Technical Overview

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Plasmid Topology Assessment Using the Agilent Fragment Analyzer Systems

Introduction

As the global demand for biotherapeutics surges, driven by the rise of in vitro transcribed (IVT) mRNA and adeno-associated virus (AAV) vector-based therapies, plasmid DNA production has expanded dramatically in recent years. As production scales, the need for high throughput, reliable analytical methods that can support modern biomanufacturing workflows has grown. Among the critical quality attributes (CQAs) evaluated during plasmid release is plasmid topology, including the percentage of DNA that is in the supercoiled isoform, a key indicator of plasmid integrity and suitability for downstream applications.

The Agilent Plasmid Topology kit (p/n 5191-6627) for the Agilent Fragment Analyzer systems enables analysis of supercoiled, linear, and open circular plasmid DNA isoforms. Designed primarily for quality control (QC) and sizing of supercoiled DNA, the kit is validated for use with the 5200 and 5300 Fragment Analyzer systems using the 12- and 48-capillary arrays, respectively. As part of continued product development, an updated vent valve is required for this assay and is available for installation on existing instruments. This new valve maintains performance specifications and is compatible with current and future chemistries.[1]

The Plasmid Topology kit supports a sizing range of 2,000–10,000 bp with recommended sample concentrations of 0.1–1.0 ng/µL (Table 1). Each run takes approximately 45 minutes, and sample volume requirements depend on concentration, with a minimum of 24 µL per sample. This technical overview highlights the sizing and resolution capabilities of the kit, and provides examples of QC analysis of several plasmids, including concentration and percent purity calculations.

Table 1. Specifications of the Agilent Plasmid Topology kit for the Agilent Fragment Analyzer systems*

Analytical Specifications	
DNA sizing range	2,000–10,000 bp
Recommended concentration range	0.1–1.0 ng/µL final concentration DNA in 0.3x pDNA Dilution Buffer
Physical Specifications	
Total electrophoresis run time	33 cm: 45 minutes
Samples per run	12, 48
Sample volume required	Variable, depends on input concentration (24 µL minimum final sample volume required)
Guaranteed shelf life	4 months

**Instrument with updated Vent Valve (p/n M5301A) is required to run this assay.*

Experimental

Commercial plasmid DNA samples were obtained and diluted to 20–50 ng/µL with 1X TE buffer (Agilent, p/n DNF-495-0060). Samples tested include: pUC19 (NEB, p/n N3041S), PhiX174 RF1 (NEB, p/n N3021S), M13mp18 (NEB, p/n N4018S), and Gag-Pol (pSB211-CAG-GagpolIIB, BIOVECTRA). Concentrations were measured using a NanoDrop spectrophotometer and further diluted to 0.1–1 ng/µL with 0.3x Dilution Buffer. Samples were analyzed on the Agilent 5200 and 5300 Fragment Analyzer systems using the Agilent Plasmid Topology kit (p/n 5191-6627), according to the kit guide.[2] Briefly, 24 µL of sample or pDNA Ladder was aliquoted to each well of a sample plate, and a separate marker plate contained 30 µL of pDNA Marker per well. All wells were overlaid with mineral oil and analyzed in multiple replicates. A key procedural note is that the inlet buffer tray should contain 800 µL of 1x pDNA Inlet Buffer.

Each plasmid was digested following the manufacturer's instructions to generate linear and open circular isoforms for resolution testing. For each reaction, 2 µL of enzyme and 10 µL of buffer were combined with plasmid DNA and brought to a final volume of 100 µL with nuclease free water. pUC19, PhiX174 RF1, and Gag-Pol digests used 2 µL of plasmid DNA, while the M13mp18 digest used 20 µL. Linear isoforms were generated using EcoRI HF (NEB p/n R3101S) for pUC19, M13mp18, and Gag-Pol at 37 °C for 60 minutes, or SacII (NEB p/n R0157S) for PhiX174 RF1 at 37 °C for 12 hours. Open circular isoforms were generated using Nt.BspQI (NEB p/n R0644S) for pUC19, PhiX174 RF1, and Gag-Pol at 50 °C for 1 hour, or Nb.BtsI (NEB p/n R0707S) for M13mp18 at 37 °C for 1 hour. Linear digests were heat inactivated at 65 °C for 20 minutes, and open circular digests were inactivated at 80 °C for 20 minutes.

Results and discussion

Resolution of plasmid isoforms

An important aspect of plasmid QC is the ability to resolve and detect supercoiled, linear, and open circular forms of the plasmid. To evaluate the ability of the Fragment Analyzer and the Plasmid Topology kit to resolve the different isoforms, multiple plasmids were enzymatically digested to produce mixtures of supercoiled, linear, and open circular DNA. Analysis of four plasmid samples across the sizing range of the kit each demonstrated baseline separation between all three topologies, as shown in the electropherogram overlay in Figure 1.

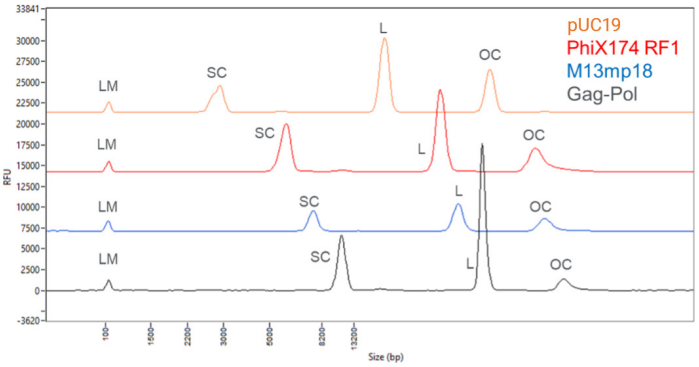


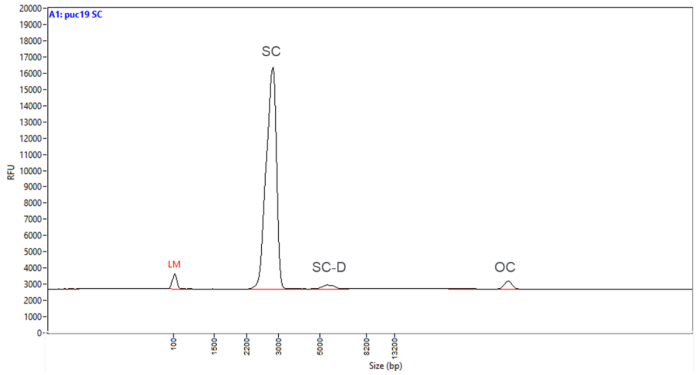
Figure 1. The Agilent Plasmid Topology kit for the Agilent Fragment Analyzer systems resolves all three isoforms of digested plasmid DNA samples of varying sizes, as demonstrated in the electropherogram overlay of pUC19, PhiX174 RF1, M13mp18, and Gag-Pol. Fragment order (from left): lower marker (LM), supercoiled (SC), linear (L), open circular (OC) forms.

Supercoiled plasmid sizing

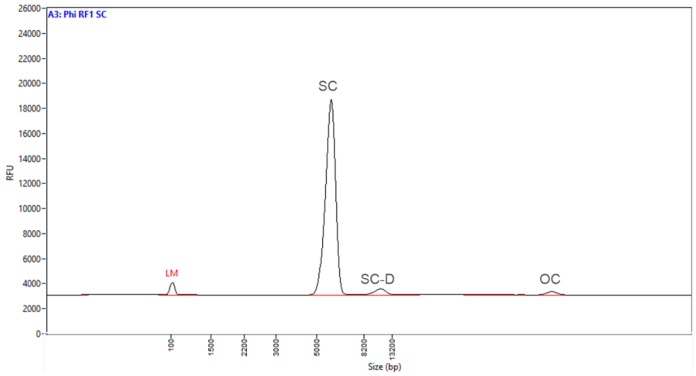
The Plasmid Topology kit can be used to size the supercoiled monomer for plasmids across the assay range. To demonstrate the functionality of the system, the sizing accuracy and precision of the supercoiled isoforms of four different DNA plasmids spanning the sizing range of the kit from 2,000 to10,000 bp were analyzed across multiple replicates and multiple instruments. Results from the Fragment Analyzer are automatically generated by the Agilent ProSize data analysis software following electrophoresis. Representative electropherogram traces and digital gel images are shown in Figure 2.

Across multiple replicates and instruments, the average measured sizes closely matched expected values for all four plasmids (Figure 3A). The pUC19 supercoiled plasmid has an expected size of 2,686 bp and showed an average size of 2,797 bp with the Fragment Analyzer (Figure 2A). PhiX174 RF1, with a known size of 5,386 bp, had an average size of 5,583 bp (Figure 2B). The expected size of M13mp18 is 7,249 bp, and displayed an average size of 7,433 bp (Figure 2C). Gag-Pol has an expected size of 9,679 bp, and had an average size of 10,079 bp (Figure 2D). Each sample showed excellent sizing accuracy, as demonstrated by the correlation between the measured and expected sizes (Figure 3A), less than 5% error (Figure 3B) and high reproducibility, with precision of 5 %CV or less (Figure 3C).

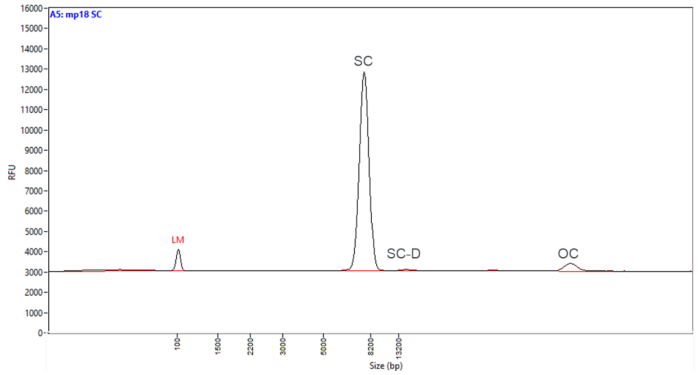
A) pUC19



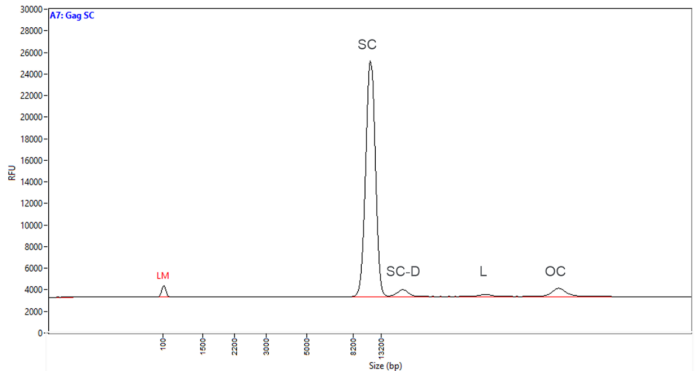
B) PhiX174 RF1



C) M13mp18



D) Gag-Pol



E) Digital gel image (SC only)

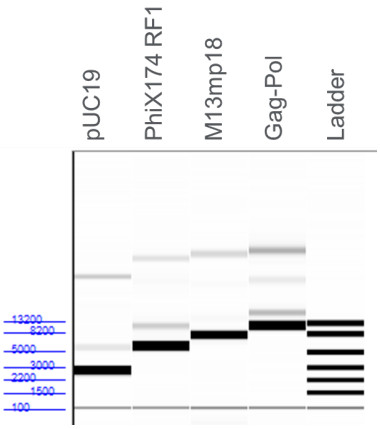
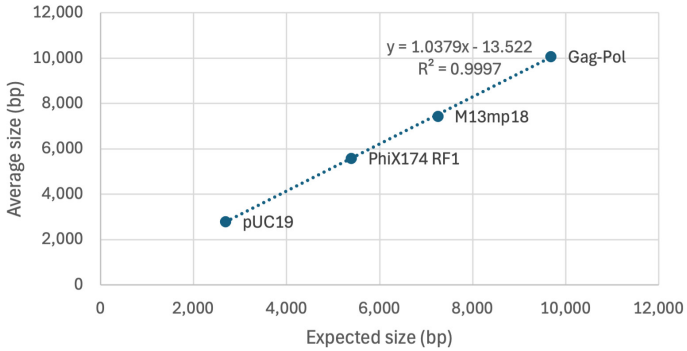
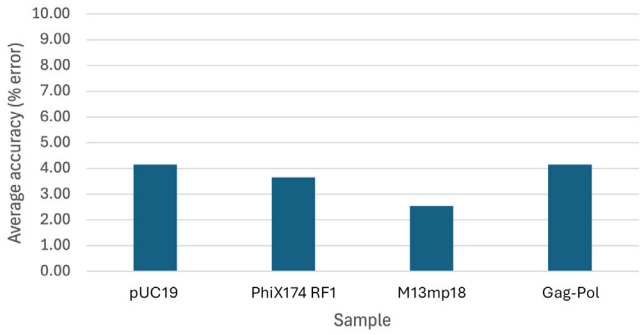


Figure 2. Plasmid DNA samples were analyzed on the Agilent Fragment Analyzer systems with the Agilent Plasmid Topology kit across the sizing range of the assay, from 2,000 to 10,000 bp. Shown are electropherogram images of (A) pUC19, (B) PhiX174 RF1, (C) M13mp18, (D) Gag-Pol, and (E) a digital gel image of each sample with the ladder. The primary peak or band shown is the supercoiled form, while the smaller peaks and bands indicate the presence of low levels of supercoiled dimers, linear, and open circular isoforms, respectively.

A) Supercoiled plasmid DNA sizing linearity



B) Supercoiled plasmid DNA sizing accuracy



C) Supercoiled plasmid DNA sizing precision

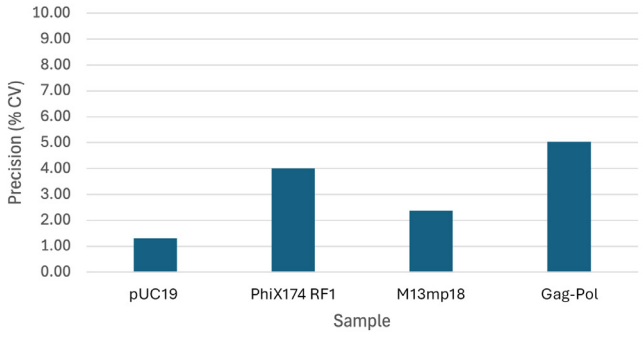


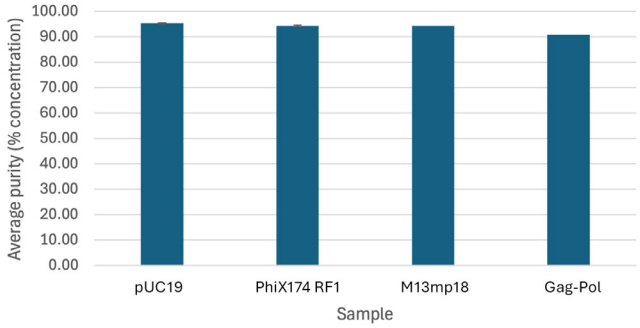
Figure 3. Plasmid DNA samples were analyzed on the Agilent Fragment Analyzer systems with the Agilent Plasmid Topology kit across the sizing range of the assay, from 2,000 to 10,000 bp. The sizing (A) linearity, (B) accuracy, and (C) precision of the supercoiled isoform are shown for each of the four samples tested. N=23

Purity assessment

Assessment of plasmid purity is critical for QC workflows. The high resolution provided by the Plasmid Topology kit enables analysis of the supercoiled isoform in relation to the total sample. The ProSize data analysis software automatically integrates each peak in the sample to determine percent purity of the supercoiled monomer relative to total DNA.

The four plasmid DNA samples were analyzed across multiple replicates with the Plasmid Topology kit (Figure 2A–D). The electropherogram images of pUC19 (Figure 2A), PhiX174 RF1 (Figure 2B), M13mp18 (Figure 2C) each show a primary peak representative of the supercoiled monomer. Analysis with the Plasmid Topology kit also enabled the detection of two smaller peaks, indicative of a supercoiled dimer and the open circular isoform. Gag-Pol also showed a small amount of linear DNA (Figure 2D). The purity of each sample was determined by the percent total of the supercoiled DNA peak. Each of the samples showed high percent purity that was consistent between runs and across multiple Fragment Analyzer systems. pUC19 had an average purity of 90.9%, PhiX174 RF1 94.4%, M13mp18 94.3%, and Gag-Pol 90.9% (Figure 4A). Notably, the precision of the purity measurements for each sample was less than 0.35%, indicating excellent reproducibility between runs and across multiple instruments (Figure 4B).

A) Supercoiled plasmid DNA purity



B) Supercoiled plasmid DNA purity precision

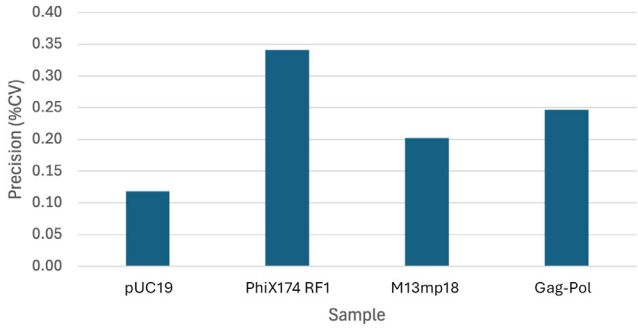


Figure 4. Four plasmid DNA samples were assessed on the Agilent Fragment Analyzer systems with the Agilent Plasmid Topology kit. The purity of the supercoiled isoform was determined using the percent total concentration reported by the Agilent ProSize data analysis software. (A) Average purity and (B) precision from multiple runs and instruments are shown (N=15). Error bars represent standard deviation.

Purity across concentration range

The ability to detect and quantify impurities is essential for accurate assessment of plasmid DNA quality in QC workflows. In addition to the primary supercoiled monomer, plasmid preparations can contain low levels of supercoiled dimers, open circular, and linear forms, or other impurities that may impact product performance. The high resolution of the Plasmid Topology kit enables reliable detection of these peaks across the recommended concentration range, allowing quantitative evaluation of minor species while maintaining accurate measurement of the primary supercoiled peak.

The Plasmid Topology kit has a recommended concentration range of 0.1 to 1 ng/µL. To demonstrate the ability of the kit to detect impurities across this range, the PhiX174 RF1 sample was analyzed across multiple runs and instruments at both 0.1 and 1 ng/µL. Supercoiled dimer (SC-D) and open circular (OC) peaks were easily detected at both sample dilutions (Figure 5A–B). Additionally, the percentage of the total concentration for each peak was consistent across both dilutions (Figure 5C). The supercoiled isoform displayed an average purity of 94.5% at both concentrations, with an excellent precision of less than 1%. The supercoiled dimer was on average 3.5%, and the open circular 2% of the total across both concentrations tested. While this sample was able to detect impurities throughout the recommended concentration range, users should determine the best concentration for their samples to ensure that the primary peak is at optimal loading conditions and impurities can be detected.

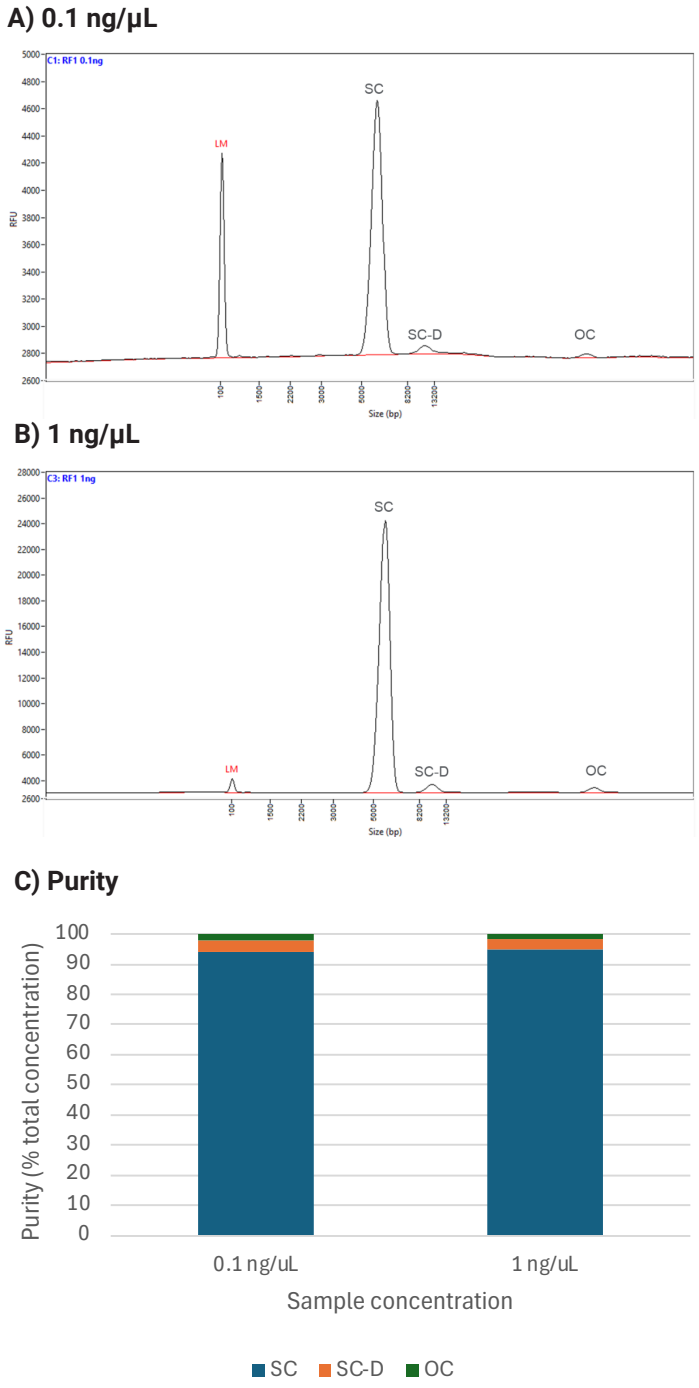
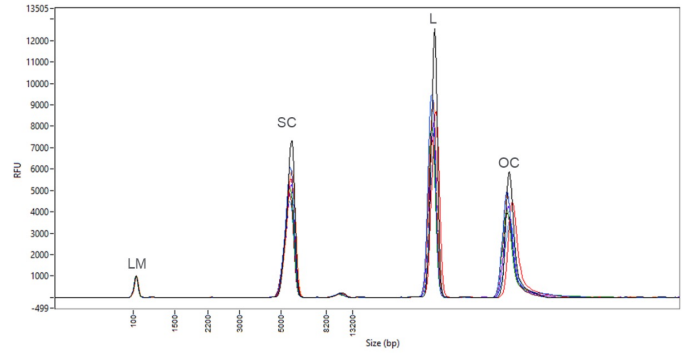


Figure 5. PhiX174 RF1 was analyzed on the Agilent Fragment Analyzer systems with the Agilent Plasmid Topology kit across the concentration range of the kit. The assay easily resolved the supercoiled (SC), supercoiled-dimer (SC-D), and open circular (OC) peaks at both (A) 0.1 and (B) 1 ng/µL. (C) The percent total of each of the peaks was consistent across both sample dilutions (N=15).

Reproducibility

To demonstrate the reliability of the Fragment Analyzer and the Plasmid Topology kit, a 1:1:1 mixture of the PhiX174 RF1 supercoiled, linear, and open circular isoforms was analyzed across multiple runs. An electropherogram overlay from the same capillary over eight successive runs shows the consistent migration time, size, and profile of each of the three isoforms (Figure 6A). The size and purity of the supercoiled isoforms were further assessed to demonstrate the reproducibility of the kit (Figure 6B). The average peak size was 5,471 bp, with a precision of 0.95 %CV, while the average purity was 30.7% with a precision of 0.30 %CV, highlighting the strong reproducibility of the system from run to run.

A)



B)

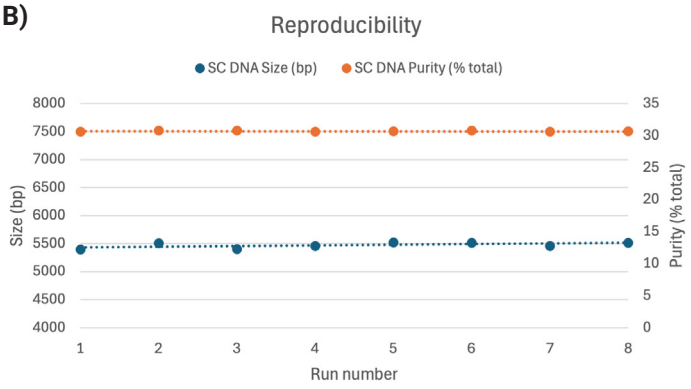


Figure 6. (A) A 1:1:1 mixture of the PhiX174 RF1 supercoiled, linear, and open circular isoforms was analyzed across multiple runs (N=8) on the Agilent Fragment Analyzer systems with the Agilent Plasmid Topology kit. The size and purity of the three isoforms were determined using the Agilent ProSize data analysis software. (B) To demonstrate the reproducibility of the assay, the sizing and purity of the supercoiled fragment over eight subsequent runs are shown.

Conclusions

The Agilent Plasmid Topology Kit provides a robust, accurate, and highly reproducible solution for characterizing plasmid DNA across key QC parameters and CQAs. When paired with the Agilent 5200 and 5300 Fragment Analyzer systems, the kit delivers accurate and precise sizing of the supercoiled form within the 2,000 to 10,000 bp range, reliable purity measurements, detection of minute impurities, and clear resolution of supercoiled, linear, and open circular isoforms. These capabilities, combined with consistent performance across instruments and runs, make the kit a powerful tool for plasmid quality assessment in research, development, and manufacturing workflows.

References

1. Vent Valve Update in Agilent Fragment Analyzer Instruments. *Agilent Technologies technical overview*, publication number 5994-8790EN, **2026**.
2. Agilent Plasmid Topology Kit. *Agilent Technologies kit quick guide*, publication number SD-AT000126, **2026**.

www.agilent.com/genomics/plasmid-dna-kit

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