0.9919 (shown as the solid orange line), indicating a good linear relationship. Therefore, the plasmid concentration range of 0.13–1.00 ng/µL was determined as a good concentration range for this plasmid sample, with the 1.00 ng/µL concentration as the optimal

concentration due to its strong signal. The same titration experiment was performed for 4 other plasmids in this technical note. For all 5 plasmids, the 1.00 ng/µL concentration was determined as the optimal concentration and was used for further analyses.

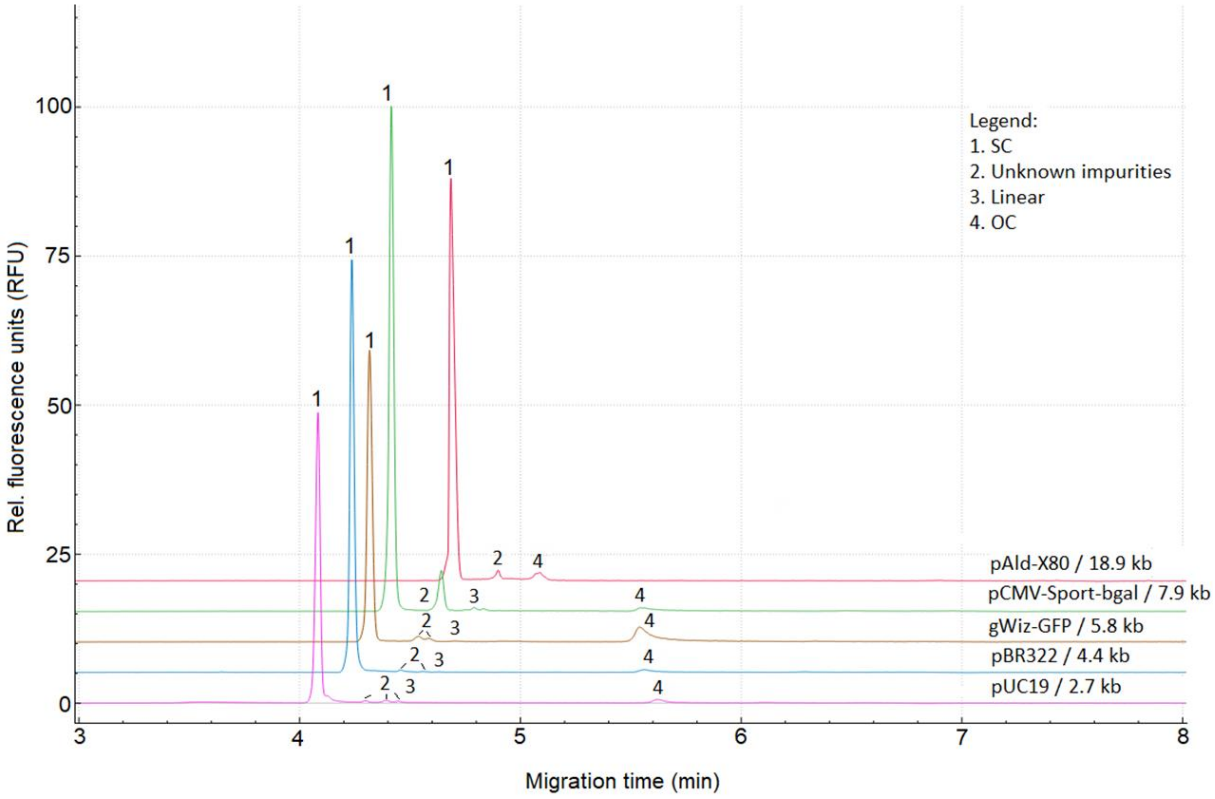

Figure 2. High-resolution separation of topological isoforms in plasmids across a wide size range (2.7–18.9 kb). Five plasmid samples were analyzed simultaneously on the BioPhase 8800 system using the DNA 20 kb Plasmid and Linear kit. Plasmid names and their sizes are labeled above each trace. Details for each peak are provided in the legend above the overlay traces.

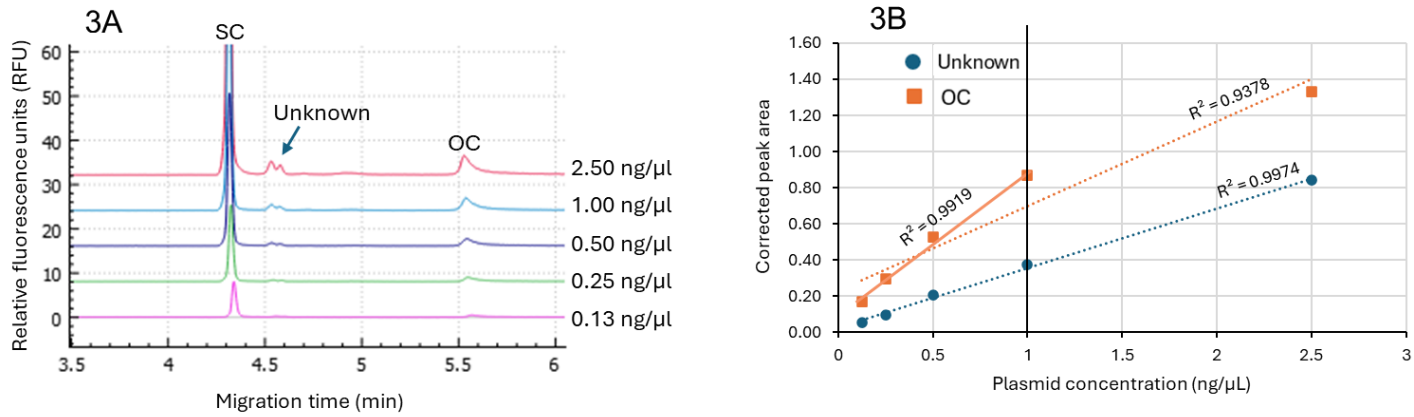

Figure 3. Determination of the optimal plasmid concentration for topological isoform analysis on the BioPhase 8800 system. The gWiz-GFP (5.8 kb) plasmid was serially diluted from 2.50 ng/µL to 0.13 ng/µL and analyzed on the BioPhase 8800 system. Panel 3A displays a zoomed-in view of the overlay of electropherograms obtained at each plasmid concentration. Panel B shows the plots of CPAs of the OC isoform (orange dotted line) and the unknown impurity peak (blue dotted line) against the plasmid concentrations from 0.13 ng/µL to 2.50 ng/µL. The solid orange line illustrates the linear plot of CPAs of the OC isoform against the plasmid concentrations from 0.13 ng/µL to 1.00 ng/µL. The black vertical line indicates that 1.00 ng/µL was the optimal plasmid concentration.

Table 1. Assay repeatability with 5 plasmids. The average CPA% for SC and OC isoforms and other impurities from all 12 injections of each plasmid, along with RSD% values for average MT and CPA% for the SC isoform, are listed.

Plasmid (Size)	SC				OC	Other impurities
	MT(min)	RSD% of MT	CPA%	RSD% of CPA%	CPA%	CPA%
PUC 19 (2.7 kb)	4.09	0.21	94.92	0.13	2.05	3.03
PBR322 (4.4 kb)	4.23	0.20	96.44	0.13	1.37	2.19
gWiz-GFP (5.8 kb)	4.32	0.19	83.05	0.65	12.47	4.48
pCMV-Sport-bgal (7.9 kb)	4.41	0.15	88.68	0.37	1.80	9.52
pAld-X80 (18.9 kb)	4.68	0.11	93.48	0.40	3.72	2.80

Table 2: Assay reproducibility: Each of the 5 plasmids was injected 24 times. The results obtained with the first and 24th injections for each of the 5 plasmid samples are summarized in this table to highlight the consistency of the MT of the SC isoform, CPA% of SC and OC isoforms, and other impurities between the two injections.

Plasmid (Size)	Injection #	SC				OC	Other impurities
		MT (min)	ΔMT%	CPA%	ΔCPA%	CPA%	CPA%
pUC 19 (2.7 kb)	Inj #1	4.14	1.21	94.41	1.25	2.30	3.29
	Inj #24	4.19		95.59		1.86	2.55
pBR322 (4.4 kb)	Inj #1	4.28	1.17	96.39	0.09	1.39	2.22
	Inj #24	4.33		96.48		1.25	2.27
gWiz-GFP (5.8 kb)	Inj #1	4.37	1.72	84.24	1.58	12.46	3.30
	Inj #24	4.45		85.57		10.95	3.48
pCMV-Sport-bgal (7.9 kb)	Inj #1	4.46	1.07	87.81	0.57	2.62	9.57
	Inj #24	4.51		88.31		2.09	9.60
pAld-X80 (18.9 kb)	Inj #1	4.74	2.42	94.54	0.05	2.96	2.50
	Inj #24	4.85		94.59		2.90	2.51

Excellent assay repeatability and reproducibility: Plasmids with sizes of around 7 kb are commonly used as raw materials for viral vector development in cell and gene therapy. Figure 1 shows the overlay of electropherograms obtained with 12 consecutive injections of a 7.9 kb plasmid from the same sample well. The inset shows a zoomed-in view of the overlay. The peak profiles are consistent across the 12 injections. The same experiment was carried out with 4 other plasmids with sizes of 2.7–18.9 kb. As shown in Table 1, the average MTs of the SC correlated well with the sizes of the plasmids, with longer MTs for bigger plasmid sizes. The SC% for pUC19 (2.7 kb), pBR322 (4.4 kb), gWiz-GFP (5.8 kb), pCMV·SPORT-βgal (7.9 kb) and pALD-X80 (18.9 kb) was 94.92%, 96.44%, 83.05%, 88.68% and 93.48%, respectively, indicating the ability of the assay to detect different purity levels in different plasmid samples with different sizes. RSDs for MT and CPA% for the SC isoform were better than 0.25% and 0.70% for all 5 plasmids in 12 injections, demonstrating excellent assay repeatability.

Another analyst conducted a separate experiment on a different day using a different BioPhase 8800 system with a full plate of plasmid samples. Each plasmid sample was added to 12 wells of the same row in a sample plate. In every run, 8 plasmid samples in 1 column were analyzed simultaneously within 17 minutes. After 1 injection per well was done for all 12 columns, the injection was repeated for each column for the second round of analysis. Only 1 set of buffer plates was required to

run 24 injections of 8 samples (192 total injections), enabling the analysis of a full plate of 96 samples with duplicate injections. Table 2 summarizes the results obtained with the 1st and 24th injections for each of the 5 plasmid samples, highlighting the consistency of the MT and CPA% of each isoform between the 2 injections. Notably, the MT and CPA% differences between the 1st and 24th injections were within 2.50% for the SC isoform for all 5 plasmids, indicating excellent assay repeatability. The MTs and CPA% values for different isoforms for 5 plasmids in this experiment were consistent with the ones obtained by the first analyst (Table 1), demonstrating high assay reproducibility in plasmid topology and purity analysis using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system. These results demonstrate that duplicate injections of a full plate of 96 samples can be analyzed in 6.8 hours with good reproducibility and minimal reagents. With the BioPhase 8800 system, the analysis time for topology isoforms was 2 minutes per data sample and 3.4 hours for a full plate of 96 samples, which is faster than the 27 minutes per data sample required by the PA 800 Plus system (data not shown) using a traditional approach with the dsDNA 1000 kit. Plasmid topology analysis on the BioPhase 8800 system is not only faster, but also maintains high resolution between different isoforms.

High-resolution analysis of linear DNA fragments over an extended size range (0.1–20 bp): Sizing analysis of double-stranded DNA (dsDNA) is a critical component in the production of nucleic acid therapeutics. US Food and Drug Administration (FDA) guidelines recommend manufacturers include a gene map featuring relevant restriction sites for gene therapy vector constructs and vector diagrams identifying the gene insert, regulatory regions and pertinent restriction endonuclease sites

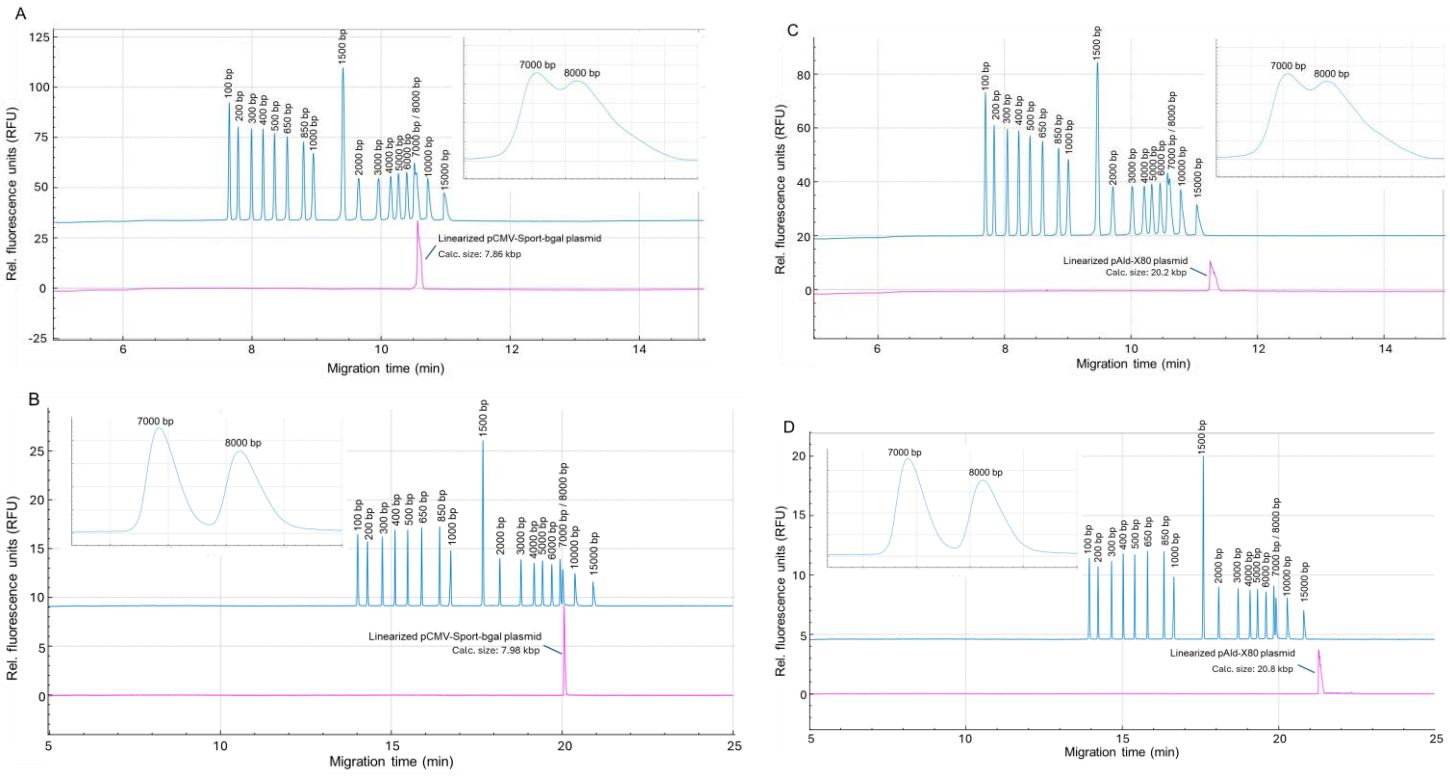

Figure 4. Size determination of the linearized 7.9 kb (A and B) and 18.9 kb (C and D) plasmids using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system. The 1 kb Plus DNA Ladder and the linearized plasmid samples were separated with a BioPhase BFS capillary cartridge - 8 x 30 cm (A and C) or BioPhase BFS capillary cartridge - 8 x 50 cm (B and D). The MT of each size standard fragment in the ladder was plotted against their corresponding sizes in base pairs (bp). The calculated size for the linearized 7.9 kb plasmid was determined as 7.86 kb (A) or 7.98 kb (B). The calculated size for the linearized 18.9 kb plasmid was determined as 20.2 kb (C) or 20.8 kb (D). The inset in each panel shows the resolution between the 7,000 bp and 8,000 bp size standard fragments.

when submitting applications for investigational new drugs (INDs).[5,6] In addition, accurate sizing of restriction fragments from vectors of various sizes often requires measurement over an extended size range. Traditional agarose gel electrophoresis has limitations, such as poor resolution of fragments, restricted size coverage, and issues with sizing accuracy and reproducibility. Figure 4A and Figure 4C demonstrate excellent resolution of the DNA size standard fragments using the BioPhase BFS capillary cartridge - 8 x 30 cm on the BioPhase 8800 system. The DNA size standard fragments in the 1 Kb Plus DNA Ladder cover sizes from 100 bp to 15 kb. A calibration curve was generated by plotting the MTs against the sizes of the standard fragments and used to estimate the size of the linearized 7.9 kb plasmid and the 18.9 kb plasmid. The calculated size for the linearized 7.9 kb plasmid was 7.86 kb based on the calibration curve, within 0.8% deviation of the theoretical size of 7.85 kb. Although the theoretical size for the 18.9 kb plasmid is out of the size range of the 1 Kb Plus DNA Ladder, the calculated size extrapolated from the calibration curve was 20.2 kb, within 9% of the theoretical size of 18.9 kb. When the BioPhase BFS capillary cartridge - 8 x 50 cm was used, the resolution was improved over the BioPhase BFS capillary cartridge - 8 x 30 cm, as demonstrated by the separation of the 7 kb and 8 kb size standard fragments in Figure 4B and Figure 4D. The peak shape for the linearized 7.9 kb and 18.9 kb plasmids also improved with the BFS capillary cartridge - 8 x 50 cm. The calculated sizes determined using the BFS capillary cartridge - 8 x 50 cm for these 2 linearized plasmids were 7.98 kb and 20.8 kb. The accuracy for size determination was within 1.7% and 10.5% for the 7.9 kb and 18.9 kb plasmid, respectively. These results demonstrate high resolution and accurate sizing analysis of linear DNA fragments over an extended size range (0.1–20 kb), indicating a valuable assay for evaluating linearized plasmid DNA samples.

Conclusions

- The ability to perform both plasmid topological isoform analysis and size determination of linear DNA fragments using the same DNA 20 kb Plasmid and Linear kit was demonstrated, providing users with streamlined operations and cost savings compared to using two separate kits
- The DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system successfully separated plasmid topological isoforms ranging from 2.7 kb to 18.9 kb, enabling accurate analysis of purity and stability for plasmids used in cell and gene therapy and nucleic acid vaccines
- High assay repeatability and reproducibility were demonstrated for plasmid isoform analysis with RSDs in MT and CPA% of <1% for 5 plasmids in 12 injections
- Fast plasmid purity analysis was verified with 2 minutes per sample analysis time and 3.4 hours for 96 samples using the DNA 20 kb Plasmid and Linear kit on the BioPhase 8800 system
- Accurate size determination of linear DNA fragments was demonstrated over an extended size range (0.1–20 kb), providing confidence in the verification of plasmid integrity

Reference

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