Technical Overview

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Plasmid Analysis Using the Agilent Fragment Analyzer Systems

Introductions

Plasmids are small, circular DNA molecules commonly found in bacteria that replicate autonomously[1]. In the molecular biology laboratory, plasmids are ideally suited for experimental protocols employing cloning, a process in which a piece of DNA is inserted into the linearized plasmid, which is then circularized and introduced into host bacteria. The plasmid replicates with the bacteria, generating multiple copies of the plasmid and the sequence of interest independent of the bacterial chromosomal DNA. These artificially constructed plasmids can be used to help study DNA sequences or genes. Downstream applications using plasmids include genetic engineering and CRISPR technologies, gene therapy, pharmaceutical development, and recombinant DNA technologies[2].

Plasmids can appear in several conformations, including supercoiled, linear, and open-circular. To help ensure robust experimental results, it is important to begin with high-quality DNA. One of the methods to ensure the quality of plasmids for downstream use is analysis with gel electrophoresis. Each conformation will run differently through a gel. Supercoiled plasmid is the native conformation, composed of an intact double helix that is over- or under-wound. This compact supercoiled plasmid will migrate fastest through a gel matrix. This form is most critical to determining the quality of a plasmid, and so a common analysis is to determine the percentage of the plasmid that is of the supercoiled form. The linear plasmid has been cut at both strands in the same place and is in the relaxed or non-coiled state. It migrates slower than the supercoiled form on a gel. The nicked open-circular form of a plasmid is cut on only one of the two DNA strands, allowing for the formation to relax slightly and causing it to migrate the slowest of the plasmid formations on a gel.

The Agilent Fragment Analyzer system is an automated capillary electrophoresis instrument that can be used for quality control of a range of samples, including plasmids. With the Agilent Plasmid DNA kit, the Fragment Analyzer utilizes an optimized method that allows for accurate detection and sizing of supercoiled plasmids. The Plasmid DNA kit may also be used to detect plasmids in the linear form. When accurate sizing of linearized plasmids is necessary, the qualitative DNA kits for the Fragment Analyzer, such as the Agilent dsDNA 930 Reagent kit, are also available. An advantage of the qualitative DNA kits for the Fragment Analyzer is that the samples are prepared and injected separately from the markers, making it possible to analyze the same sample plate with multiple kits when needed. Due to the shape and large, irregular size of nicked open-circular plasmids, this form cannot be detected with the Plasmid DNA kit.

This technical overview examines the analytical specifications of the Plasmid DNA kit, highlighting the sizing and concentration range of the kit. In addition, several supercoiled plasmids are digested with restriction enzymes to produce the linear form to compare the sizing of the supercoiled DNA using the Plasmid DNA kit to the linear form with the dsDNA 930 kit.

Experimental

Plasmid information

Supercoiled plasmids of various sizes were obtained from GenScript and Origene and analyzed on an Agilent 5200 Fragment Analyzer system (p/n M5310AA) equipped with an Agilent FA 12-Capillary Array Short, 33 cm (p/n A2300-1250-3355). The plasmids were diluted to 0.5 ng/µL with 1x TE and prepared according to the Agilent DNF-940 Plasmid DNA Kit (p/n DNF-940-K0500) manual[3]. The plasmids used are summarized in Table 1.

Serial dilutions

The capabilities of the Plasmid DNA kit were examined through a series of serial dilutions to cover the concentration range of the kit. Each plasmid was diluted to 1 ng/µL with 1x TE, followed by two-fold dilutions down to 0.125 ng/µL. Multiple replicates of each dilution were analyzed with the Plasmid DNA kit.

Plasmid digests

One microgram of the 4.6 kb plasmid was digested with 1 µL *ScaI* (Thermo Fisher Scientific, p/n ER0431) following manufacturer's instructions. 1 µg of the 5.4, 6.2, and 7.6 kb plasmids were digested with 1 µL *BamHI-HF* (New England BioLabs, p/n R3136) following manufacturer's instructions. Aliquots of the supercoiled and the linearized plasmids were diluted to 0.5 ng/µL with 1x TE and analyzed on the Fragment Analyzer with both the Plasmid DNA kit and the Agilent dsDNA 930 Reagent kit (75-20000 bp) (p/n DNF-930)[4].

Table 1. Plasmid sample information.

Plasmid ID	Plasmid Name	Company	p/n	Size (bp)
3.1 kb	pUC57 plasmid with 409 bp insert	GenScript	NA - custom synthesis	3,119
4.5 kb	pGuide-EF1a-GFP	Origene	GE100044	4,564
5.4 kb	pRS shRNA Vector	Origene	TR20003	5,430
6.2 kb	PCMVMIR MicroRNA Expression Vector	Origene	PCMVMIR	6,219
7.6 kb	pGFP-V-RS shRNA Vector	Origene	TR30007	7,584
8.5 kb	pRFP-CB-shLenti shRNA Vector	Origene	TR30032	8,491
10 kb	pCas-Scramble-EF1a-GFP	Origene	GET100021	10,469

Results and discussion

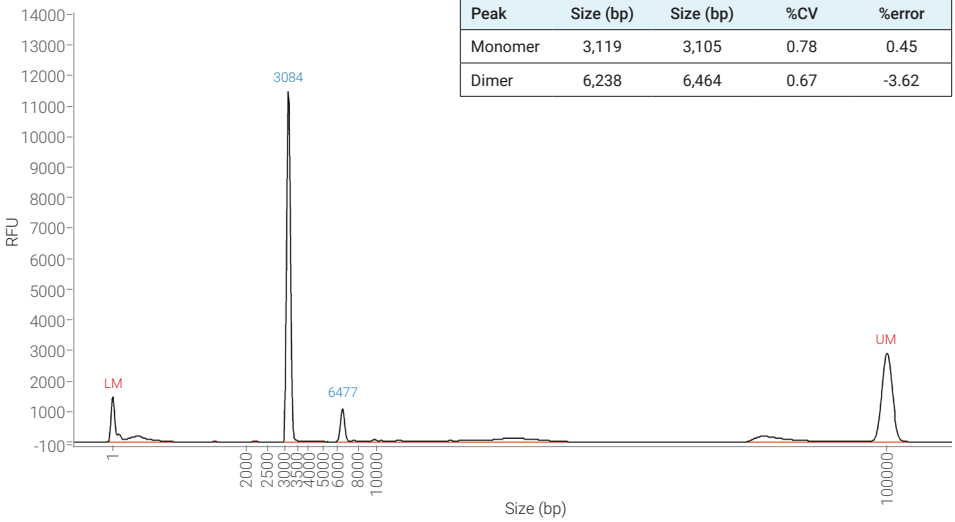
Plasmid analysis

The Plasmid DNA kit was designed for the analysis of supercoiled plasmid DNA. The sizing ladder developed for the Plasmid DNA kit is comprised of supercoiled plasmids, allowing for accurate quality assessment and precise sizing of samples between 2,000 and 10,000 bp. Linear plasmids can also be analyzed for quality assessment (but not for sizing) using the kit. Relative concentration can be achieved for both forms of plasmids.

To demonstrate the capabilities of the Plasmid DNA kit, commercially available plasmids of various sizes ranging from 3 to 10 kb (Table 1) were analyzed on the Fragment Analyzer. The primary purpose of plasmid analysis is to confirm the size of the supercoiled plasmid and to examine the purity of the plasmid. Figure 1A shows a 3.1 kb plasmid, with most of the sample observed as a single sharp peak at 3,084 bp and a shorter fragment to the right of the fragment at 6,477 bp. The size of the main fragment is consistent with the size expected for the supercoiled plasmid, while the secondary fragment is likely a dimer concatemer. Concatemers occur when multiple copies of the monomeric plasmid link together as a result of homologous recombination during bacterial replication[2]. The size of the second peak in this example is approximately double that of the monomer peak, confirming that it is a dimer concatemer. The electropherogram gives a visual representation of the purity of the sample while the data analysis software provides data such as the percent total of the peak, to further aid in determining the purity of a sample. In this example, the 3.1 kb peak represents 90.5% of the total sample.

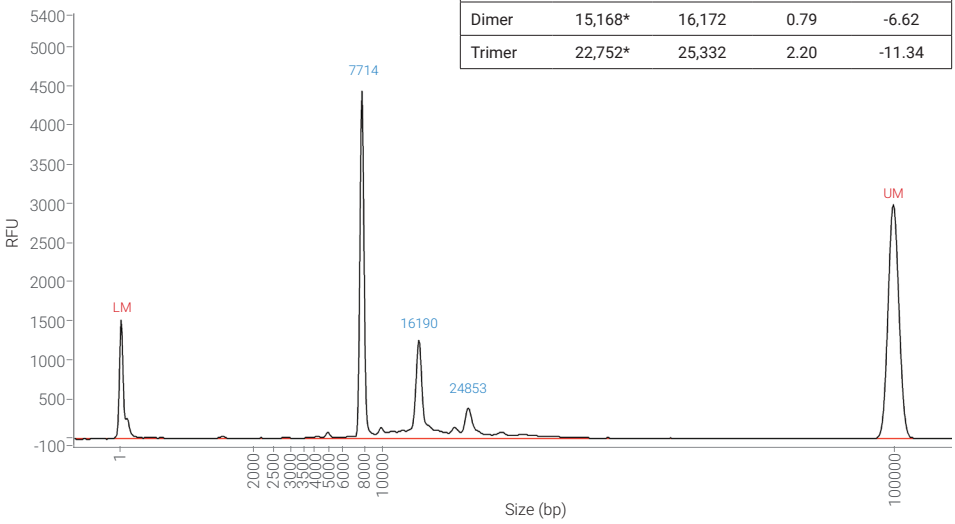
A. 3.1 kb plasmid

Peak	Expected Size (bp)	Average Size (bp)	Precision %CV	Accuracy %error
Monomer	3,119	3,105	0.78	0.45
Dimer	6,238	6,464	0.67	-3.62



B. 7.6 kb plasmid

Peak	Expected Size (bp)	Average Size (bp)	Precision %CV	Accuracy %error
Monomer	7,584	7,720	0.10	-1.80
Dimer	15,168*	16,172	0.79	-6.62
Trimer	22,752*	25,332	2.20	-11.34



C. 10 kb plasmid

Peak	Expected Size (bp)	Average Size (bp)	Precision %CV	Accuracy %error
Monomer	10,469*	10,922	0.93	-4.33
Dimer	20,938*	2,1042	1.93	-0.50
Trimer	31,407*	31,142	0.32	0.84

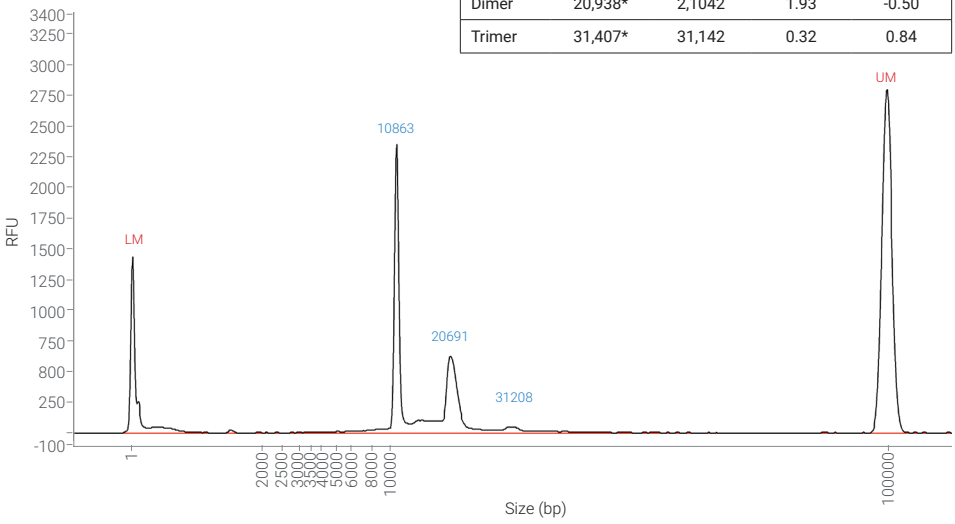


Figure 1. Supercoiled plasmids analyzed on the Agilent Fragment Analyzer system with the Agilent Plasmid DNA kit. A) A 3.1 kb plasmid with a single monomer concatemer. B) A 7.6 kb plasmid with dimer and trimer concatemers. C) A 10 kb plasmid with dimer and trimer concatemers. Inset tables indicate the size of the expected plasmid and the theoretical sizes of the concatemers, as well as the average size of each peak as reported by the Fragment Analyzer (n=4). **outside of kit specifications*

In another example, a 7.6 kb plasmid displayed 3 sharp peaks at 7,714, 16,190, and 24,853 bp (Figure 1B). These sizes are consistent with the presence of the monomer form of the supercoiled plasmid, as well as dimer and trimer concatemers. Similarly, the 10 kb plasmid also displayed two sharp peaks at 10,863 and 20,691 bp, as well as a small smear at 31,208 bp (Figure 1C). While the larger sized peaks in both the 7.6 and 10 kb plasmids are consistent with the presence of dimer and trimer concatemers, it is important to note that these larger fragments are outside of the sizing range of the kit, and the reported sizes may be less accurate. Thus, to examine the sizing accuracy of the Plasmid DNA kit, only the first peak, or monomer, of each of the plasmids was further analyzed. The sizing reported by the Fragment Analyzer was consistent with the expected size of the supercoiled plasmid, with a high accuracy and less than 4.4% error for each plasmid. Additionally, an R^2 of 0.9995 indicated an excellent correlation between the expected plasmid size and that reported by the Fragment Analyzer (Figure 2).

Instrument comparison

The sizing accuracy of the Plasmid DNA kit was compared across two Fragment Analyzer instruments. Seven plasmids were analyzed in duplicate on each instrument, and the average size of the primary plasmid peak (excluding any concatemers) was compared across instruments. The sizing was consistent between each instrument. Each of the plasmids displayed a high accuracy, with less than 4.6% error across both instruments, well within the kit specifications of 10%. In addition, each instrument displayed a %CV of less than 2.7% for each fragment, indicating excellent sizing precision between runs (Figure 3).

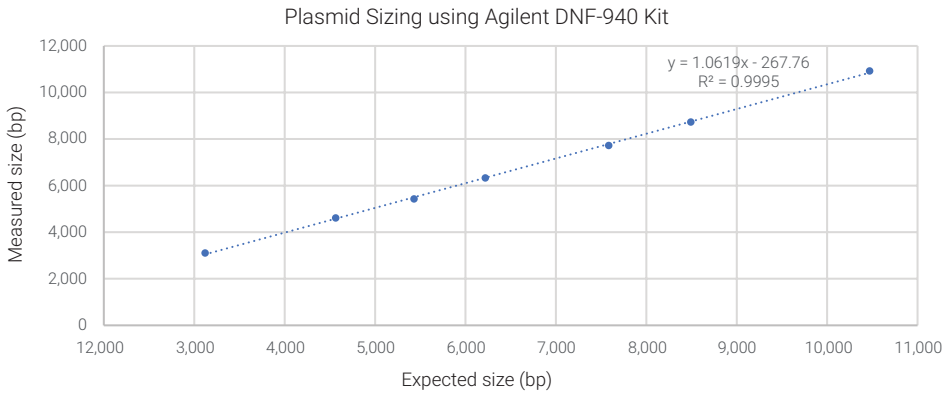
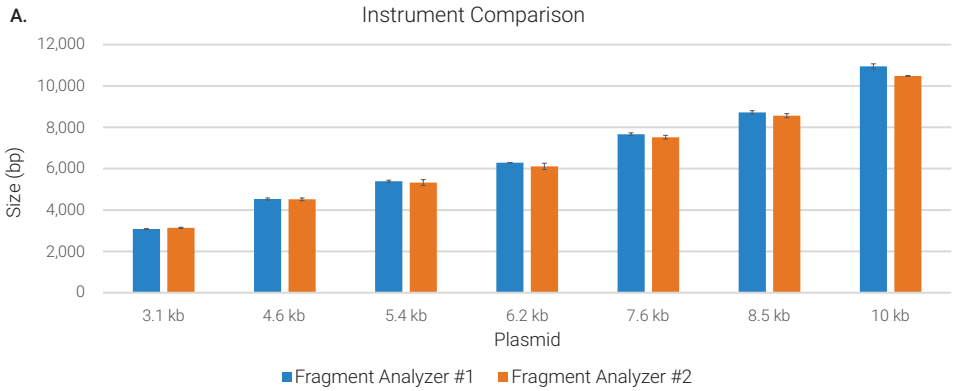


Figure 2. A selection of supercoiled plasmids ranging in size from 3.1 to 10 kb at a concentration of 0.5 ng/µL were analyzed on the Agilent Plasmid DNA kit with the Agilent Fragment Analyzer system. The average size of the primary plasmid peak reported by the Fragment Analyzer was compared to the known size of the plasmid. The measured size correlated well with the expected size of the supercoiled plasmid (n=4).



B.	Average Size (bp)		Precision %CV		Accuracy %error	
Plasmid ID	Fragment Analyzer #1	Fragment Analyzer #2	Fragment Analyzer #1	Fragment Analyzer #2	Fragment Analyzer #1	Fragment Analyzer #2
3.1 kb	3,084	3,137	0.00	0.81	1.12	-0.58
4.6 kb	4,536	4,518	1.12	1.46	0.61	1.02
5.4 kb	5,394	5,331	0.93	2.63	0.67	1.82
6.2 kb	6,287	6,110	0.00	2.52	-1.09	1.75
7.6 kb	7,667	7,520	0.88	1.21	-1.09	0.85
8.5 kb	8,723	8,562	0.90	1.16	-2.73	-0.83
10 kb	10,949	10,482	1.11	0.18	-4.58	-0.12

Figure 3. Various supercoiled plasmids ranging in size from 3.1 to 10 kb at a concentration of 1 ng/µL were analyzed on the Agilent Plasmid DNA kit using two Agilent 5200 Fragment Analyzer instruments.
A) The reported size of each plasmid was consistent between instruments. Error bars indicate standard deviation. B) The size, %CV, and %error for each plasmid are shown (n=2 per instrument).

Concentration range of the Plasmid DNA kit

A two-fold serial dilution of each plasmid was performed to investigate the impact of concentration on sizing. The plasmids were diluted from 1 to 0.125 ng/µL and analyzed on the Fragment Analyzer system with the Plasmid DNA kit. Shown in Figure 4 are overlays of the resulting electropherograms. The primary plasmid peak and any concatemers were visualized across the entire concentration range, highlighting the sensitivity of the Fragment Analyzer to detect samples and impurities at even low concentrations. Furthermore, the size of the primary peak for each of the seven plasmids tested remained consistent throughout the dilution series (Figure 4C).

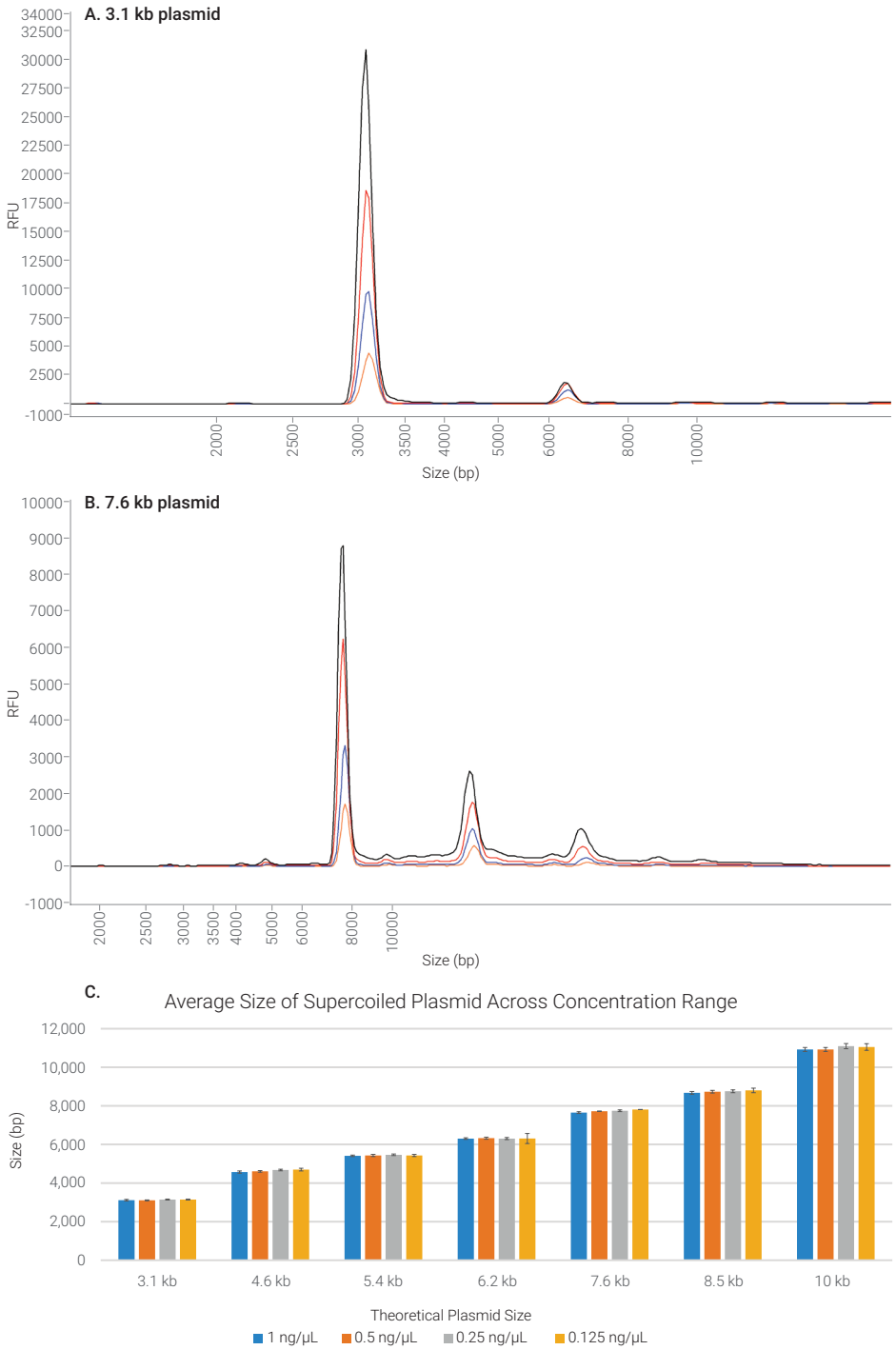


Figure 4. Serial dilutions of each plasmid from 1 to 0.125 ng/µL were analyzed with the Agilent Plasmid DNA kit on the Agilent Fragment Analyzer. Electropherograms are zoomed-in to highlight the main plasmid and any impurities. Shown are examples of A) a 3.1 kb plasmid with a single, small concatemer, and B) a 7.6 kb plasmid with two prominent concatemers. All peaks were observed in each sample across all concentrations tested. C) The size of the primary peak of each plasmid was compared across all concentrations. The average size of the fragment remained consistent across each concentration. n=4 replicates per concentration.

Comparison of supercoiled and linear plasmid sizing

As previously mentioned, the Plasmid DNA kit can be utilized for the analysis of supercoiled and linear DNA, with accurate sizing of only the supercoiled form. Linearized plasmids can be detected and examined for purity using the Plasmid DNA kit. For accurate sizing, linearized plasmids can be analyzed on the Fragment Analyzer using a range of qualitative DNA kits, each with different sizing ranges ideal for analysis of DNA fragments from 35 to 20,000 bp.

To demonstrate the abilities of the Fragment Analyzer to accurately size plasmid DNA, the supercoiled plasmids were analyzed using the Plasmid DNA kit, and then linearized through single restriction digest. The linearized plasmid was then analyzed using the dsDNA 930 Reagent kit. Shown in Figure 5 are examples of the 4.6 kb supercoiled and linear plasmids analyzed on their respective kits. Figure 5A is an overlay of a 4.6 kb supercoiled (black) and linearized (red) plasmid analyzed on the Plasmid DNA kit. While the supercoiled form displays the expected plasmid size of approximately 4.6 kb, the size of the digested linear plasmid is not accurate (Figure 5A). In contrast, the linearized plasmid analyzed with a qualitative DNA kit reports a size of 4.5 kb, and a 0.2% error indicative of high accuracy (n=4) (Figure 5B). The supercoiled plasmid can be detected but does not size accurately on the qualitative DNA kits (data not shown).

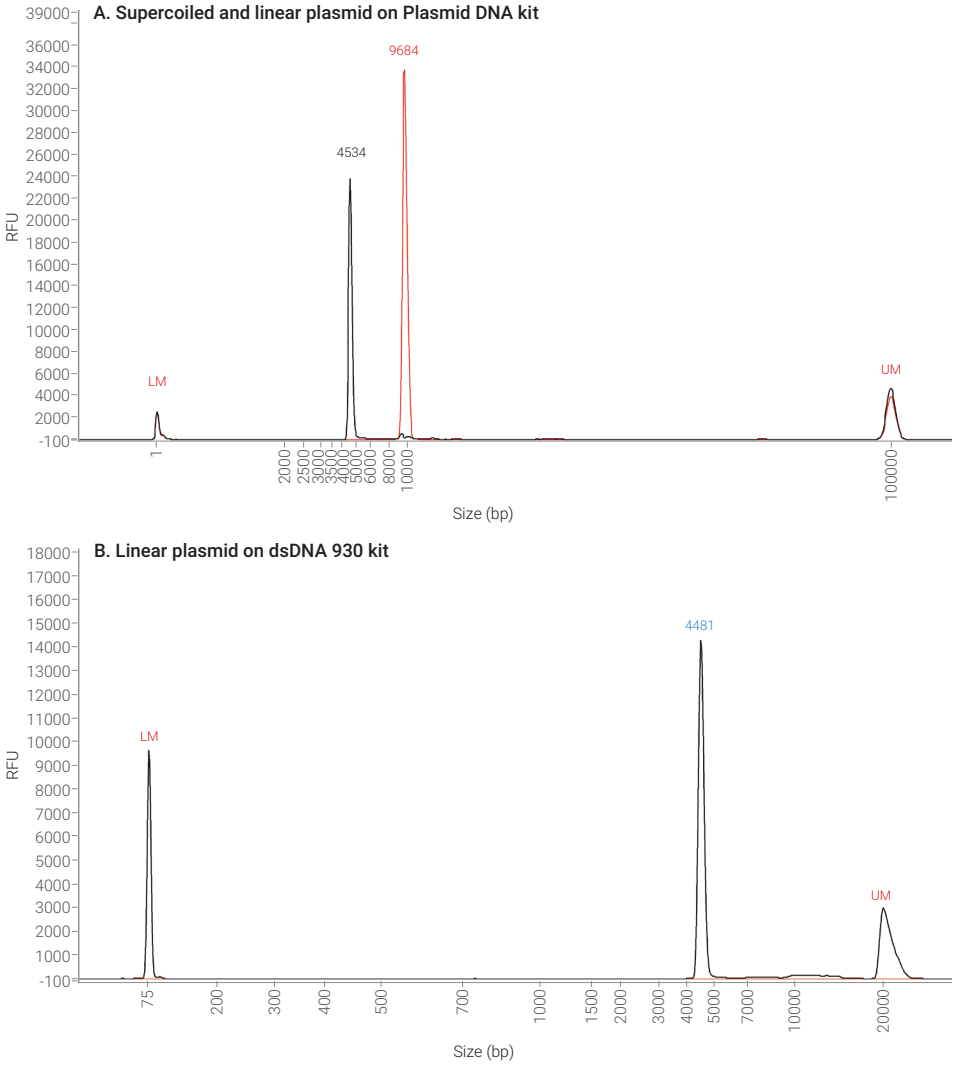


Figure 5. A 4.6 kb plasmid was analyzed on the Agilent Fragment Analyzer. A) The supercoiled form (black trace) can be accurately sized on the Agilent Plasmid DNA kit, while the linearized form (red trace) can be detected, but not sized. B) The digested form the of the plasmid can be accurately sized using a qualitative DNA kit, the Agilent dsDNA 930 Reagent kit.

To provide further evidence that the extra peaks evident in some of the plasmids are concatemers, the 5.4, 6.2, and 7.2 plasmids were linearized using a single restriction digest. The enzyme successfully digested each of the concatemers, resulting in a single peak in the linear form of the plasmid. The linear plasmids were sized with the dsDNA 930 Reagent kit and were consistent with the size of the monomer peak of the supercoiled plasmid. Figure 6 shows the supercoiled (A) and linear (B) forms of the 5.4 kb plasmid.

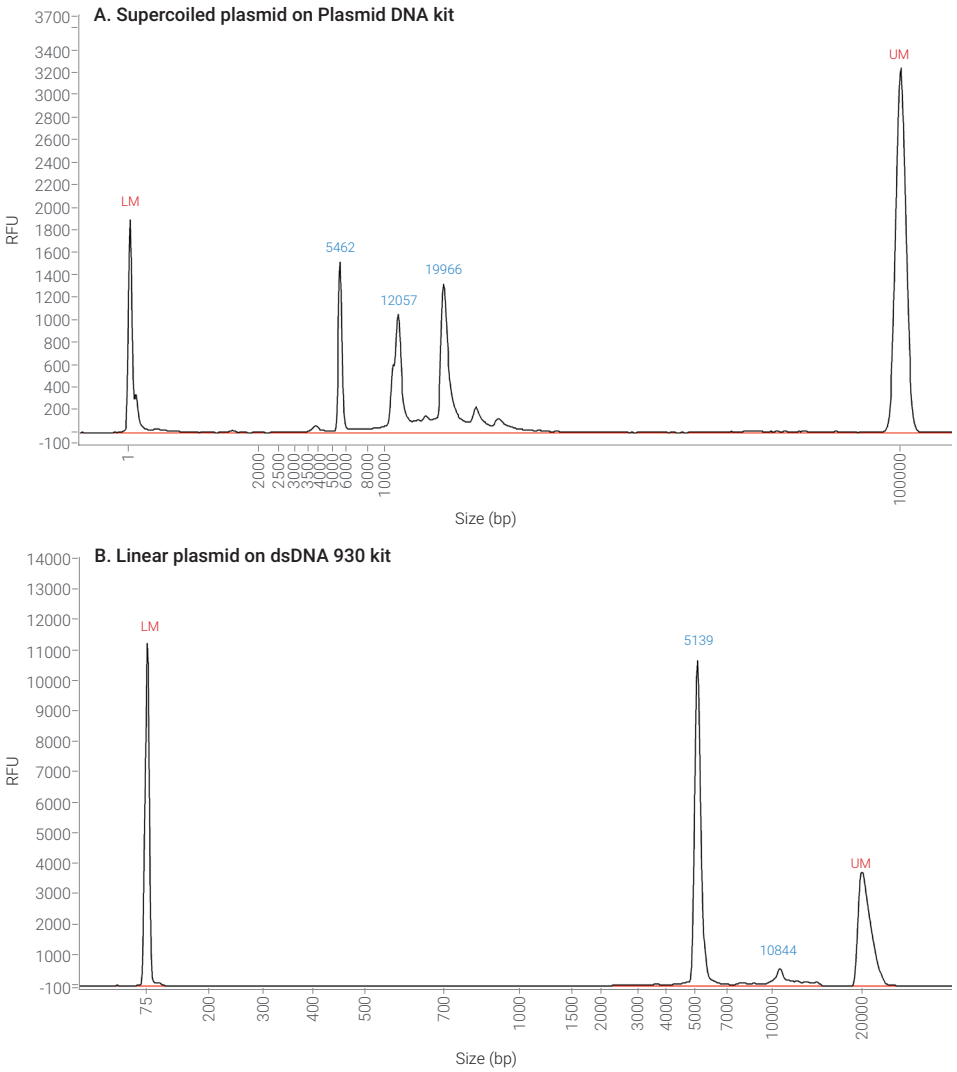


Figure 6. A 5.4 kb plasmid was analyzed on the Agilent Fragment Analyzer. A) The supercoiled form of the plasmid on the Agilent Plasmid DNA kit shows monomer, dimer, and trimer concatemers. B) The plasmid was digested with *BamHI* and the linear form analyzed on the Agilent dsDNA 930 kit.

Conclusion

Automated electrophoresis with the Agilent Fragment Analyzer instrument along with the Agilent Plasmid DNA kit provides a quick and easy solution to analyze plasmids. The Plasmid DNA kit enables accurate sizing of supercoiled plasmid DNA up to 10 kb over a broad concentration range. While the Plasmid DNA kit is optimized for supercoiled DNA, detection of linearized plasmids is also possible with the kit. Accurate sizing of the linear plasmid can be achieved with the Agilent qualitative kits for the Fragment Analyzer systems.

References

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