Technical Overview

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Separation Resolution Capabilities of the Agilent ZAG 135 and 110 dsDNA Kits and the Agilent Fragment Analyzer dsDNA 935 and 915 Reagent Kits

Introduction

The ability to distinguish between two closely sized nucleic acid fragments using electrophoretic-based technology is often referred to as "*separation resolution*". The terms "*separation*" and "*resolution*" are often used interchangeably when describing fragment analysis in electrophoresis[1]. Liquid chromatography-based applications define resolution of a fragment as a quantitative measure of how well two elution peaks can be differentiated. The separation of two peaks is more subjective in nature and refers to the ability to visually distinguish two peaks unequivocally on an electropherogram.

Fragment analysis often requires the separation of closely sized DNA fragments. For example, fragment separation is especially critical for distinguishing homozygous and heterozygous alleles in plant genotyping[2] or when analyzing simple sequence repeats[3]. Resolution of fragments is also important when characterizing the genetics of phenotypes in animals and when studying human donor-recipient chimerism. Synthetic biology applications rely on the ability to resolve fragment digestion patterns in DNA building block assembly[4]. In addition, many genetics-based workflows require quality control of fragments, for instance, restriction digestion of plasmids[5] and PCR amplicons. Fragment analysis is used in many different applications across a wide variety of areas and requires reliable separation of closely sized fragments.

Traditional agarose or polyacrylamide slab gel electrophoresis techniques are commonly used for many fragment analysis applications. These techniques can be labor-intensive, requiring significant monitoring and manual data annotation that may be prone to human error. Conventional gel-based systems also lack the ability to resolve fragments close in size. Automated capillary electrophoresis provides many advantages over gel electrophoresis, including higher resolution of fragments, conservation of sample with low sample volume and concentration requirements, and reducing human error with digital results. The Agilent ZAG DNA Analyzer and Agilent Fragment Analyzer systems are considerably more discerning than gel electrophoresis, allowing for separation of closely sized fragments, conservation of sample, and screening for minute amounts of contamination or nonspecific peaks. The unique gel chemistry utilized by these instruments allows for separation of closely sized DNA fragments, providing sample results previously unattainable with the old gel techniques. In addition, the Agilent ProSize Data Analysis software offers automated fragment sizing and flagging options.

To demonstrate the resolution capabilities of each platform and congruity of fragment analysis between the ZAG and the Agilent 5300 Fragment Analyzer systems, samples consisting of mixed known fragment sizes were analyzed. Both the ZAG and 5300 Fragment Analyzer systems are capable of simultaneous analysis of 96 samples. The Fragment Analyzer systems hold three plates and have both quantitative and qualitative kits for DNA and RNA analysis. Alternatively, the ZAG accommodates nine plates for qualitative DNA analysis kits, with faster run times and is ideal for laboratories requiring ultrahigh sample throughput[6]. Several of the ZAG and Fragment Analyzer kits have the same analytical specifications, allowing for seamless comparison between instruments (Table 1). The wide concentration range offered by both kits often eliminates a dilution step and enables direct processing of samples with unknown concentrations, saving time in the workflow.

Experimental

Fragment preparation

Fragments were prepared from Lambda DNA (Thermo Fisher Scientific, p/n SD0011) with specific primers which added an MssI site to both ends (IDT, Integrated DNA Technologies) and amplified with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, p/n F530S) according to manufacturer's instructions. PCR conditions were as follows: initial denaturation (98 °C; 30 seconds), denaturation (98 °C; 10 seconds), annealing (60 °C; 30 seconds) extension (72 °C; 15–30 seconds per kilobase) for 30 cycles and final extension (72 °C; 10 minutes). PCR products were digested with FastDigest MssI (Thermo Fisher Scientific, p/n FD1344) resulting in blunt end fragments of known size. The digested fragments were separated on a 1% gel and then purified with the Zymoclean Gel DNA Recovery kit (Zymo Research, p/n D4007). Fragment concentration was measured by the Qubit 4.0 and the 1X dsDNA High Sensitivity kit (Thermo Fisher Scientific, p/n Q33230) and diluted to approximately 8 ng/µL with 1X TE.

Fragment analysis

The fragments and mixes of the fragments were analyzed on the Agilent ZAG DNA Analyzer system (p/n M5320AA) with the ZAG 135 dsDNA kit (1–1500 bp) (ZAG 135 kit) (p/n ZAG-135) and the ZAG 110 dsDNA kit (35–5000 bp) (ZAG 110 kit) (p/n ZAG-110). For comparison, the same samples were analyzed on the Agilent 5300 Fragment Analyzer system (p/n 5311AA, respectively) with the dsDNA 935 Reagent kit (1–1500 bp) (Fragment Analyzer 935 kit) (p/n DNF-935) and the dsDNA 915 Reagent kit (35–5000 bp) (Fragment Analyzer 915 kit) (p/n DNF-915). The ProSize Data Analysis software (ProSize software) displays a digital gel image, electropherogram and peak table with sizing for both the ZAG and Fragment Analyzer systems.

Table 1. Specification comparisons for the Agilent ZAG 135 dsDNA (1–1500 bp) and the Fragment Analyzer dsDNA 935 Reagent kits (1–1500 bp) and the ZAG 110 dsDNA (35–5000 bp) and the Fragment Analyzer dsDNA 915 Reagent kits (35–5000 bp).

ZAG DNA Analyzer Kits	Fragment Analyzer Kits	Sizing Range	Sizing Accuracy	Sizing Precision	Sizing Resolution	Concentration Range
ZAG 135 dsDNA kit	dsDNA 935 Reagent kit	100-1,500 bp	5%	2%	100-1,500 bp ≤ 10%	0.5-50 ng/µL
ZAG 110 dsDNA kit	dsDNA 915 Reagent kit	35-5,000 bp	5%	2%	35-100 bp ≤ 10% 100-1,000 bp ≤ 5% 1,000-5,000 bp ≤ 10%	0.5-50 ng/µL

Results and discussion

Separation and resolution

Separation and resolution are often used interchangeably when describing electropherogram images. Although resolution can be defined with an equation and numerical value, separation relies on visual discrimination. Resolution is calculated by the difference in retention times of the two peaks, divided by the combined full width or width at half maximum ($W_{0.5}$) for each elution peak[1] (Figure 1). There can be varying degrees of separation between two fragments, often referred to as peaks in electropherograms. For our purposes, we define the different degrees of separation as follows:

1. **Complete separation** – the return to baseline or separation below half-width ($W_{0.5}$) between the two peaks.
2. **Partial separation** – the visual separation at approximately half-width to 1/3 from the top of each of the two peaks. The ProSize software will recognize them as two separate peaks.
3. **Limited separation**– just the tops of the two peaks are distinct from one another. The ProSize software most likely will not select them as separate peaks.
4. **Shouldering** (to the left) – bump on the left side of a peak widening the base, which can be due to merging of two peaks, amongst other factors.
5. **Tailing** (to the right) – elongation on the right side of a peak widening the base, which can be due to merging of two peaks, amongst other factors.

Agilent provides an adaptable solution for improving resolution by varying migration lengths with different capillary arrays. A longer migration length is one way to allow for better separation and resolution of similarly sized DNA fragments[7]. As fragment resolution improves, the area between two fragments increases. In turn, two partially resolved peaks will shift towards two separate peaks with complete baseline separation. Agilent offers three different capillary array lengths for the Fragment Analyzer: ultrashort (22 cm effective, 47 cm total), short (33 cm effective, 55 cm total), and long (55 cm effective, 80 cm total), while the ZAG can accommodate the short and long capillary arrays. The long array will provide the best resolution with a longer run time, while the ultrashort array has the shortest run times, but compromises on resolution. In the middle is the short array, which has the advantages of suitable resolution and run times, meeting most user's needs. The arrays can be exchanged within the instruments to accommodate the user's requirements.

To determine the separation capabilities of the ZAG and Fragment Analyzer systems, mixes of two fragments differing by a known number of base pairs were analyzed with the equivalent separation kits for each instrument. The ZAG 135 and Fragment Analyzer 935 kits were specifically designed for accurate sizing of fragments less than 1,500 bp, while the ZAG 110 and Fragment Analyzer 915 kits size up to 5,000 bp. Both sets of kits utilize specially formulated gels that enhance separation of similarly sized fragments. Common applications for these kits include genotyping, the analysis of PCR amplicons and microsatellites (also known as simple sequence repeats).

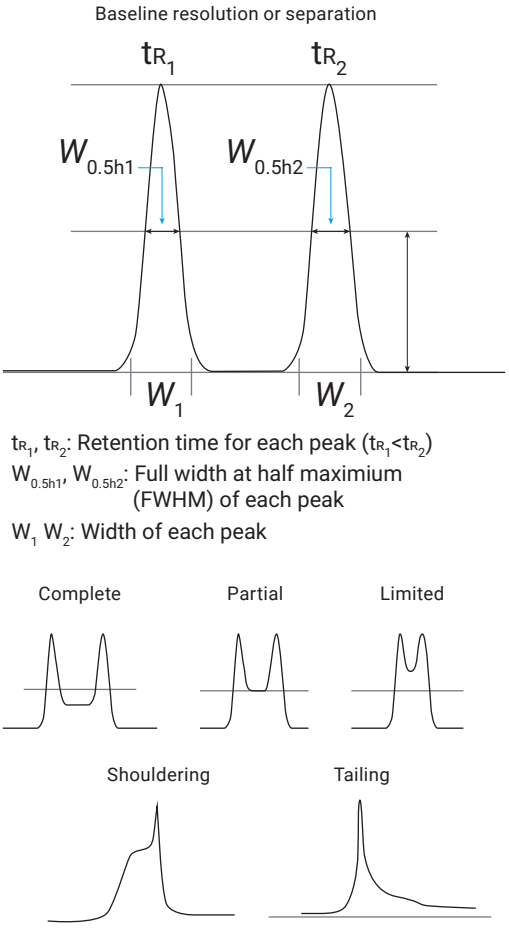


Figure 1. Differing degrees of separation between two peaks in electropherograms.

Separation on the ZAG 135 and Fragment Analyzer 935 kits

Resolution of 400 bp fragment

Resolution at 400 bp was investigated by mixing 400 and 425 bp fragments. The 25 bp (or 6.3%) difference is below the 10% resolution specification of both the ZAG 135 kit and the Fragment Analyzer 935 kit. Both kits provided complete separation of the two fragments (Figure 2). Sizing of the fragments, when analyzed separately or mixed, resulted in very accurate and similar sizing (Table 2). At the smaller base pair end of the kits, better resolution can be achieved than the 10% resolution specified in the kits.

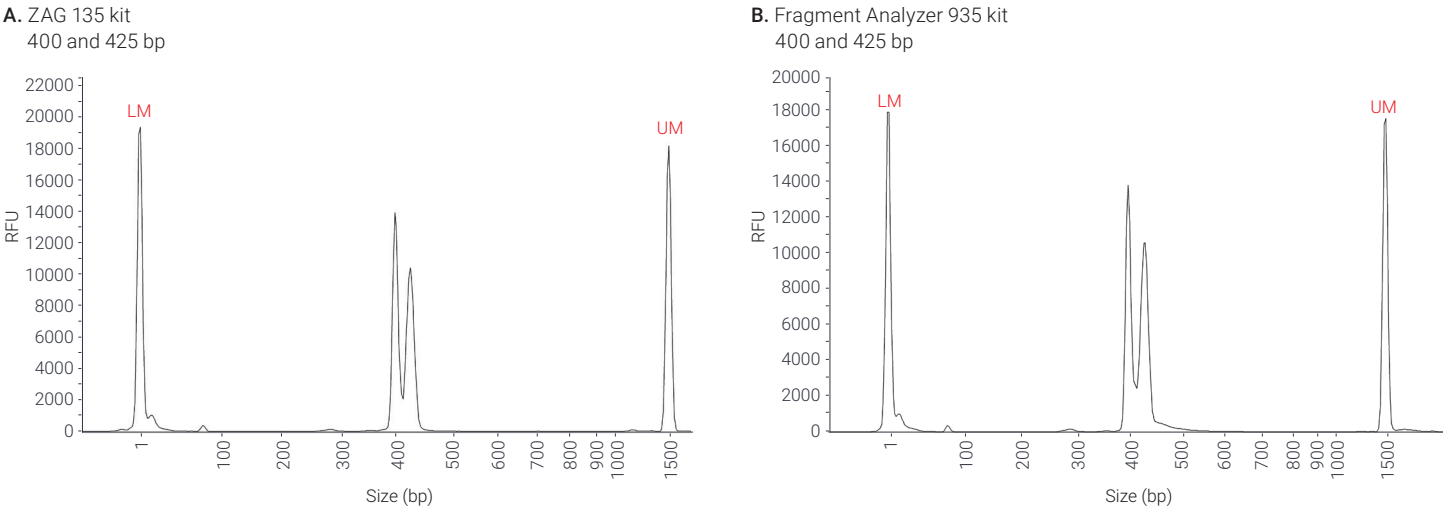


Figure 2. Resolution of 400 and 425 bp fragment mix separated on the (A) Agilent ZAG DNA Analyzer with the ZAG 135 dsDNA kit (1–1500 bp) and (B) the Agilent Fragment Analyzer system with the dsDNA 935 Reagent kit (1–1500 bp). LM = lower marker; UM = upper marker.

Resolution of 1,000 bp fragment

Resolution for both kits is 10%, with resolution at 1,000 bp requiring a ±100 bp difference. To test the resolution, the 1,000 and 1,050 bp fragment mix (a 5% difference) and 1,000 and 1,100 bp fragment mix (a 10% difference) were analyzed on both instruments. The 1,000 and 1,050 bp mix resulted in similar electropherograms for both instruments (Figure 3A and B). The 1,000 and 1,050 bp peaks are distinguishable from each other visually, however, they are not considered resolved from each other because there is limited separation space between the peaks. In addition, the two fragments were not identified as separate peaks by the ProSize software. ProSize software allows the user to "*add peak or split peak*" in order for a size to be assigned to both fragments. This ProSize software option was utilized for the 1,000 and 1,050 bp fragment mix in order to obtain the size of each fragment. In comparison, the 1,000 and 1,100 bp are easily separated, with clear separation between the peaks on both instruments (Figure 3C and D).

At a 5% difference the resolution specification of 10% is clearly observed in the electropherograms when comparing the 1,000 and 1,050 bp mix to the 1,000 and 1,100 bp mix. Even though the 1,000 and 1,050 bp mix had limited separation, sizing of the mixed fragments was accurate and similar to the completely resolved 1,000 and 1,100 bp mix (Table 2). Sizing accuracy and precision for both mixes and the single fragments were far below the kit specifications of 5% and 2%, respectively. The 1,050 and 1,100 bp mix was also analyzed with both kits, resulting in one wide peak on both electropherograms (data not shown). This was to be expected since 10% resolution at 1,100 bp would require a 110 bp difference. Sizing and separation of the fragment mixes was similar on both the ZAG and 5300 Fragment Analyzer systems (Table 2), demonstrating that regardless of the instrument, reliable sizing will be reported.

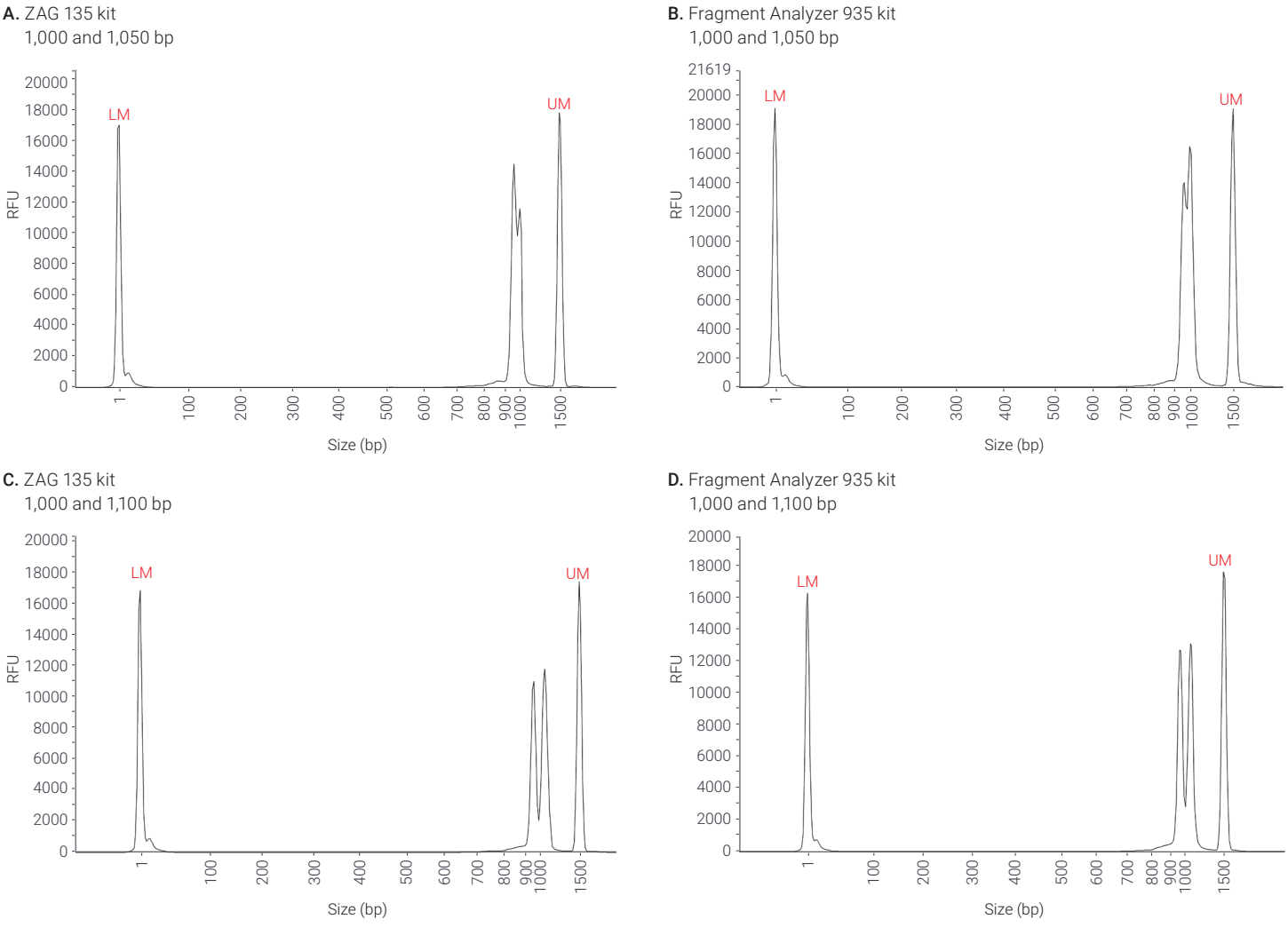


Figure 3. Resolution of DNA fragment mixes separated on the Agilent ZAG DNA Analyzer and Fragment Analyzer systems. (A) 1,000 and 1,050 bp fragments with the ZAG 135 dsDNA kit; (B) 1,000 and 1,050 bp fragments with the dsDNA 935 Reagent kit (1–1500 bp); (C) 1,000 and 1,100 bp fragments with the ZAG 135 dsDNA kit; (D) 1,000 and 1,100 bp fragments with the dsDNA 935 Reagent kit (1–1500 bp). LM = lower marker; UM = upper marker.

Resolution from the upper marker

The upper marker (UM) is a 1,500 bp fragment used for sizing and alignment of the ladder and samples on both the ZAG 135 dsDNA and the Fragment Analyzer 935 kits. To determine the resolution and accuracy of sizing apart from the upper marker, fragments at 10% (1,350 bp) and 5% (1,425 bp) base pair difference from the upper marker were separated. The 1,350 bp fragment displayed complete separation from the upper marker with extremely accurate sizing (Figure 4A). However, the 1,425 bp fragment had limited separation from the upper marker and required manual selection of the peak in the ProSize software, allowing for an accurate size to be assigned (Figure 4B).

A mix of the 1,350 and 1,425 bp fragments resulted in shouldering, where the two fragments merged into one peak with a bump on the left side that, on occasion, may present as a tiny peak (Figure 4C and D). Prior knowledge as to the number of fragments present in the sample would be necessary to unequivocally determine if the split peak represented one or two fragments. In this instance, two fragments were mixed and manual selection of the two peaks was necessary, allowing ProSize software the capability of assigning accurate sizes for both fragments (Table 2).

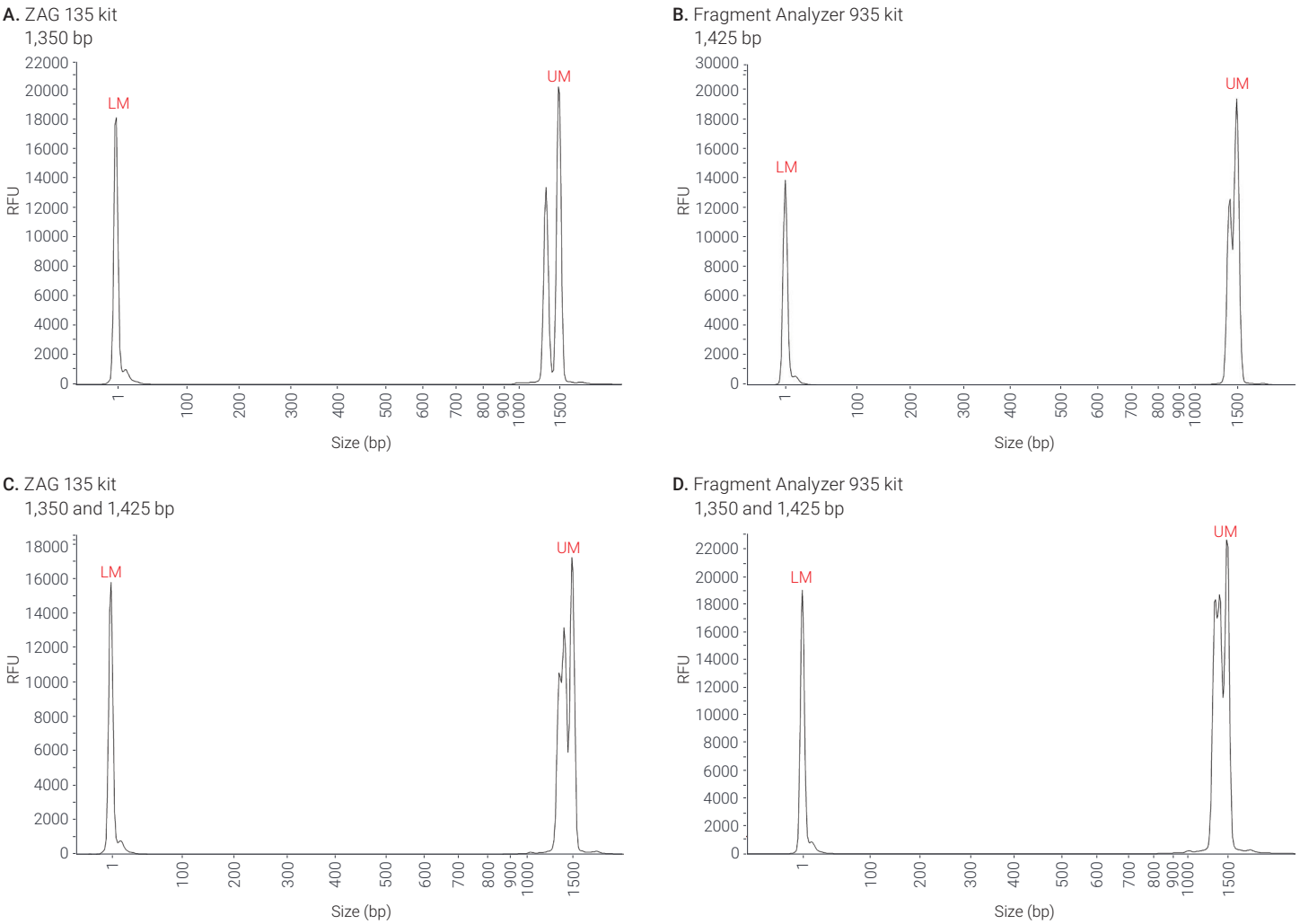


Figure 4. Resolution of single DNA fragments and mixes with the upper marker (UM) on the Agilent ZAG DNA Analyzer and Fragment Analyzer system. (A) 1,350 bp fragment with the ZAG 135 dsDNA kit (1–1500 bp); (B) 1,425 bp fragment with the dsDNA 935 Reagent kit (1–1500 bp); (C) 1,350 and 1,425 bp fragments with the ZAG 135 dsDNA kit (1–1500 bp); (D) 1,350 and 1,425 bp fragments with the dsDNA 935 Reagent kit (1–1500 bp). LM = lower marker; UM = upper marker.

Separation with the ZAG 110 kit and the Fragment Analyzer 915 kit

Resolution of 306, 311, 400, and 425 bp fragments

Resolution in the smaller base pair range of the two kits was explored with the 306 and 311 bp mix and the 400 and 425 bp mix. Limited separation was achieved with the 306 and 311 bp mix (Figure 5A and B). This was to be expected due to the limited sizing difference of 2% or 5 bp, which is outside of the percent resolution kit specifications. The 400 and 425 bp mix has a 6% difference in sizing and therefore is within kit specifications for complete resolution. Indeed, the electropherograms displayed complete separation from one another with both kits (Figure 5C and D). Accurate sizing was provided for both the single fragments and the mixes, with the percent error below 1% in all instances (Table 2). The ZAG 110 and Fragment Analyzer 915 kits can provide partial and limited separation with accurate sizing below the specified 5% resolution and complete separation at or above the specified resolution of 5%.

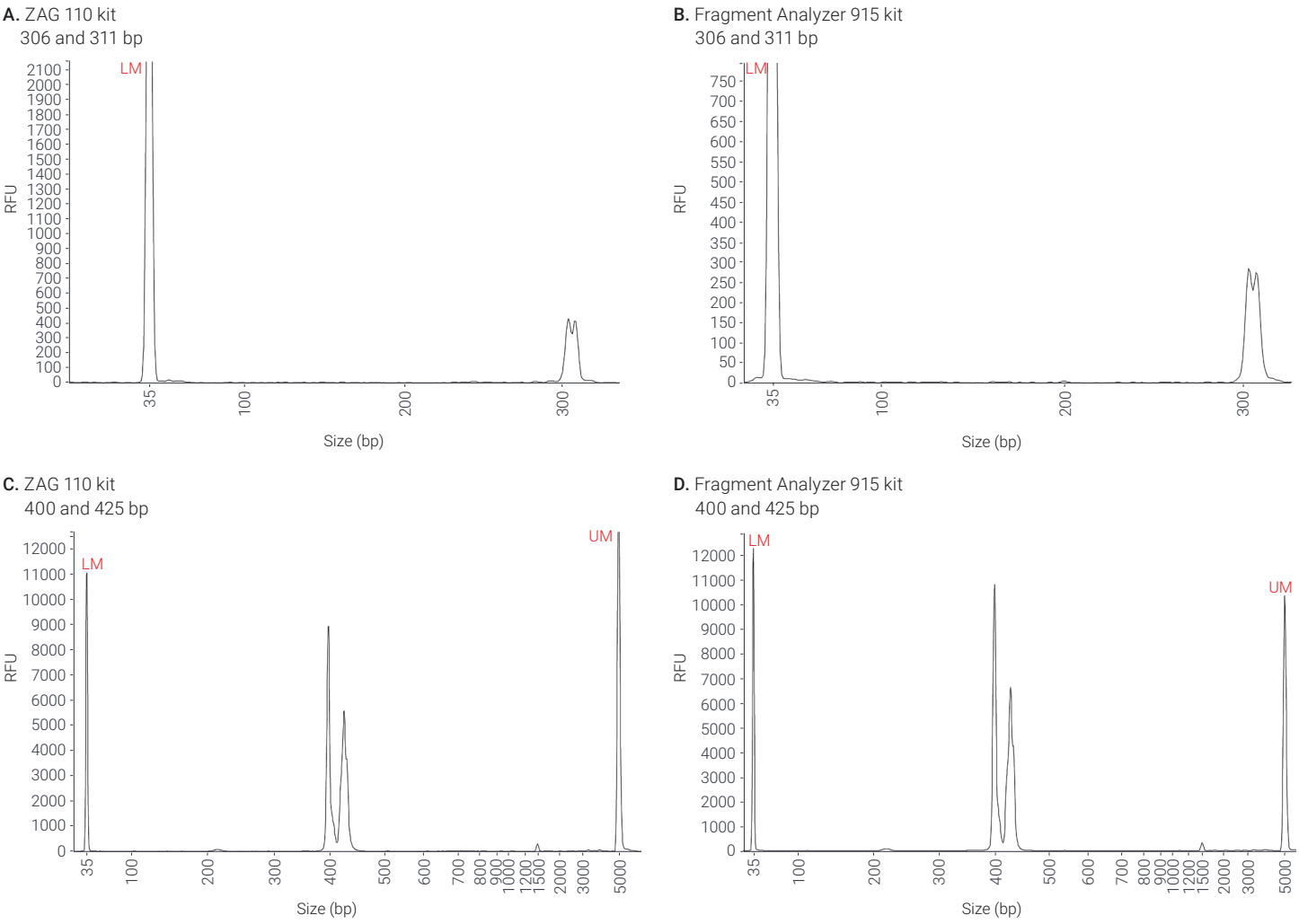


Figure 5. Resolution of fragment mixes separated on the Agilent ZAG DNA Analyzer and Fragment Analyzer systems. (A) 306 and 311 bp mix analyzed with the ZAG 110 dsDNA kit (1–5000 bp) (B) 306 and 311 bp mix analyzed with the dsDNA 915 Reagent kit (1–5000 bp), (C) 400 and 425 bp with the ZAG 110 dsDNA kit (1–5000 bp), (D) 400 and 425 bp with the dsDNA 915 Reagent kit (1–5000 bp). LM = lower marker; UM = upper marker.

Resolution of 1,000, 1,050, and 1,100 bp fragments

Mixes of the 1,000 and 1,050 bp fragments were similar on both instruments and displayed slightly better separation than with the ZAG 135 and Fragment Analyzer 935 kits (Figure 3A and B), with partial peak separation and ProSize software identifying both peaks (Figure 6A). The 1,000 and 1,100 bp fragment mix had a similar complete separation profile (Figure 6B) as seen on the ZAG 135 and Fragment Analyzer 935 kits (Figure 3C and 3D) with very accurate sizing (Table 2). In order to determine the extent of the resolution capabilities of the ZAG 110 and Fragment Analyzer 915 kits at the 1,000 bp range, all three fragments were mixed and analyzed. All three peaks were separated visually, with ProSize software identifying and assigning accurate sizes (Figure 6C and D). The percent resolution at the 1,000 bp range is 5%, meeting the specifications of the kits.

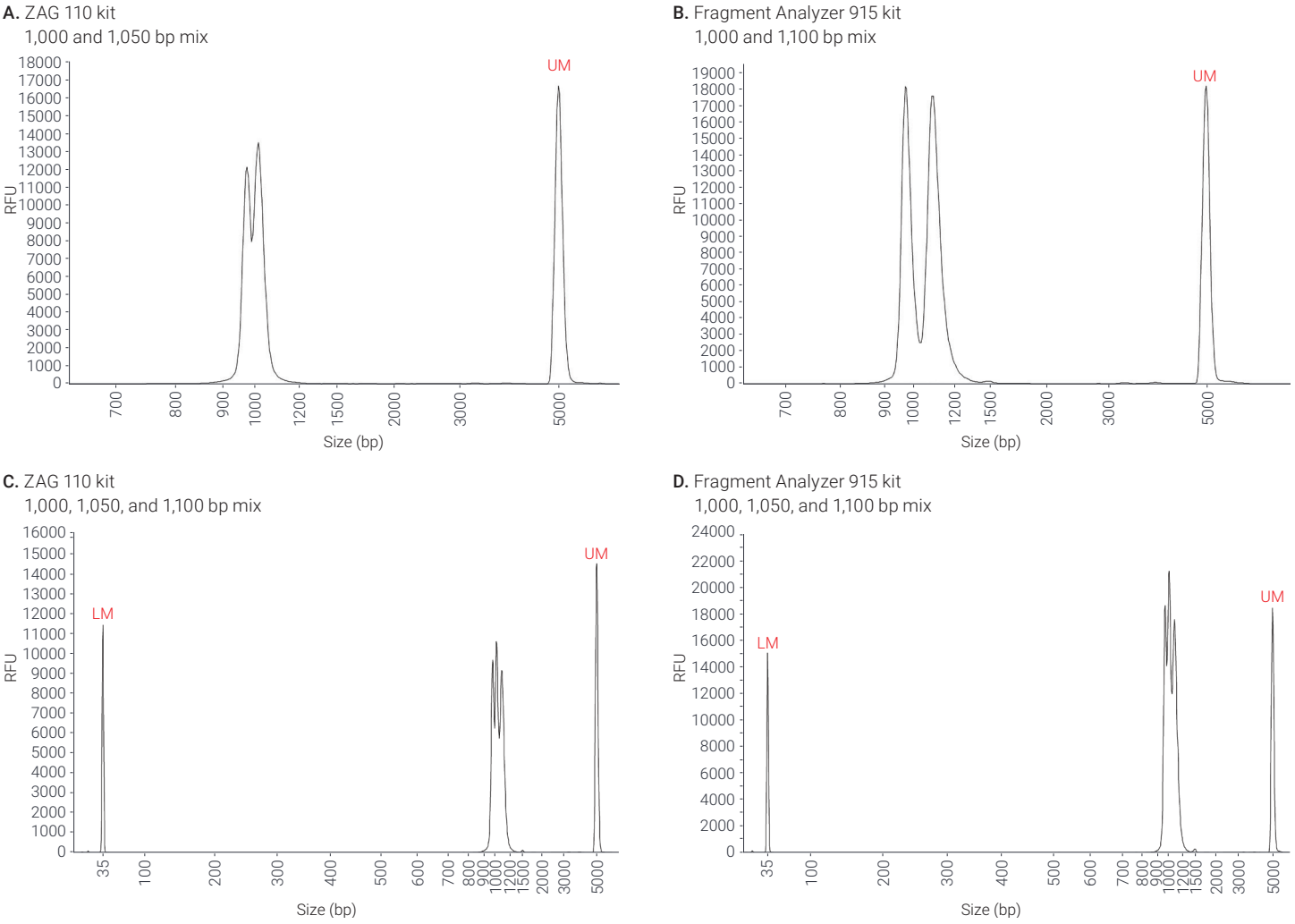
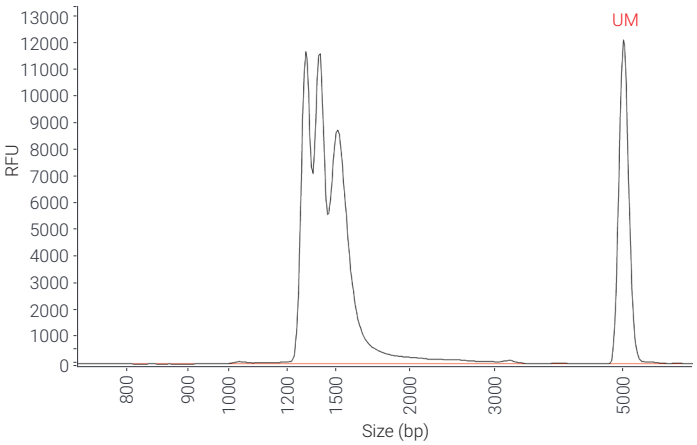


Figure 6. Resolution at the 1,000 bp fragment with various fragment mixtures displayed similar results with both the Agilent ZAG DNA Analyzer and the Fragment Analyzer systems. (A) 1,000 and 1,050 fragment mix on the ZAG DNA Analyzer with the ZAG 110 dsDNA kit (1–5000 bp), (B) 1,000 and 1,100 fragment mix on the Fragment Analyzer system with the dsDNA 915 Reagent kit (1–5000 bp). Resolution of 1,000, 1,050, and 1,100 bp fragment mix separated on the (C) ZAG DNA Analyzer with the ZAG 110 dsDNA kit (1–5000 bp) and (D) the Fragment Analyzer system with the dsDNA 915 Reagent kit (1–5000 bp). LM = lower marker; UM = upper marker.

Resolution of 1350, 1,425, and 1,500 bp fragments

The ZAG 110 and Fragment Analyzer 915 kits specification for percent resolution changes at the 1,000 bp size from 5% to 10% resolution. The fragments 1,350, 1,425, and 1,500 bp are just outside the 100 to 1,000 bp range, allowing for investigation into the transition area. Analysis of the 1,425 and 1,500 bp fragment mix, a 5% difference, resulted in a partial separation, while the 1,350 and 1,500 bp mix, a 10% resolution, displayed a complete separation (electropherograms not shown) with ProSize software assigning accurate sizing for all fragments. The 1,350 and 1,500 bp mix resulted in the same separation, while the 1,425 and 1,500 bp resulted in a slightly better partial separation than the ZAG 135 and Fragment Analyzer 935 kits, respectively (Figure 4A and 4B). A mix of all three fragments (1,350, 1,425 and 1,500 bp) resulted in a partial separation (Figure 7) with ProSize software identifying all three peaks and assigning accurate sizes on both kits (Table 2). A partial separation between fragments in the 1,000 to 1,500 bp range can be achieved below the specified 10% resolution on the ZAG 110 and Fragment Analyzer 915 kits.

A. ZAG 110 kit
1,350, 1,425, and 1,500 bp



B. Fragment Analyzer 915 kit
1,350, 1,425, and 1,500 bp

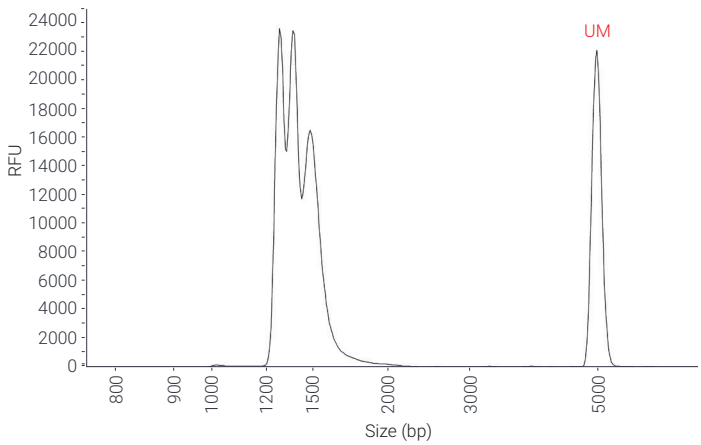


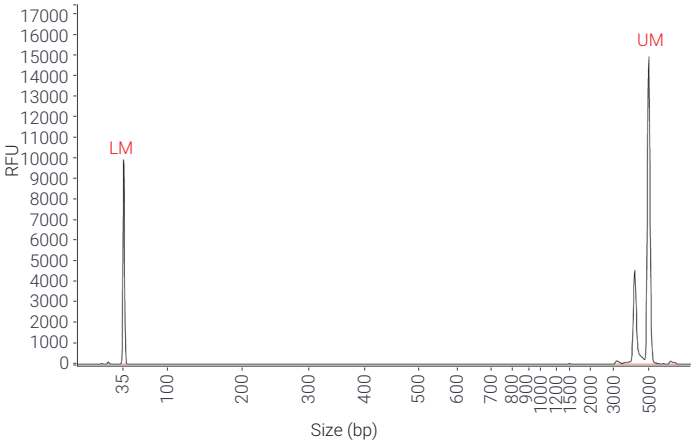
Figure 7. Resolution of 1,350, 1,425, and 1,500 bp fragment mix separated on the (A) Agilent ZAG DNA Analyzer with the ZAG 110 dsDNA kit (1–5000 bp) and (B) the Agilent Fragment Analyzer system with the dsDNA 915 Reagent kit (1–5000 bp). LM = lower marker; UM = upper marker.

Resolution From the upper marker

Resolution from the 5,000 bp upper marker on the ZAG 110 and Fragment Analyzer 915 kits was analyzed with the 4,500 bp fragment. The 4,500 bp fragment displayed complete separation from the upper marker on both kits (Figure 8).

The 10% size difference from the upper marker meets both kit specifications of 10% resolution for complete separation. A partial separation from the upper marker is possible with fragments larger than 4,500 bp.

A. ZAG 110 kit
4,500 bp



B. Fragment Analyzer 915 kit
4,500 bp

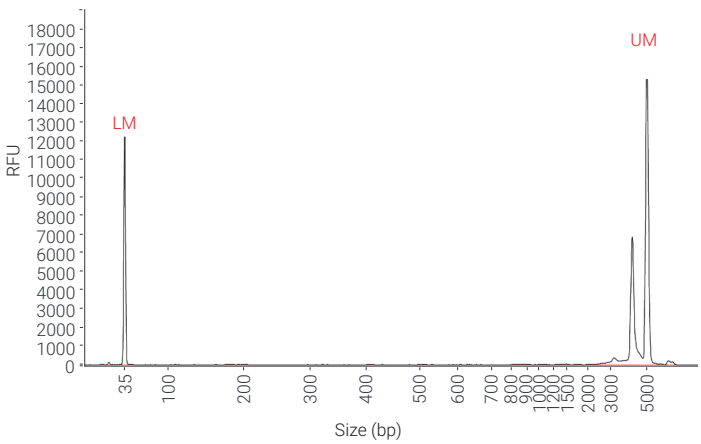


Figure 8. . Resolution of 4,500 bp fragment from the upper marker (UM) on the (A) Agilent ZAG DNA Analyzer with the ZAG 110 dsDNA kit (1–5000 bp) and (B) the Agilent Fragment Analyzer system with the dsDNA 915 Reagent kit (1–5000 bp). LM = lower marker; UM = upper marker.

Table 2. Sizing of single fragments and mixes of fragments on the Agilent ZAG DNA Analyzer and Fragment Analyzer systems with the ZAG 135 dsDNA kit (1–5000 bp) and dsDNA 935 Reagent kit (1–5000 bp), respectively. All fragments are within the sizing accuracy specifications of the kits. UM = upper marker, *n = 6.

	Reported Fragment Sizes			
Theoretical Fragment Size (bp)	ZAG 135 kit (bp)	Fragment Analyzer 935 kit (bp)	ZAG 110 kit (bp)	Fragment Analyzer 915 kit (bp)
306	-	-	302	305
311	-	-	308	310
Mix 306	-	-	305	305
311	-	-	309	309
400	406	400	398	400
425	435	429	428	428
Mix 400	403	400	398	398
425	431	428	427	428
1,000	968	972	983	978
1,050	1,018	1,015	1,026	1,022
1,100	1,066	1,062	1,099	1,095
Mix 1,000	975	970	986	981
1,050	1,015	1,013	1,029	1,021
1,100	-	-	1,099	1,091
Mix 1,000	976	973	985	978
1,100	1,077	1,066	1,100	1,095
1,350	1,356	1,347	1,297	1,289
1,425	1,422	1,415	1,393	1,389
1,500	UM	UM	1,499	1491
Mix 1,350	1,360	1,362	1,306	1,295
1,425	1,414	1,410	1,390	1,382
1,500	UM	UM	1,502	1,492
Mix 3,000	-	-	2,943	2,932
4,000	-	-	4,022	3,911
4,500	-	-	4,225	4,131

Conclusion

The Agilent ZAG 135 dsDNA and Fragment Analyzer dsDNA 935 Reagent kits delivered comparable results for resolution and sizing of single fragments and fragment mixes, as did the Agilent ZAG 110 dsDNA and the Fragment Analyzer dsDNA 915 Reagent kits when compared. All four kits provided complete fragment separation at the specified percent resolution of the kits. When complete resolution between peaks was not achievable, Agilent ProSize Data Analysis software was still capable of recognizing and assigning accurate sizes for partially separated fragment mixtures. While there is an overlap in the sizing range between the kits, the percent resolution is lower at the 100– 1,000 bp range of the ZAG 110 dsDNA and Fragment Analyzer 915 Reagent kits, allowing for the resolution requirements of the user to be met. Both instruments provide automated sizing and fragment resolution that is difficult to achieve with conventional slab gels. The Agilent ZAG DNA Analyzer and Fragment Analyzer systems with their respective kits can be used interchangeably to provide high resolution and accurate sizing for fragment analysis.

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PR7000-8052

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Printed in the USA, July 15, 2021
5994-3638EN

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