Application Note

Genomics

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Performance of the Agilent 55 and 165 kb BAC Kits on the Agilent Femto Pulse System

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Abstract

Bacterial artificial chromosomes (BAC) are a stable and versatile tool used for hosting entire gene(s) and their associated regulatory elements. Successful downstream results rely on proper gene integration. Verification of gene insertion into BACs is an essential quality control checkpoint for all BAC applications. Agilent has developed a reliable and swift analysis method for verification of gene insertion by sizing of digested BAC fragments. The Agilent Femto Pulse system, along with the 55 kb BAC kit and the 165 kb BAC kit, provides unparalleled fragment resolution, sensitivity, and accurate sizing of high molecular weight DNA fragments.

Introduction

Bacterial artificial chromosomes (BAC) were developed for cloning and maintenance of high molecular weight (HMW) DNA (100 to 350 kb) in *Escherichia coli*. BACs can contain an entire gene (or genes) and all its associated regulatory elements or loci to control expression. They are versatile tools used to easily manipulate gene expression in specific cell types and a plethora of disease models. BAC libraries have also been constructed to isolate genes coding for disease resistance in plants using a map-based cloning approach. BAC clones are often used in *de novo* genome assembly, reducing sequence assembly down to 40 to 200 kb instead of an entire genome. After sequencing of the digested clone, overlay of fragments and alignment of different restriction digest points allows for assembly of the molecular backbone of the gene without a reference sequence for alignment. The ability of BAC clones to house large genes has made them the best option for whole genome mapping and *de novo* sequencing projects for microbes, plants, animals, and humans.

Accurate sizing of BAC clones is an important step in quality control of associated workflows. This ensures that the gene has been inserted in its entirety and correct downstream libraries are produced. Pulsed-field gel electrophoresis (PFGE), a classic method for size and integrity assessment of gDNA and BAC clones, requires a minimum of 12 hours to complete, with limited sensitivity and a large amount of sample input. The Femto Pulse system presents a technological leap in sizing and quality assessment of high molecular weight DNA and BAC fragments by delivering superior fragment resolution in 90 minutes, while requiring only picogram levels of sample. The Femto Pulse system smoothly integrates into gDNA and BAC workflow processes by increasing throughput, eliminating long run times, and reducing labor and costs.

Experimental

Separation on the Agilent Femto Pulse system was performed with the Agilent 55 kb BAC kit (p/n FP-1003-0275) and the 165 kb BAC kit (p/n FP-1004-0275).

Individual fragments

The 55 kb BAC kit was used to analyze the 1,000, 20,000, and 48,500 bp NoLimits Individual DNA fragments (Thermo Fisher Scientific, p/n SM1671, SM1541, and SD0011, respectively), while the 165 kb BAC kit was used to analyze the 20,000 bp NoLimits Individual DNA fragment (Thermo Fisher Scientific, p/n SM1541) and the 83,000 bp BAC digestion fragment (Amplicon express, custom order).

Multifragment samples

The 55 kb BAC kit was used to analyze the Lambda DNA/HindIII Marker (Thermo Fisher Scientific, p/n SM0101), while the 165 kb BAC kit was used to analyze the digestion of the T4 GT7 DNA (FUJIFILM Wako Pure Chemicals, p/n 318-03971) with restriction enzymes PmeI and SacII (New England Biolabs, p/n R0560S and R0157S, respectively).

Ladders

The Agilent 55 kb BAC ladder (p/n FP-7003-U035) and 165 kb BAC ladder (p/n FP-7004-U035) were analyzed on the Femto Pulse system.

Results and discussion

Kit overview

Agilent offers two kits for analysis of digested BAC samples on the Femto Pulse system (Table 1). The 55 kb BAC kit is designed for accurate sizing of fragments ranging from 75 to 48,500 bp, while the 165 kb BAC kit is intended for samples with a wide range of sizes between 75 to 165,000 bp. The two kits utilize different pulsing conditions and different separation times, 90 minutes for the 55 kb BAC kit and an extended run time of 170 minutes for the 165 kb BAC kit.

55 kb BAC kit

The 55 kb BAC kit offers exceptional sizing and separation resolution for enzyme-digested BAC samples and DNA samples with single or multiple fragments less than 55 kb. A dilution series covering the single fragment concentration range of 1.6 to 25 pg/µL on the 55 kb BAC kit was performed with a 1,000 bp fragment (Figure 1). The average size over the dilution series on two different Femto Pulse systems was 1,021 bp with very high precision of 3.8% (% CV) and accuracy of 2% (% error).

A dilution series of the multifragment sample Lambda HindIII was separated on three different Femto Pulse systems with the 55 kb BAC kit (Figure 2). The Lambda HindIII sample has eight fragments with expected sizes of 564, 2,027, 2,322, 4,361, 6,557, 9,416, 23,130, and 27,491 bp. All eight fragments displayed baseline resolution, including the closely sized 2,027 and 2,322 bp fragments. A high precision and accuracy, below 5% for both, was reported for all fragments across the multifragment concentration range (6.3, 12.5, 25, 50, and 100 pg/µL) of the 55 kb BAC kit.

Table 1. Overview of the Agilent 55 kb BAC kit and the 165 kb BAC kit.

Specification	55 kb BAC Kit	165 kb BAC Kit
DNA Sizing Range	75 to 48,500 bp	75 to 165,000 bp
DNA Fragment Concentration Range*		
Multiple Fragment Concentration	6.3 to 100 pg/µL	12.5 to 100 pg/µL
Single Fragment Concentration ≤48,500 bp	1.6 to 25 pg/µL	1.6 to 25 pg/µL
Single Fragment Concentration ≥48,500 bp	-	3 to 50 pg/µL
Sizing Precision	15% CV	15% CV
Sizing Accuracy	±15%	±15%
Pulsed Field Separation Time	90 minutes	170 minutes

*Determined using 1,000 bp; 20,000 bp; 48,500 bp; 83,000 bp fragments, BAC samples, and commercial ladders as samples.

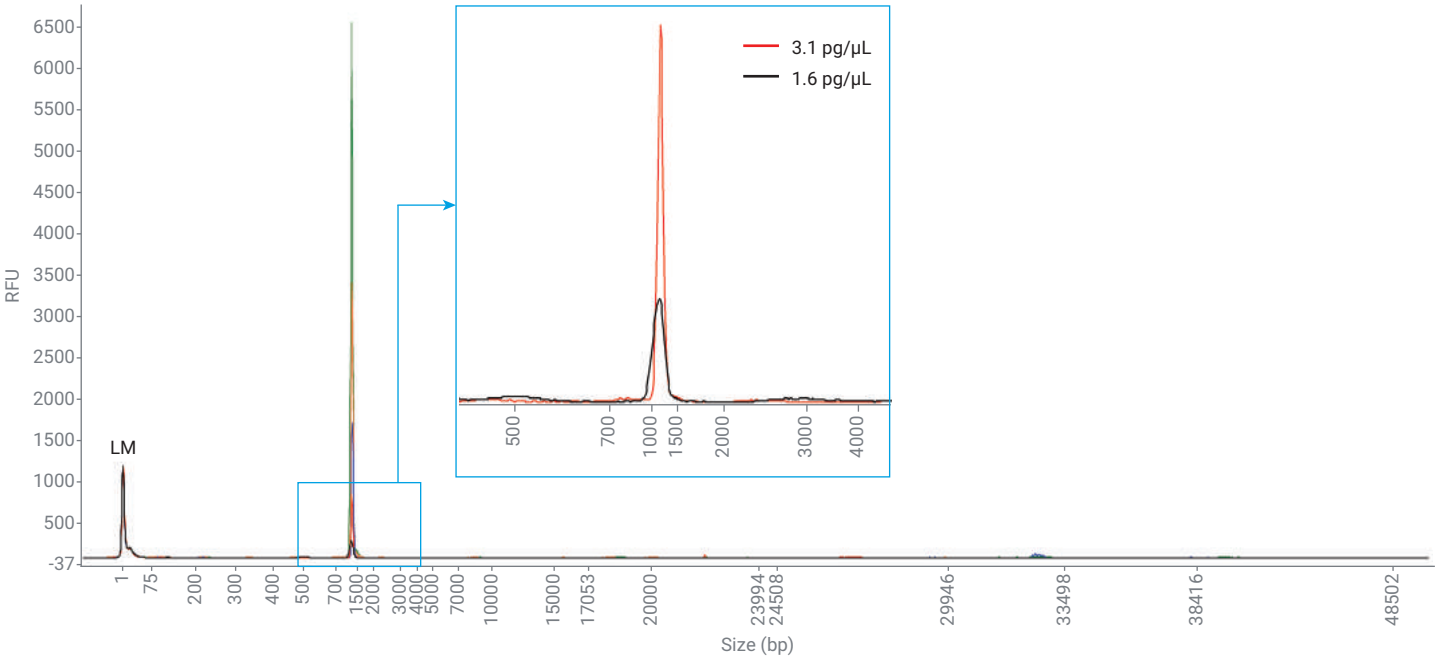


Figure 1. A dilution series from 1.6 to 25 pg/µL of the 1,000 bp DNA fragment separated on the Agilent Femto Pulse system over the concentration range of the Agilent 55 kb BAC kit. The average size is 1,021 bp. LM = lower marker; n = 6.

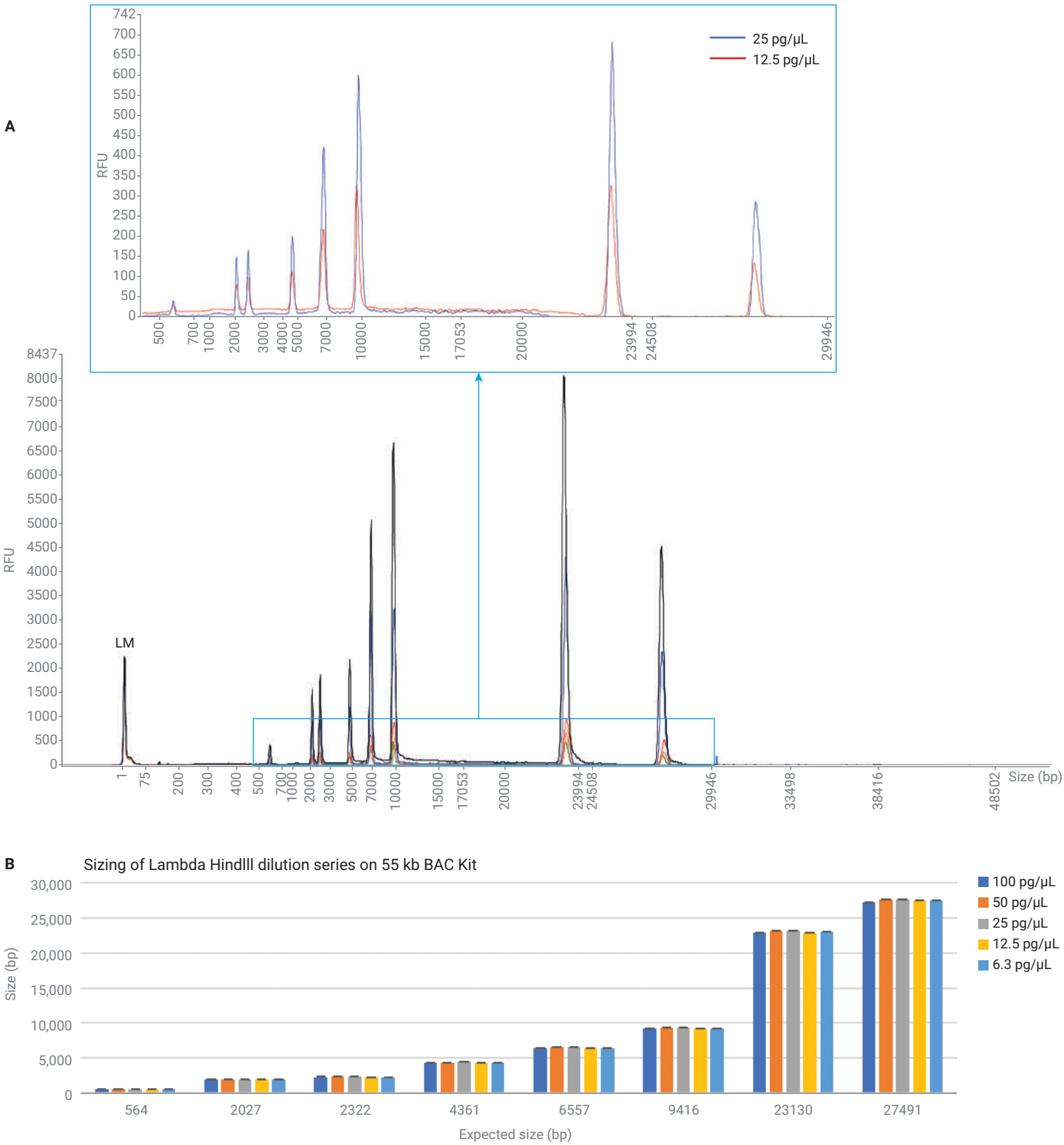


Figure 2. Dilution series from 6.3 to 100 pg/µL of Lambda HindIII multifragment sample separated on the Agilent Femto Pulse system with the 55 kb BAC kit. (A) Electropherogram overlay of dilution series. (B) Average size of each fragment over the dilution series. LM = lower marker; n = 9.

165 kb BAC kit

The 165 kb BAC kit is intended for samples containing fragments with a wide range of sizes from 75 to 165,000 bp. A dilution series covering the entire single fragment concentration range (3 to 50 pg/µL) of the 165 kb BAC kit was performed with the 83,000 bp BAC fragment (Figure 3). The average size of the 83,000 bp BAC fragment over the dilution series, on four different Femto Pulse systems, was 92,244 bp with 2.2% CV and 11% error. However, the 50 pg/µL concentration provided the most accurate sizing at 87,539 bp, with a percent error of 5.5%. Sample concentrations ranging from 3 to 25 pg/µL had an average percent error of 12.6%. Sizing of large fragments becomes more accurate the closer their concentration is to the concentration of the sizing ladder. The concentration of the 165 kb Ladder is 100 pg/µL. A concentration of 50 pg/µL for single fragments over 48,500 bp on the 165 kb BAC kit will provide extremely accurate sizing.

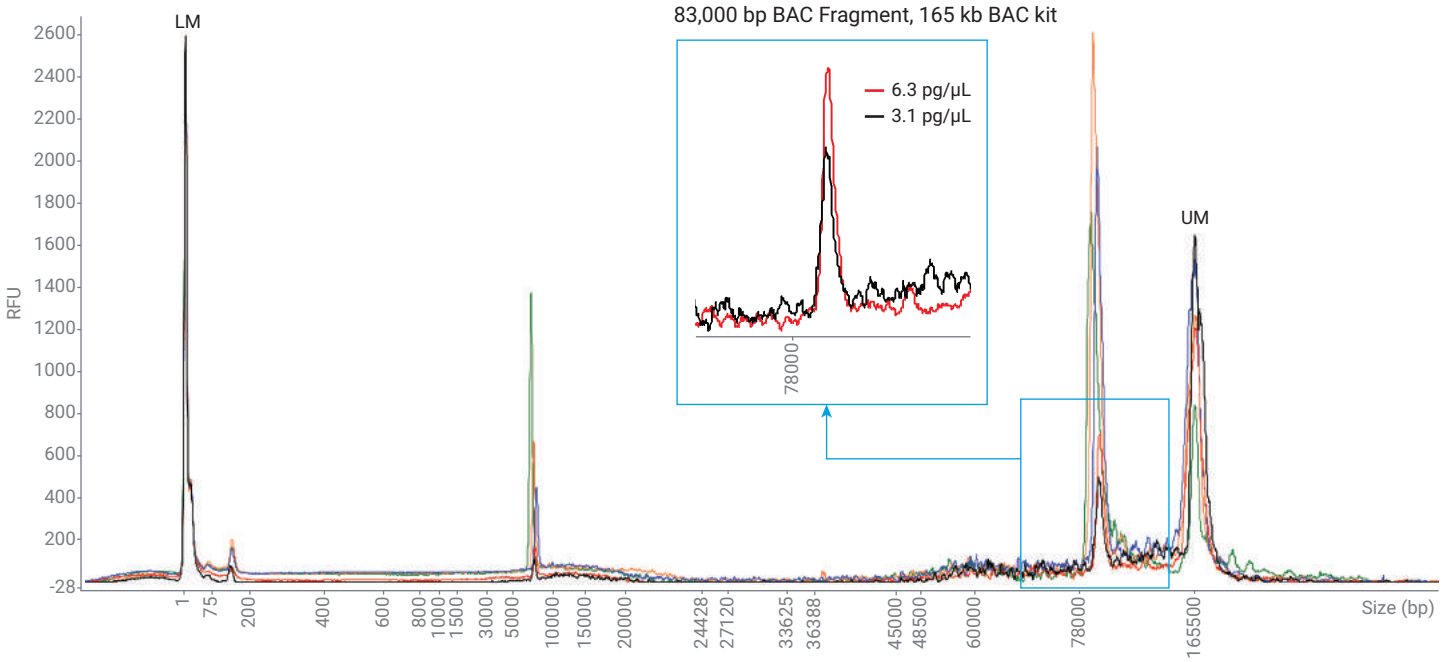


Figure 3. A dilution series from 3 to 50 pg/µL of the 83,000 bp DNA fragment separated on the Agilent Femto Pulse system over the concentration range of the Agilent 165 kb BAC kit. The average size is 92,244 bp. The BAC clone without the gene inserted is observed around 8,000 bp. LM = lower marker; UM = upper marker; n = 16.

The multifragment sample, T4 GT7 DNA PmeI and SacII digest, was separated on two different Femto Pulse systems with the 165 kb BAC kit (Figure 4). A T4 GT7 DNA PmeI and SacII digest results in five fragments with expected sizes of 4,928, 29,182, 34,110, 44,843, and 86,707 bp. All five fragments were observed and displayed baseline resolution. All fragments across the multifragment concentration range reported a percent precision and accuracy well below the specifications of the 165 kb BAC kit (Table 1).

Comparison of the 55 kb and 165 kb BAC kits

The 55 kb BAC and the 165 kb BAC kit have an overlapping sizing range from 75 to 48,500 bp. The 20,000 and 48,500 bp (lambda) fragments were analyzed with both kits to compare sizing accuracy between the kits. The analysis was performed in triplicate on four different Femto Pulse systems throughout the appropriate concentration range for each single fragment.

The 20 kb fragment was chosen for comparison to demonstrate the accuracy of the lower sizing range of the 165 kb BAC kit. Throughout the single fragment concentration range for fragments less than 48,500 bp, of 1.6 to 25 pg/µL, the 20 kb fragment averaged 20,005 and 19,636 bp for the 55 kb and 165 kb kits, respectively (Figure 5).

Both kits provided precision and accuracy well below the specifications of the kits.

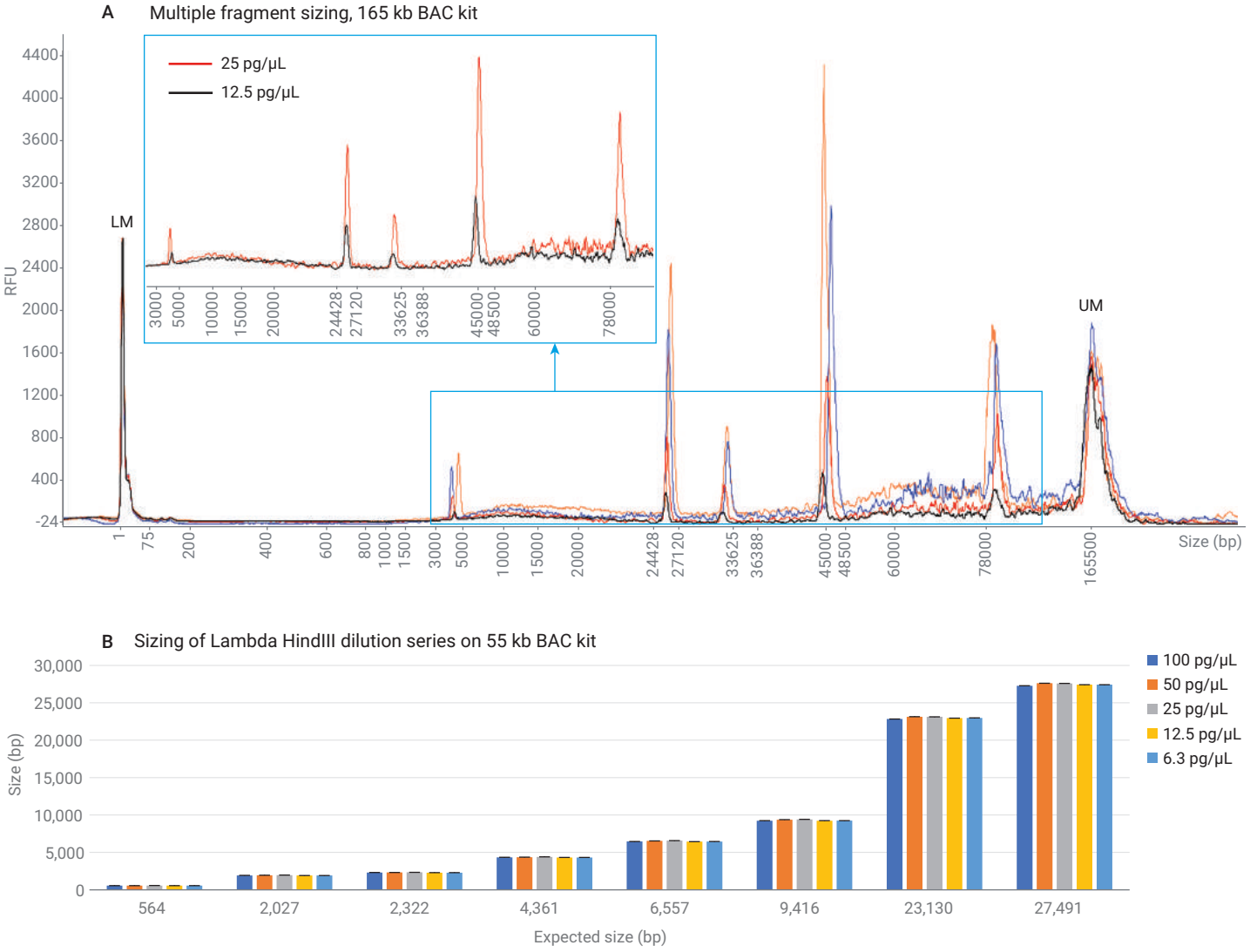
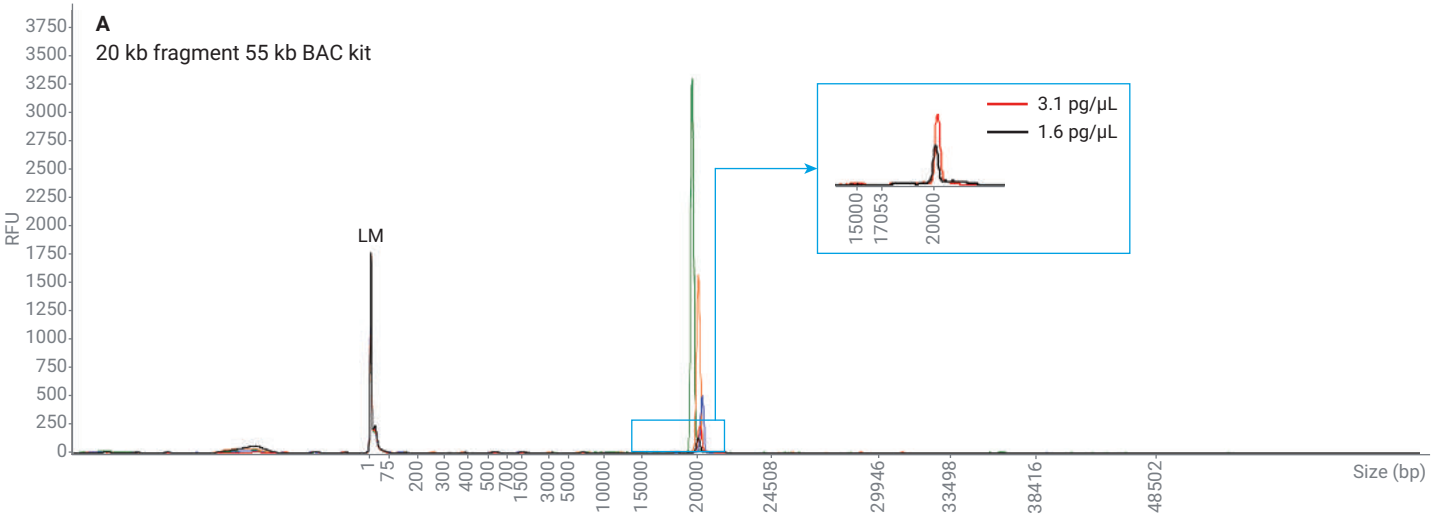
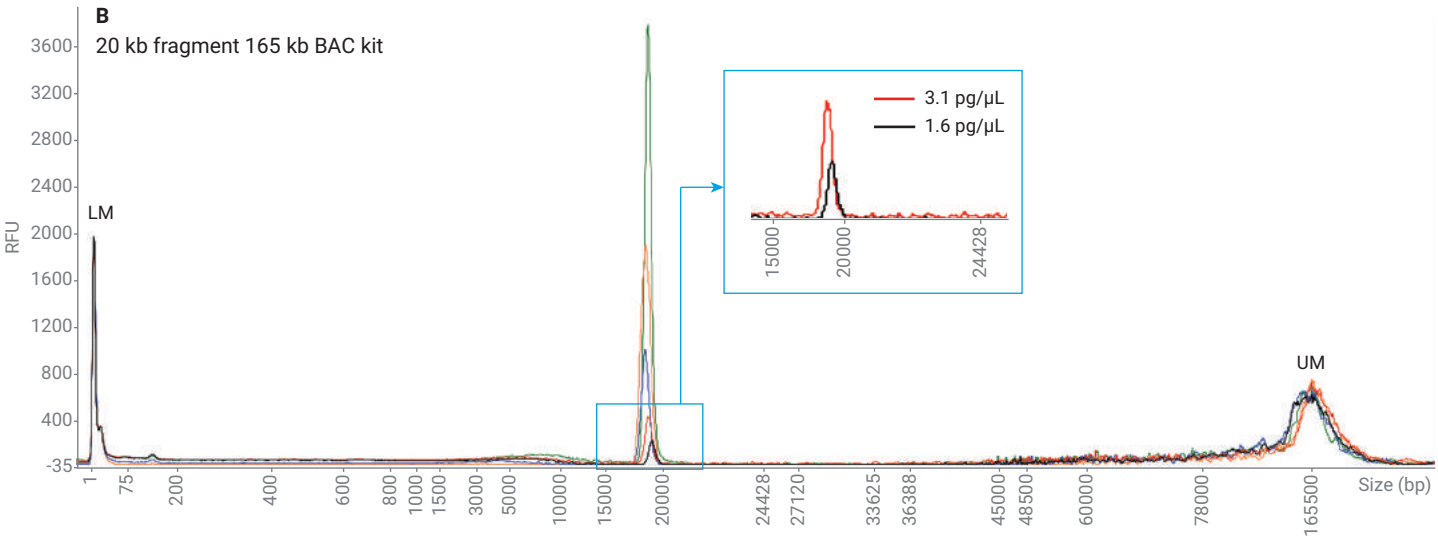


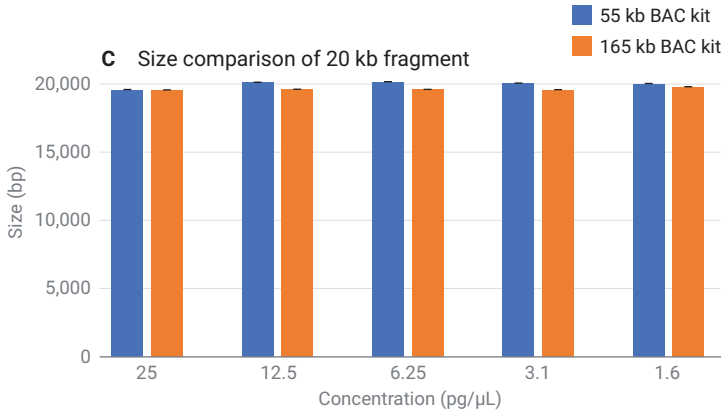
Figure 4. Multiple fragment sample separated on the Agilent Femto Pulse system with the 165 kb BAC kit. (A) Electropherogram overlay through the concentration range of 12.5 to 100 pg/µL. (B) Average size of the multiple fragment sample through the concentration range of the kit. LM = lower marker; UM = upper marker; n = 6.

The average percent precision and accuracy for the 55 kb BAC kit was lower than the 165 kb BAC kit (Figure 5D).

This was expected due to the different pulsing methods of each kit. The 55 kb BAC kit has a pulsing method focused on separation of lower molecular weight fragments under 48,500 bp compared to the 165 kb BAC kit, which is directed toward separation of the higher molecular weight fragments.





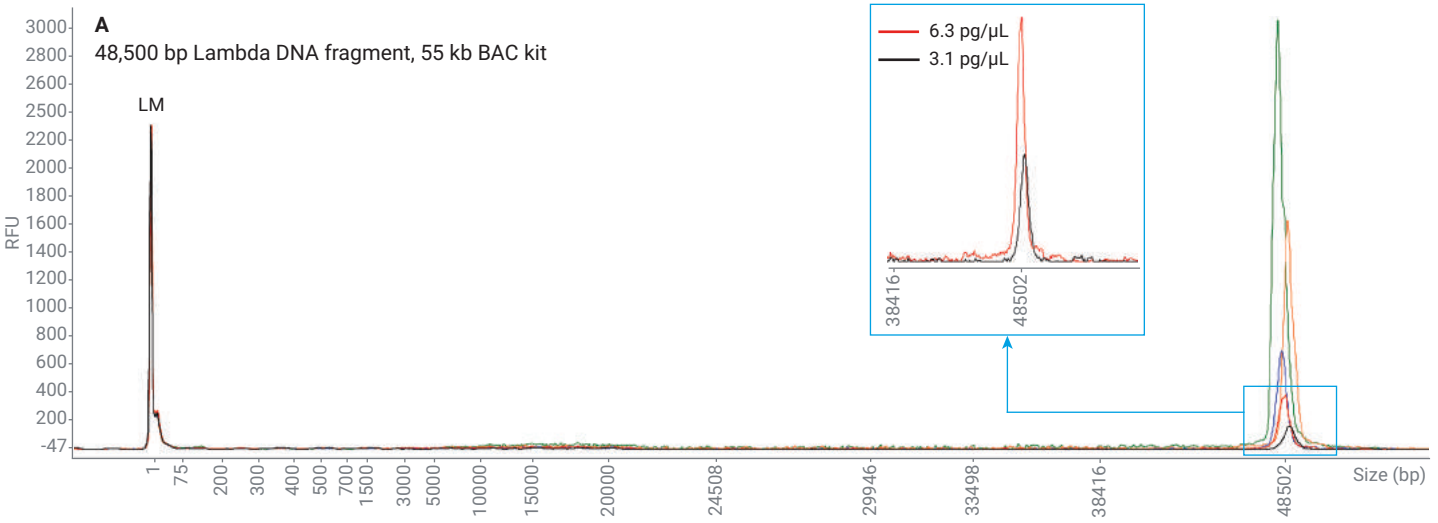


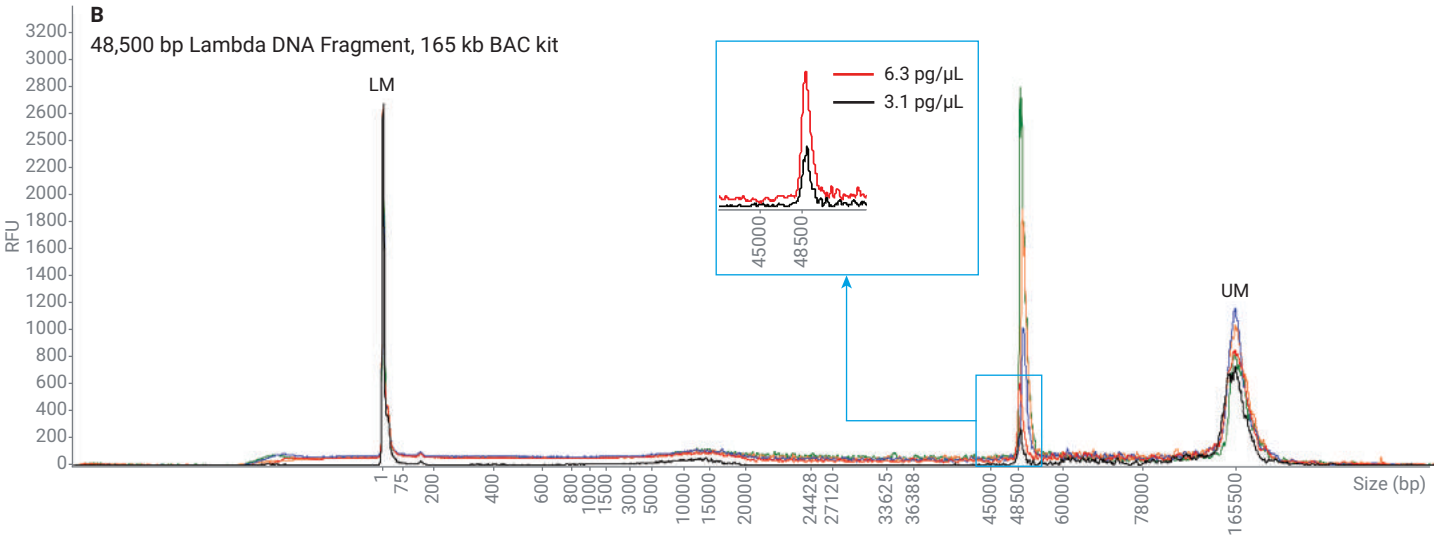
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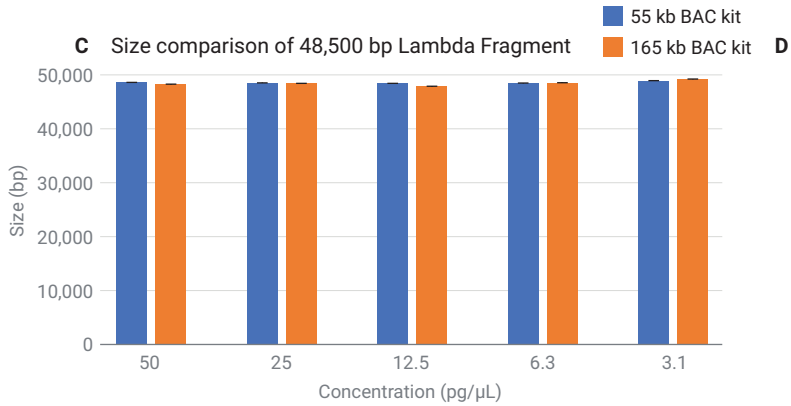
20,000 kb Fragment						
Concentration (pg/µL)	55 kb BAC Kit			165 kb BAC Kit		
	Average Size (bp)	% CV	% Error	Average Size (bp)	% CV	% Error
25	19,601	0.7%	-2.0%	19,570	3.1%	-2.1%
12.5	20,136	0.5%	0.7%	19,617	4.3%	-1.9%
6.3	20,173	1.2%	0.9%	19,603	4.7%	-2.0%
3.1	20,074	0.7%	0.4%	19,584	3.1%	-2.1%
1.6	20,040	0.3%	0.2%	19,803	1.9%	-1.0%
1.6 to 25	20,005	0.7%	0.02%	19,636	3%	-1.8%

Figure 5. The 20,000 bp fragment analyzed on the Agilent Femto Pulse system with the (A) Agilent 55 kb BAC kit and (B) Agilent 165 kb BAC kit. (C) Comparison of the average size over the single fragment concentration range of 1.6 to 25 pg/µL. (D) Table comparing the size, precision (% CV), and accuracy (% error). LM = lower marker; UM = upper marker; n = 12.

The 48,500 bp lambda fragment was chosen for comparison to demonstrate the accuracy of the higher sizing range of the 55 kb BAC kit and the lower end of the 165 kb BAC kit. Throughout the single fragment concentration range of 3.1 to 50 pg/µL, the 48,500 bp lambda fragment averaged 48,492 and 48,396 bp for the 55 kb BAC and 165 kb BAC kits, respectively (Figure 6).







D

48,500 bp Lambda Fragment						
Concentration (pg/µL)	55 kb BAC Kit			165 kb BAC Kit		
	Average Size (bp)	% CV	% Error	Average Size (bp)	% CV	% Error
50	48,589	1.8%	0.2%	48,268	2.3%	-0.5%
25	48,518	2.6%	0.0%	48,409	1.8%	-0.2%
12.5	48,421	2.2%	-0.2%	47,891	1%	-1.3%
6.3	48,472	1.7%	-0.1%	48,546	0.2%	0.1%
3.1	48,933	1.4%	0.9%	49,206	2.3%	1.5%
3.1 to 50	48,492	2%	-0.02%	48,396	2%	-0.2%

Figure 6. The 48,500 bp lambda fragment analyzed on the Agilent Femto Pulse system with the (A) Agilent 55 kb BAC kit and (B) Agilent 165 kb BAC kit. (C) Comparison of the average size over the single fragment concentration range of 3.1 to 50 pg/µL. (D) Table comparing the size, precision (% CV), and accuracy (% error). LM = lower marker; UM = upper marker; n = 12.

Both kits provided similar percent precision and accuracies that were well below the specifications for the kits.

A sample of mixed fragments covering the sizing range of both the 55 kb BAC and the 165 kb BAC kits was analyzed to determine sizing linearity. Both kits provided a linear sizing range as determined by the slope and an R^2 value close to one (Figure 7). Both the 55 kb BAC and 165 kb BAC kits provided accurate and reliable sizing. Accurate sizing with excellent precision can be achieved with either kit for fragments that overlap the sizing range of both kits.

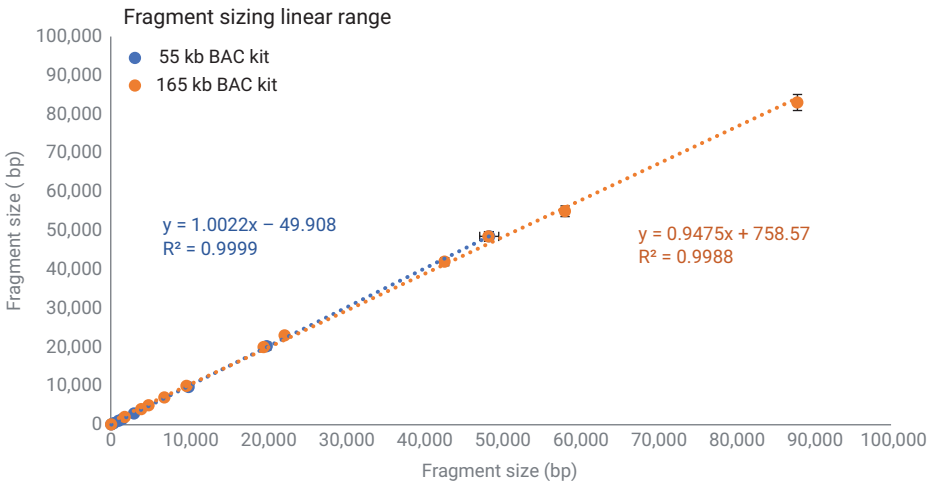


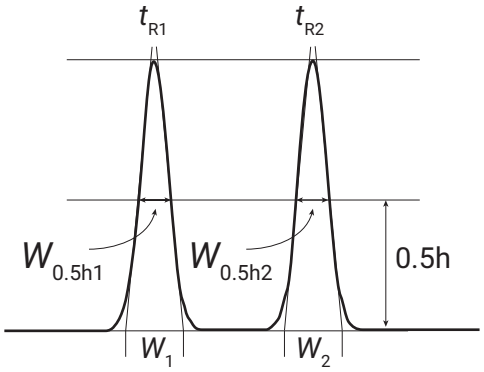
Figure 7. Multiple fragment sample run on the Agilent Femto Pulse system with the 55 kb BAC and the 165 kb BAC kits. A linear sizing range was displayed for both kits.

Ladder resolution

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 equations are commonly used for calculating resolution (Figure 8). Peaks are usually successfully differentiated when resolution is greater than one.

Equation 1: $$R = \frac{t_{R2} - t_{R1}}{1/2(W_1 + W_2)}$$

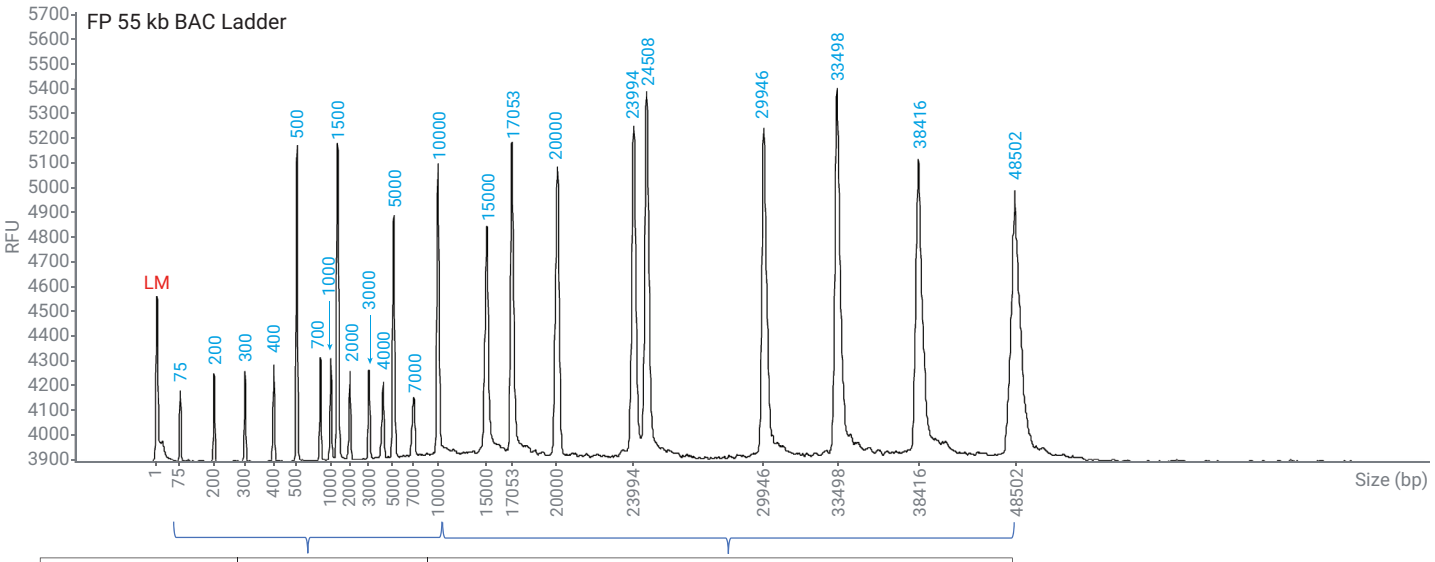
Equation 2: $$R = 1.18\left(\frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}}\right)$$



t_{R1}, t_{R2}	Retention time for each peak ($t_{R1} < t_{R2}$)
$W_{0.5h1}$, $W_{0.5h2}$	Full width at half maximum (FWHM) of each peak
W_1, W_2	Width of each peak

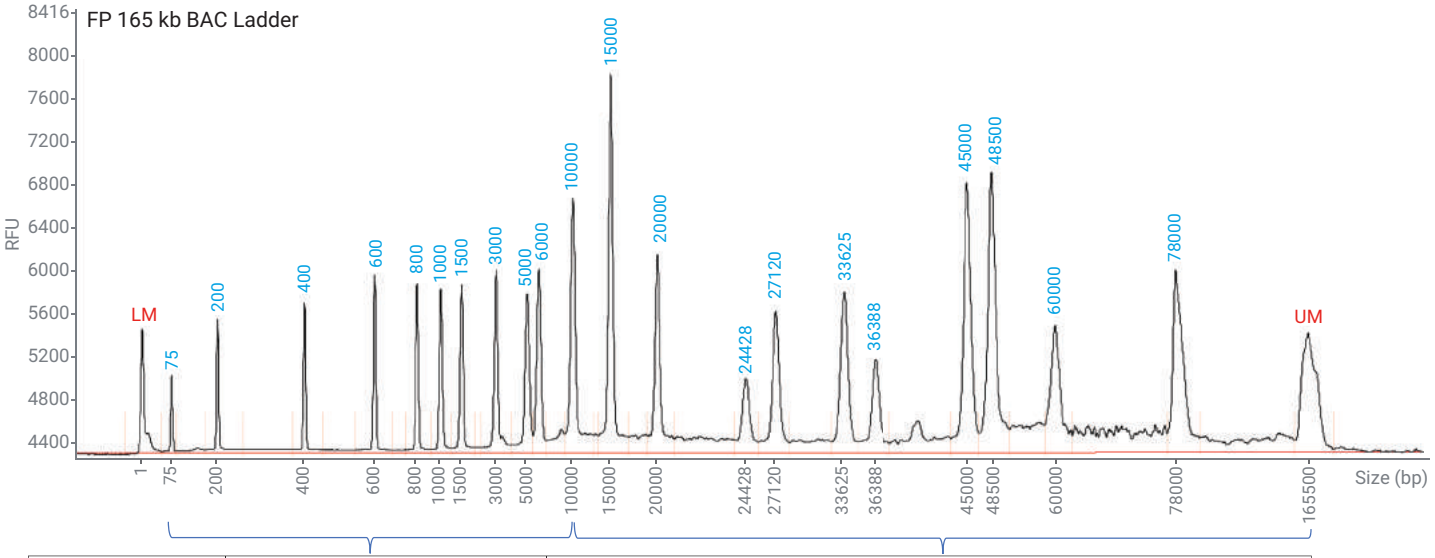
Figure 8. Equations and diagram for calculating resolution (R).

A lower percent resolution indicates better separation between the ladder fragments. To provide a more in-depth view of the resolution throughout the large sizing range of the BAC kits, the percent resolution was divided into two sizing ranges representing the lower and higher molecular weight DNA fragments. The 55 kb and 165 kb BAC kits reported similar percent resolutions of 7.0 and 7.5%, respectively, for the lower molecular weight range of 75 to 10,000 bp (Figures 9 and 10). The higher molecular weight ranges, 10 to 55 kb and 10 to 165 kb, provided a lower percent resolution of 2.1 and 4.7%, respectively, indicating better resolution than the lower sizing range. This is due to the pulsed-field methods that were developed especially for exceptional resolution for the larger molecular weight fragments found in BAC samples.



Size Range (bp)	75 to 10,000	10,000 to 48,500
Percent Resolution	7.0%	2.1%
Standard Deviation	4.4	1.0

Figure 9. The Agilent FP 55 kb BAC Ladder separated on the Agilent Femto Pulse system with the 55 kb BAC kit. LM = lower marker.



Size Range (bp)	75 to 10,000	10,000 to 165,500
Percent Resolution	7.5%	4.7%
Standard Deviation	4.4	2.2

Figure 10. The Agilent FP 165 kb BAC Ladder separated on the Agilent Femto Pulse system with the 165 kb BAC kit. LM = lower marker; UM = upper marker.

Conclusion

The Femto Pulse system offers excellent separation resolution and reliable sizing of digested BAC clones with the 55 kb BAC kit and the 165 kb BAC kit. The 55 kb BAC kit is designed for samples with fragments between 75 bp and 48.5 kb, while the 165 kb BAC kit is ideal for samples with a wide range of DNA fragment sizes between 75 bp to 165 kb. Both kits have a linear sizing range with excellent sizing accuracy in the overlapping sizing range.

Analysis of digested samples occurs within 90 minutes with the 55 kb BAC kit and 170 minutes with the 165 kb BAC kit, a fraction of the time required with traditional gel analysis.

www.agilent.com/genomics/femto-pulse

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