Application Note

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Highly Resolved Separation of DNA Fragments on the Agilent 5200 Fragment Analyzer System

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Abstract

Complete analysis of PCR amplicons requires an instrument capable of delivering reliable sizing and quality control parameters. The Agilent 5200 Fragment Analyzer system with the Agilent dsDNA 905 Reagent kit (1-500 bp) and Agilent HS Small Fragment kit offers exceptional quantification, qualification, and separation of known DNA fragment sizes with a three base pair difference.

Introduction

The ability to distinguish between two closely sized fragments is referred to as separation resolution in electrophoresis systems. Resolution of fragments is critical for accurate DNA sizing and identification of extra fragments when studying small PCR products. Traditional agarose gel-based systems lack the ability to resolve fragments close in size. The Agilent 5200 Fragment Analyzer system utilizes a unique gel chemistry, which allows for separation of closely sized DNA fragments, providing better insight into nucleic acid sample size. The Agilent HS Small Fragment kit and the Agilent dsDNA 905 Reagent kit (1-500 bp) are ideal for separating and sizing small PCR products (Table 1).

Table 1. Overview of sizing and fragment concentration ranges for the HS Small Fragment kit and the dsDNA 905 reagent kit (1-500 bp).

	Size range	Fragment concentration range	Kit type
HS Small Fragment kit	50 to 1,500 bp	5 to 500 pg/µL	Quantitative
dsDNA 905 Reagent kit (1–500 bp)	35 to 500 bp	0.5 to 50 ng/µL	Qualitative

Experimental

The experiments in this study were performed using a 5200 Fragment Analyzer system and can be replicated with comparable results on Agilent 5300 and 5400 Fragment Analyzer systems.

306, 307, 308, 309, 310, and 311 bp gBlocks (Integrated DNA Technologies) were designed such that each contained blunt end restriction sites (*HaeIII* and *EcoRV*), allowing for the creation of various known sized products differing by a single base pair at around 300, 200, and 100 bp. Each gBlock was amplified using Phusion DNA polymerase (Thermo Fisher Scientific, #F530S). Digestion of the 200 ng PCR product was achieved with *HaeIII* (Thermo Fisher Scientific, #FD0154) or *EcoRV* (Thermo Fisher Scientific, #FD0303) according to manufacturer instructions. To clean up the digested fragments, digests were separated on a 2 % agarose gel and the gel fragment was purified using the Zymoclean gel DNA recovery kit (Zymo Research, #D400). The purified fragment was quantified using Qubit high sensitivity dsDNA kit (Thermo Fisher Scientific, #32854) and diluted to 250 pg/µL using 1× TE. The fragments of various lengths (306 to 311 bp, 201 to 205 bp, and 101 to 105 bp) and mixes of the fragments were analyzed on the 5200 Fragment Analyzer system with the Agilent FA 12-Capillary Array Ultrashort, 22 cm (ultrashort array) (p/n A1600-1250-2247), the Agilent FA 12-Capillary Array Short, 33 cm (short array) (p/n A2300-1250-3355), and the Agilent FA 12-Capillary Array Long, 55 cm (long array) (p/n A2300-1250-5580) using the dsDNA 905 Reagent kit (1-500 bp) (p/n DNF-905) and the HS Small Fragment kit (p/n DNF-477).

Results and discussion

Capillary electrophoresis (CE)

The 5200 Fragment Analyzer system is a parallel capillary electrophoresis (CE) instrument paired with fluorescence detection designed for the separation and detection of DNA and RNA. Gel, intercalating dye, and sample flow through the capillaries and pass the detection window. Light passes from the LED excitation light source through the detection window, exciting the intercalated samples as they pass by. The emitted fluorescence from the intercalating dye bound to the sample passes through the camera lens and into the CCD detector. The 5200 Fragment Analyzer system enables real-time detection of nucleic acid separations that are displayed as an electropherogram. DNA migration through a gel matrix is size-dependent, with larger fragments moving slower through the gel than smaller fragments. The length of the capillary plays an important role in the separation of fragments. A shorter migration length can hinder fragment resolution, preventing the complete separation of DNA fragments. Thus, similar sized fragments will cross the detection window in close succession, becoming indistinguishable from one another and appearing as a single broad peak on the electropherogram. A longer migration length allows for better separation of similarly sized DNA fragments and may be displayed as two peaks or two partially resolved peaks often referred to as a split peak. As fragment resolution improves, the separation 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 5200 Fragment Analyzer system: ultrashort, short, and long. The arrays can be exchanged within the 5200 Fragment Analyzer system to accommodate user requirements.

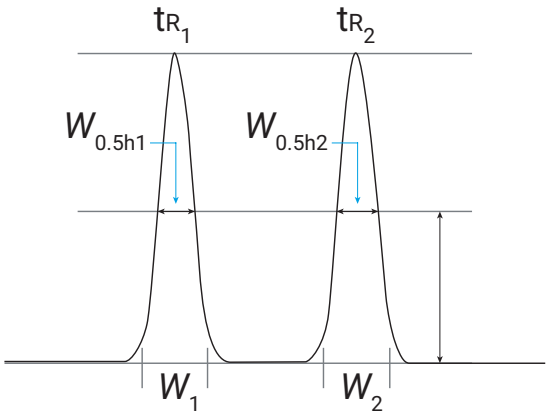
Separation and resolution

To determine the separation capabilities of the 5200 Fragment Analyzer system with the different length arrays, mixes of two fragments differing by a known number of base pairs were analyzed with two different separation kits. The dsDNA 905 Reagent kit (1-500 bp) was specifically designed for accurate sizing of fragments less than 500 bp and utilizes a specially formulated gel that enhances separation of similarly sized fragments. Common applications of this kit include genotyping, the analysis of PCR amplicons, and microsatellites/ simple sequence repeats. The HS Small Fragment kit is intended for sizing and quantification of small, low-concentrated DNA fragments and smears from 50 to 1,500 bp. This kit is ideal for the evaluation of small RNA NGS libraries, genotyping, quantitative analysis of PCR amplicons, and microsatellites.

The creation of fragments through enzyme restriction sites allows us to unequivocally produce fragments of known base pair sizes. A series of known fragment sizes were mixed and analyzed with the dsDNA 905 Reagent kit (1-500 bp) and HS Small Fragment kit. The 100 bp series included: 101/102, 101/103, 101,104, and 101/105 bp. The 200 bp series included: 201/202, 201/203, 201/204, and 201/205 bp. The 300 bp series included: 306/307, 306/308, 306/309, 306/310, and 306/311 bp. Table 2 summarizes the minimum base pair difference required to see two partially separated peaks for both the dsDNA Reagent kit (1-500 bp) and HS Small Fragment kit with the long, short, and ultrashort arrays.

$$R = \frac{t_{R_2} - t_{R_1}}{½(W_1 + W_2)}$$

$$R = 1.18\left(\frac{t_{R_2} - t_{R_1}}{(W_{0.5h1} + W_{0.5h2})}\right)$$



t_{R_1}, t_{R_2}: Retention time for each peak (t_{R_1}<t_{R_2})
$W_{0.5h1}$, $W_{0.5h2}$: Full width at half maximium (FWHM) of each peak
W_1 W_2: Width of each peak

Resolution and separation are often used interchangeably when describing electropherograms. However, in liquid chromatography applications, resolution of an elution is a quantitative measure of how well two elution peaks can be differentiated. It is defined as the difference in retention times of the two peaks, divided by the combined full width or width at half maximum for each elution peak. Both formulas are commonly used for calculating resolution. Peaks are usually successfully differentiated when resolution is greater than one. The second calculation is used for resolution specifications for the 5200 Fragment Analyzer system.

dsDNA 905 Reagent kit (1 to 500 bp)

Fragment mixes of known sizes (101/104 bp, 201/204 bp, and 306/309 bp) were analyzed with the dsDNA 905 Reagent kit (1-500 bp) on the short and long array (Figure 1). The long array performed a complete baseline resolution of the 101/104 bp fragments while displaying two partially separated peaks for the 201/204 bp and 306/309 bp fragment mixes. The short array demonstrated the 3 bp separation as two partially resolved peaks for all three fragment mixes. Separation with the dsDNA 905 Reagent kit (1-500 bp) on the ultrashort array exhibited a 3 bp separation as two partially resolved peaks for the 101/104 bp but was unable to separate the 201/204 and 306/309 bp fragment mixes. Separation on the ultrashort array was achieved at 4 bp for the 201/205 bp and 5 bp for the 306/311 bp fragment mixes, as two partially resolved peaks.

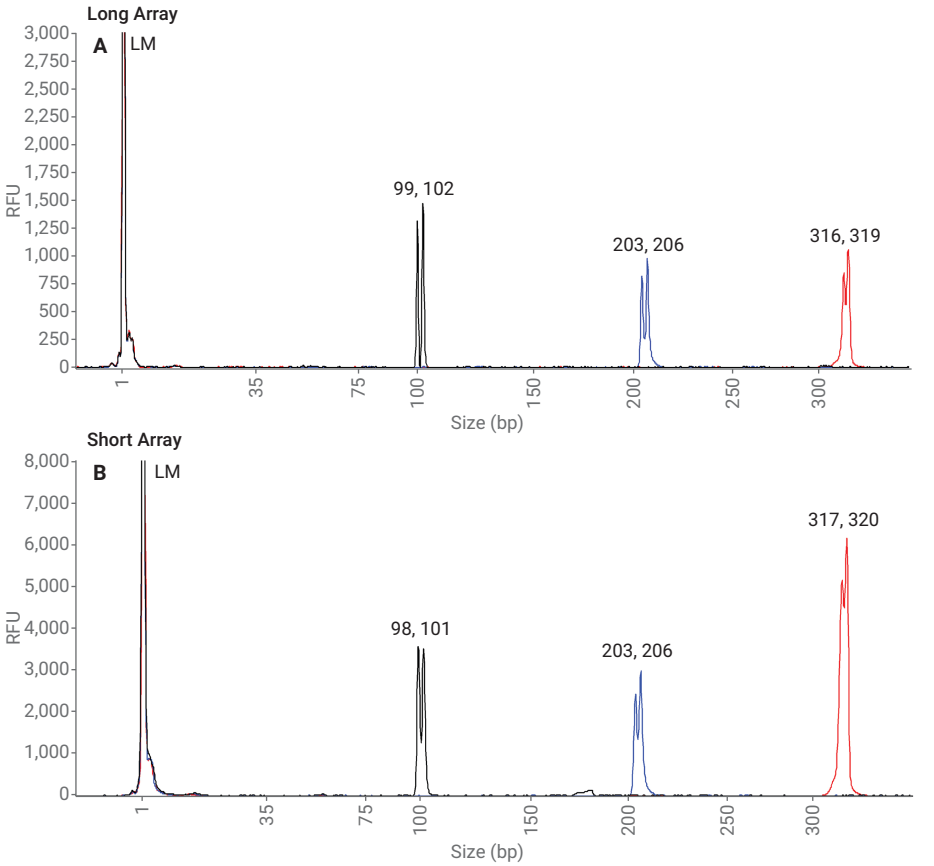


Figure 1. Separation of fragment mixes on the 5200 Fragment Analyzer system with the dsDNA 905 Reagent kit (1-500 bp); known sizes: 101/104 bp, 201/204 bp, and 306/309 bp on the (A) long array, (B) short array. The long array achieved complete baseline resolution at 3 bp for the 101/104 bp fragment mix. Average sizes in fragment mixes displayed, n = 3. LM = lower marker.

HS Small Fragment kit

The same fragment mixes of known sizes were analyzed with the HS Small Fragment kit on the long, short, and ultrashort arrays. Separation on the HS Small Fragment kit with the long array resulted in a 3 bp separation for the 101/104 bp, 201/204 bp, and 306/309 bp fragment mix as two partially separated peaks. The short array exhibited separation for the 101/104 bp, 201/205 bp, and 306/310 bp fragment mixes as two partially resolved peaks, indicating a 3 bp, 4 bp, and 4 bp separation, respectively (Figure 2). The ultrashort array displayed similar results as the short array except for achieving separation for the 306/311 bp fragment mix indicating a 5 bp separation (Figure 3).

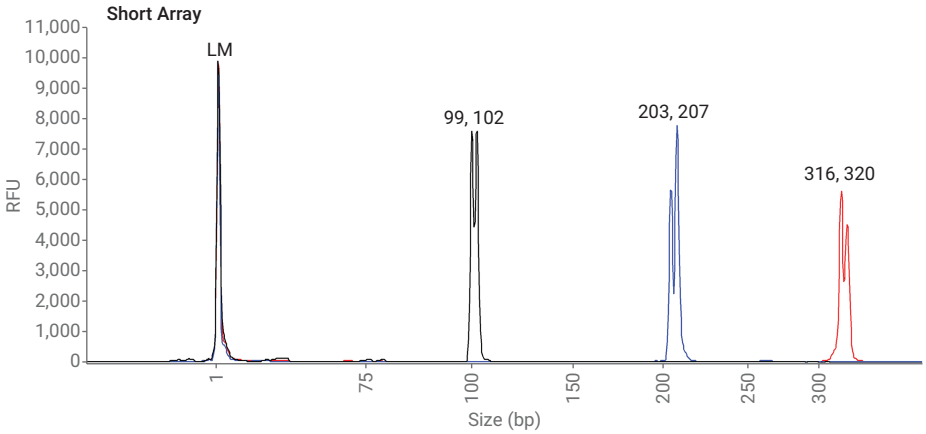


Figure 2. Separation of fragment mixes on the 5200 Fragment Analyzer system with the HS Small Fragment kit on the short array; known sizes: 101/104 bp, 201/205 bp, 306/310 bp, (3 bp, 4 bp, and 4 bp difference, respectively). Average sizes in fragment mixes displayed, n = 3. LM = lower marker.

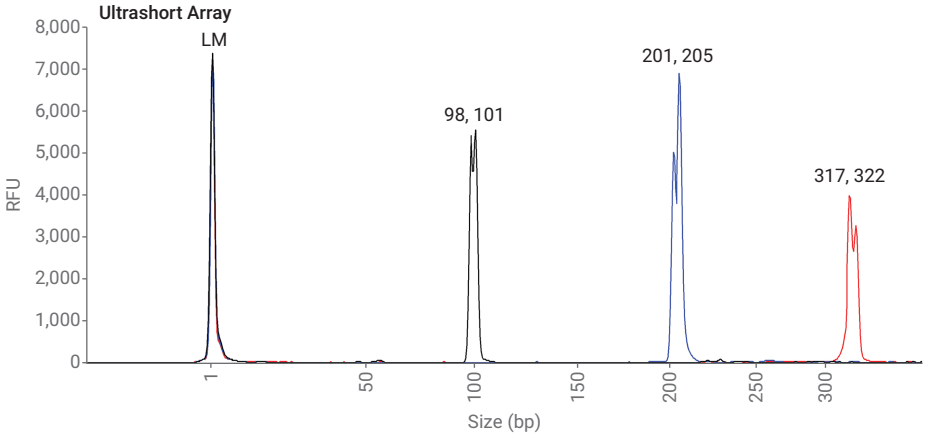


Figure 3. Separation of fragment mixes on the 5200 Fragment Analyzer system with the HS Small Fragment kit on the ultrashort array; known sizes: 101/104 bp, 201/205 bp, 306/311 bp (3 bp, 4 bp, and 5 bp difference, respectively). Average sizes in fragment mixes displayed, n = 3. LM = lower marker.

Sizing

DNA size affects separation resolution because base pair resolution is proportional to the percent of the fragment size. Larger fragments run slower through the gel due to impediment from size and gel matrices, resulting in larger fragments separating closer together (Figure 4). In addition, larger DNA fragments separate with a wider base width that can result in similar sized fragments over lapping and requiring a larger difference in size to be resolved from each. A longer separation length (array) aids in the separation of larger DNA by providing a greater amount of time for fragments to resolve. This is clearly displayed in the 306 bp mix with the HS Small Fragment kit and the dsDNA 905 Reagent kit (1-500 bp), in which a longer array resulted in better separation resolution for the larger DNA (Table 2).

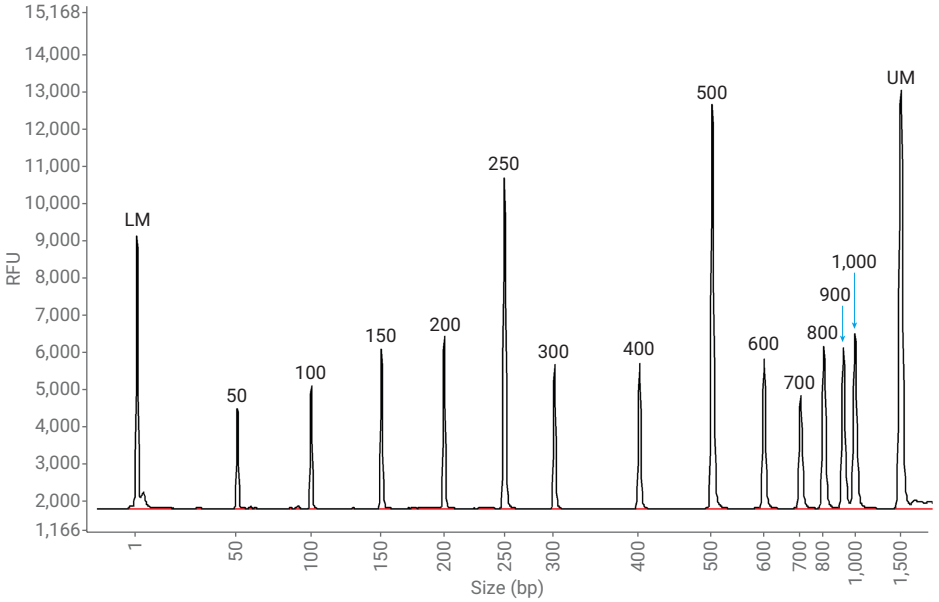


Figure 4. HS Small Fragment DNA Ladder separated on the 5200 Fragment Analyzer system with the short array. LM = lower marker; UM = upper marker.

Table 2. Minimum base pair fragment separation on the HS Small Fragment kit and the dsDNA 905 Reagent kit (1-500 bp) with the long, short, and ultrashort arrays. n = 3.

	Minimum base pair separation*					
	HS Small Fragment kit			dsDNA 905 Reagent kit (1-500 bp)		
	Long array (55 cm)	Short array (33 cm)	Ultrashort array (22 cm)	Long array (55 cm)	Short array (33 cm)	Ultrashort array (22 cm)
101 bp mix	3	3	3	3	3	3
201 bp mix	3	4	4	3	3	4
306 bp mix	3	4	5	3	3	5

* Includes partially resolved peaks

Both the dsDNA 905 Reagent kit (1-500 bp) and the HS Small Fragment kit with all arrays (ultrashort, short, and long array) provided fragment sizing within 5 % of the expected value, demonstrating the high sizing accuracy provided by the 5200 Fragment Analyzer system. The low standard deviation of each fragment in Table 3 demonstrates the excellent sizing precision of the Fragment Analyzer system. The sizing precision of all the single fragments was within the 2 % CV specifications stated for both kits. The average size, for each fragment in the fragment mixes, was displayed on the electropherograms in Figures 1, 2, and 3.

Conclusions

The 5200 Fragment Analyzer system offers exceptional separation of similarly sized fragments as demonstrated with fragments of known sizes. Both the qualitative dsDNA 905 Reagent kit (1-500 bp) and the quantitative HS Small Fragment kit provided a 3 bp separation with fragment sizing accuracy within 5 % of the expected value and sizing precision within 2 % CV. The 5200 Fragment Analyzer system demonstrated 3 bp separation with both the long and short array.

Table 3. Average sizing ± standard deviation for the known sizes of single fragments on the (A) dsDNA 905 Reagent kit (1 to 500 bp) and (B) HS Small Fragment kit. n = 3.

A	dsDNA 905 Reagent kit (1-500 bp)		
	Array		
Known size	Ultrashort	Short	Long
101	98 ± 0.0	98 ± 0.0	99 ± 0.0
102	99 ± 0.0	99 ± 0.0	100 ± 0.0
103	100 ± 0.0	100 ± 0.6	102 ± 0.0
104	101 ± 0.0	101 ± 0.0	103 ± 0.6
105	103 ± 0.0	103 ± 0.0	104 ± 0.0
201	202 ± 1.0	203 ± 0.6	205 ± 1.2
202	203 ± 1.0	205 ± 0.6	206 ± 1.2
203	204 ± 1.0	206 ± 1.0	207 ± 1.2
204	205 ± 0.0	206 ± 0.6	208 ± 1.2
205	206 ± 1.0	208 ± 0.6	209 ± 1.2
306	319 ± 0.0	315 ± 1.9	316 ± 0.0
307	319 ± 0.0	316 ± 1.3	316 ± 1.2
308	321 ± 1.0	316 ± 2.9	318 ± 0.6
309	323 ± 1.2	317 ± 3.2	319 ± 0.6
310	324 ± 0.0	321 ± 0.5	320 ± 0.0
311	324 ± 0.6	321 ± 1.0	321 ± 0.6

B	HS Small Fragment kit		
	Array		
Known size	Ultrashort	Short	Long
101	98 ± 0.0	98 ± 0.6	99 ± 0.6
102	98 ± 0.6	99 ± 0.6	100 ± 0.6
103	99 ± 0.6	100 ± 0.0	102 ± 1.7
104	100 ± 0.6	101 ± 0.0	103 ± 0.0
105	102 ± 0.6	103 ± 0.0	104 ± 0.0
201	201 ± 1.0	202 ± 1.7	203 ± 0.6
202	202 ± 0.0	203 ± 2.1	203 ± 2.3
203	203 ± 0.0	206 ± 0.6	205 ± 0.6
204	203 ± 1.7	207 ± 0.6	205 ± 0.6
205	206 ± 0.6	207 ± 1.5	206 ± 0.0
306	317 ± 0.0	317 ± 3.0	317 ± 0.0
307	317 ± 0.6	314 ± 3.5	318 ± 0.0
308	319 ± 0.6	319 ± 1.0	320 ± 0.0
309	321 ± 0.6	320 ± 0.0	320 ± 0.6
310	321 ± 0.6	319 ± 2.3	321 ± 0.0
311	323 ± 0.0	321 ± 2.1	322 ± 0.0

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