

Comparison of BAC Fragment Sizing with the Agilent Femto Pulse System and Pulsed-Field Gel Electrophoresis

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Abstract

Bacterial artificial chromosomes (BAC) are a stable and versatile tool used for hosting an entire gene (or genes) and their associated regulatory elements. Quality control analysis of BAC clones ensures proper integration of genes and their regulatory elements. Agilent has developed a reliable and swift analysis method for verification of gene insertion by sizing digested BAC fragments. The Agilent Femto Pulse system along with the 55 kb BAC kit and the 165 kb BAC kit provides unparalleled separation speed, resolution, and sensitivity of high molecular weight DNA fragments compared to the classical method of pulsed-field gel electrophoresis (PFGE).

Introduction

BACs were developed for cloning and maintenance of high molecular weight DNA (100 to 350 kb) in *Escherichia coli*. BACs can contain an entire gene (or genes) and all its associated regulatory elements or locus to control expression. Therefore, BACs can serve as a loci source for predictable vector expression of infectious viruses or high-level production of recombinant proteins. They are a versatile tool 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, 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 an indispensable tool 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. PFGE, a classic method for size and integrity assessment of gDNA and BAC clones, requires 12 to 18 hours to complete with limited sensitivity, while requiring a large amount of sample.

The Femto Pulse system presents a technological leap in sizing and quality assessment of high molecular weight (HMW) 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 clone workflow processes by increasing throughput, eliminating long run times, and reducing labor and costs.

Experimental

DNA samples were separated on the Agilent Femto Pulse system (p/n M5330AA) with the Agilent 55 kb BAC kit (p/n FP-1003-0275), the Agilent 165 kb BAC kit (p/n FP-1004-0275), and by PFGE on the Pippin Pulse (Sage Science) with the 5 to 80 kb method for 12 hours. The Agilent FP 55 kb BAC Ladder (p/n FP-7003-U035) and the FP 165 kb BAC Ladder (p/n FP-7004-U035) were used with both separation methods. Various types of samples were analyzed including ladders, enzyme digested BAC samples, and blends of different-sized fragments. BAC sample no. 1 was the FP 165 kb BAC Ladder, no. 2 and 3 are

custom BAC digested samples, no. 7 was Lambda DNA *Hind* III (Thermo Fisher, p/n SM0101), no. 8 (custom mix of BAC fragments and Lambda DNA *Hind* III), no. 9 was a 75 kb lambda ladder plus a 20 kb fragment (Thermo Fisher, p/n SM1541), and no. 10 was the FP 55 kb BAC Ladder. Expected sizes are based on reported sizes from commercial products and enzyme restriction sites for custom digestions.

Results and discussion

55 kb BAC kit and 165 kb BAC kit

There are 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 pulsed-field capillary electrophoresis separation methods specifically designed for the different size ranges of the two kits, with a separation time of 90 minutes for the 55 kb BAC kit and an extended run time of 170 minutes for the 165 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 Fragments' Concentration Range*: Multiple Fragment Concentration	6 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	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; 55,000 bp; 83,000 bp fragments, BAC samples, and commercial ladders as samples.

55 kb BAC kit

The 55 kb BAC kit offers exceptional sizing and separation resolution for enzyme-digested BAC samples and DNA samples with multiple fragments less than 55 kb. Separation of the FP 55 kb BAC Ladder was compared between the Femto Pulse system and PFGE. The FP 55 kb BAC Ladder has 24 fragments including the lower marker. Analysis of the FP 55 kb BAC Ladder (sample no. 10) on the Femto Pulse system provided easily identifiable sharp peaks with excellent separation between each peak (Figure 1D). However, separation of the ladder on the PFGE resulted in visualization of half of the ladder fragments (Figure 1E). PFGE was unable to separate the two fragments with sizes of 24 and 24.5 kb, while the lower concentrated fragments sized at 2,000, 3,000, 4,000, and 7,000 bp are only visible after color inversion (not shown). In addition, the nine fragments from 75 to 1,500 bp are no longer present on the PFGE gel. The Femto Pulse system offers the advantage of providing separation of a wide range of

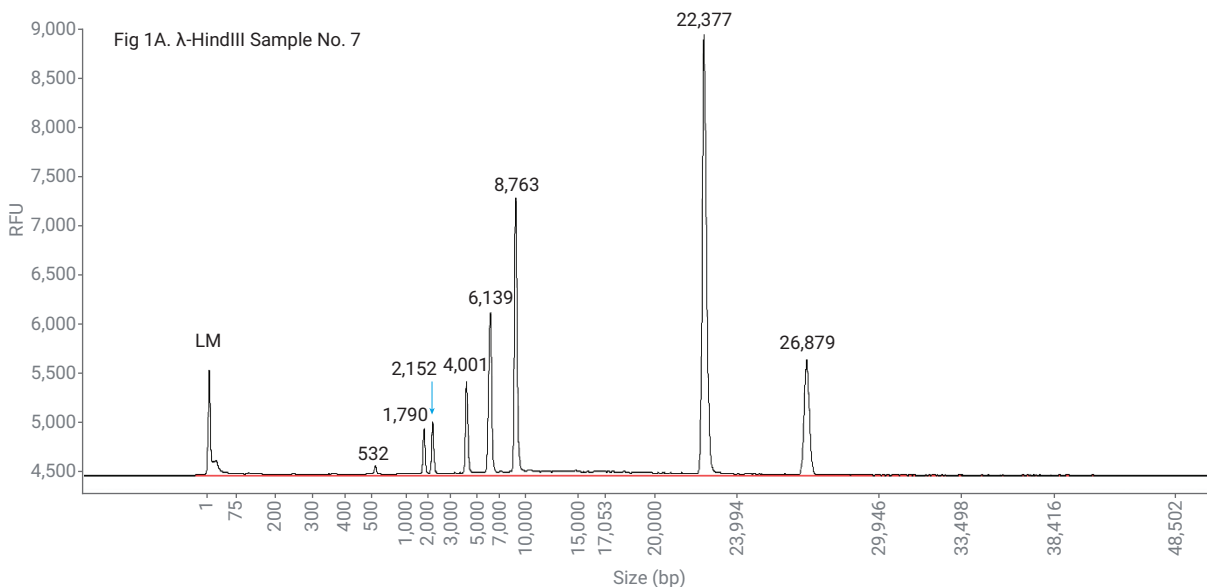
fragment sizes, at low concentrations, with excellent resolution.

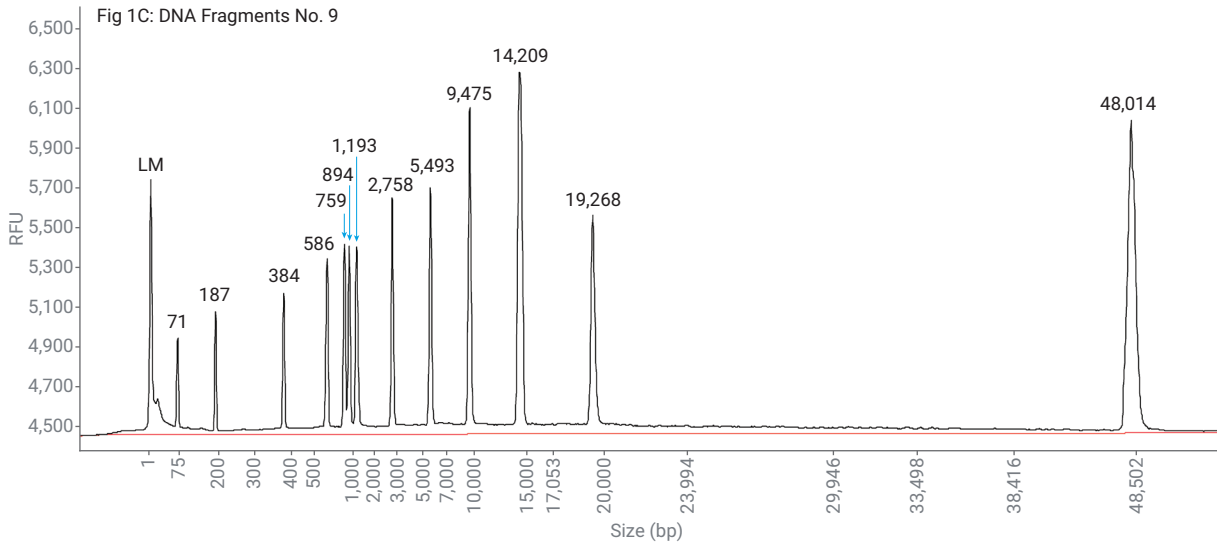
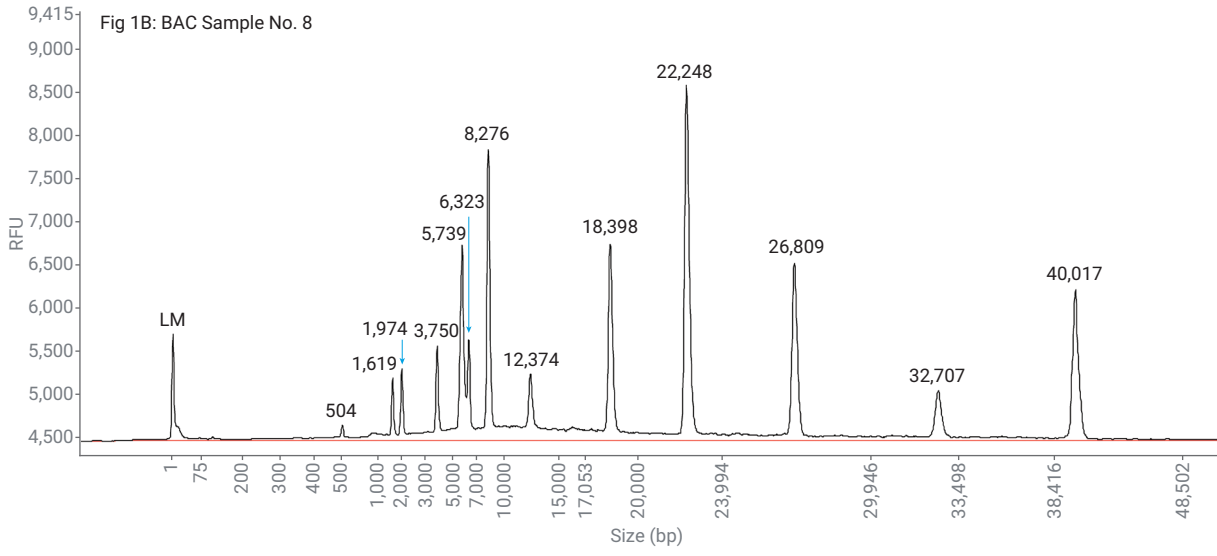
Lambda DNA digested with *Hind* III (λ -HindIII, sample no. 7) was separated on the Femto Pulse with the 55 kb BAC kit (Figure 1A). The electropherogram displayed sharp peaks with excellent baseline resolution between each peak. The same sample separated by PFGE showed peaks 2 through 8 with similar sizes as the Femto Pulse system. Unfortunately, smaller sized fragments run off the end of PFGE gels, and are lost in the process of separating the HMW fragments. This incidence was noted with the loss of the smaller 564 bp fragment on the PFGE gel. The Femto Pulse system has the advantage of being able to separate and size a wide range of molecular weight fragments all at the same time. Sizing of each λ -HindIII fragment was very precise and accurate on the Femto Pulse system as seen by the small percent CV (indicating high precision) and percent error (indicating high accuracy). The same sample was compared on four different Femto

Pulse systems, and resulted in very tight percent CV and small percent error, demonstrating reliable separation and sizing across multiple instruments (Table 2).

BAC sample no. 8 contained 13 expected fragments. Analysis on the Femto Pulse system with the 55 kb BAC kit displayed all 13 peaks, even separating the closely sized fragments of 6,557 and 6,800 bp (Figure 1B). The PFGE separation allowed for visualization of 9 of the 13 fragments. The lower concentration and smaller fragments with expected sizes from 564 to 4,361 bp were not visible on the PFGE gel. Sample no. 8 was compared on four different Femto Pulse systems, and provided precision (% CV) and accuracy (% error) (Table 3) within the specifications of the kit (Table 1).

BAC sample no. 9 consisted of 13 expected fragments. Analysis on the Femto Pulse system with the 55 kb BAC kit displayed all 13 peaks, even separating the closely sized fragments of 800 and 1,000 bp (Figure 1C). The PFGE separation displayed the six





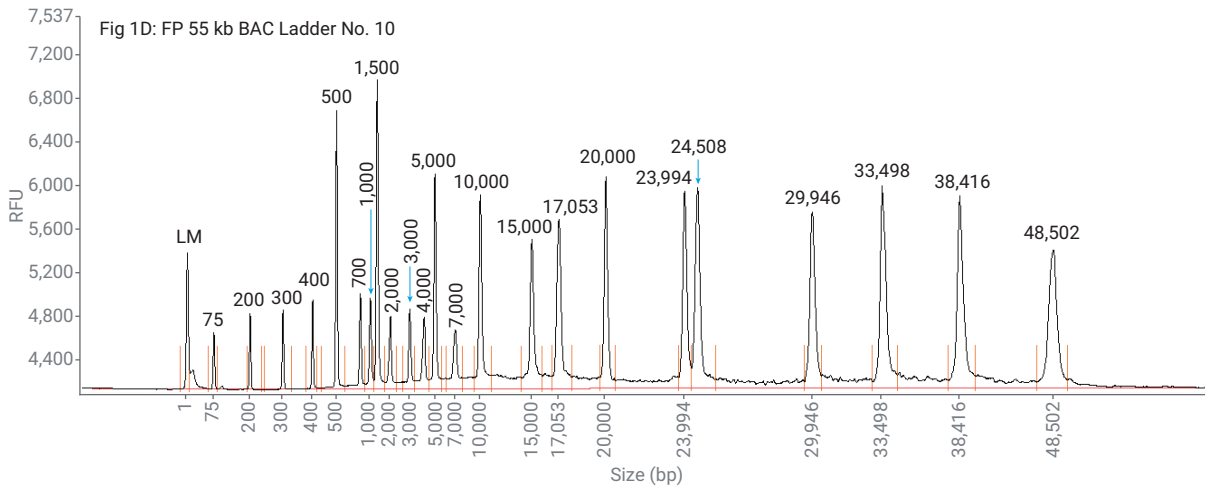


Figure 1A-D. DNA samples were separated on the Agilent Femto Pulse System with the 55 kb BAC kit. Agilent FP 55 kb BAC Ladder, sample no.10. LM = lower marker.

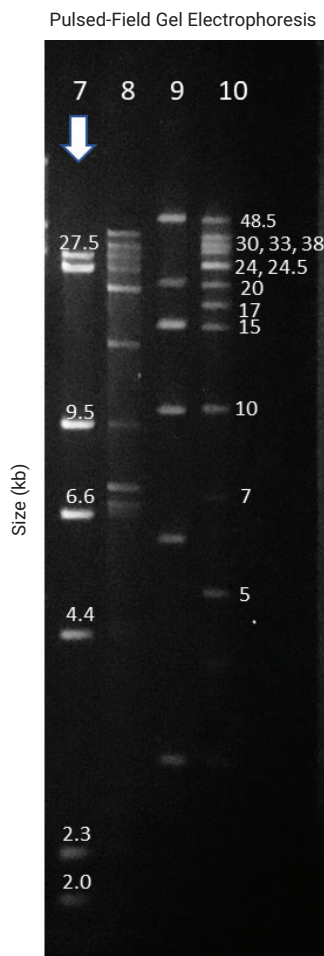


Figure 1E. PFGE separation of sample no. 7, 8, 9, and 10.

Table 2. λ -HindIII sample no. 7 was separated on four different Agilent Femto Pulse systems with the 55 kb BAC kit, resulting in tight precision (% CV) and accuracy (% error). *n = 12 for each instrument.

Peak Number	Expected Size (bp)	55 kb BAC kit λ -HindIII Sample no. 7				Average Size (bp)	% CV	% Error
		Average Peak Size (bp)*						
		#1	#2	#3	#4			
1	564	574	543	567	579	565	2.8%	0.3%
2	2,027	2,029	1,866	1,984	2,024	1,976	3.8%	-2.6%
3	2,322	2,452	2,236	2,385	2,450	2,381	4.3%	2.5%
4	4,361	4,589	4,204	4,456	4,569	4,455	4.0%	2.1%
5	6,557	6,768	6,350	6,634	6,728	6,620	2.8%	1.0%
6	9,416	9,685	9,094	9,458	9,651	9,472	2.9%	0.6%
7	23,130	23,398	22,700	23,014	23,074	23,047	1.2%	-0.4%
8	27,491	28,035	27,246	27,602	27,630	27,628	1.2%	0.5%

highest molecular weight fragments. The smaller fragments with expected sizes from 75 to 1,474 bp were not present on the PFGE gel. Sample no. 9 was compared on four different Femto Pulse systems, and resulted in precision (% CV) and accuracy (% error) within the specifications of the kit (Table 4).

165 kb BAC kit

The 165 kb BAC kit is intended for samples containing DNA fragments with a wide range of sizes from 75 to 165,000 bp. Separation of the FP 165 kb BAC Ladder was compared between the Femto Pulse system and PFGE. The FP 165 kb BAC Ladder has 23 fragments including the lower and upper marker. Analysis of the FP 165 kb BAC Ladder (sample no. 1) on the Femto Pulse system provided easily identifiable sharp peaks with excellent separation between the peaks (Figure 2A). Separation of the ladder on the PFGE resulted in visualization of 12 to 13 of the 23 fragments (Figure 2D). PFGE was unable to separate fragments 24 and 27 kb from each other, and separation of the 45 and 48.5 kb fragments was questionable. In addition, the seven fragments from 75 to 1,500 bp migrated off the PFGE gel, with the 3,000 bp fragment just barely visible at the bottom. The Femto Pulse system offers the advantage of providing separation of a wide range of fragment sizes at low concentrations with excellent resolution.

Digested BAC samples no. 2 and no. 3 were separated on the Femto Pulse system with the 165 kb BAC kit (Figure 2B and C). Both samples exhibited all of the expected peaks, with sample no. 2 displaying eight peaks and sample no. 3 separating six peaks. Excellent separation resolution between each peak was seen with all fragments from both samples. The same samples separated by PFGE exhibited

fewer peaks. Sample no. 2 on the PFGE displayed seven fragments instead of eight. A wider band around 36,000 bp on the PFGE may encompass both the third and fourth fragment in sample no. 2. In sample no. 3, the last fragment at 20,000 bp was difficult to spot on the PFGE. This is due to a low concentration, which could result in the fragment being overlooked. In contrast, the 20,000 bp fragment was easily identified on the Femto Pulse system. Comparison of the techniques revealed unparalleled resolution and sensitivity with the Femto Pulse system compared to the PFGE separations. Automatic sizing of BAC sample no. 2 and 3 using the Agilent ProSize data analysis software resulted in excellent precision (% CV) and accuracy (% error) (Tables 5 and 6).

Incomplete enzymatic digestion

Enzymatic digestion is a highly reliable and efficient way to cleave DNA. Although on occasion, the expected number of fragments from enzymatic digestion can differ from the actual results. The cause is often due to incomplete digestion. Enzymes are easily inactivated from temperature, salt concentration, pH, and inhibitors. In addition, their activity can decrease greatly due to enzyme concentration, co-factor concentrations, inaccessible cleavage sites, and age. Adding to the sensitivity of enzymes is the very nature of DNA. DNA fragments with sticky ends can reanneal or bind together creating one long fragment instead of two. Frequently, the size of an unexpected longer fragment is the sum of two expected smaller fragments with the possibility of all three being present in the sample. It is not uncommon to separate out a different number of DNA fragments than expected after enzymatic digestion.

Table 3. BAC sample no. 8 was separated on four different Agilent Femto Pulse systems with the 55 kb BAC kit, resulting in tight precision (% CV) and accuracy (% error). *n=48 over four instruments.

55 kb BAC kit BAC Sample no. 8				
Peak No.	Expected Size (bp)	Average Size (bp)*	% CV	% Error
1	564	523	4.0%	-7.2%
2	2,027	1,752	5.4%	-13.6%
3	2,322	2,138	5.8%	-7.9%
4	4,361	3,970	4.9%	-9.0%
5	6,557	5,952	4.6%	-9.2%
6	6,800	6,600	4.1%	-2.9%
7	9,416	8,654	3.7%	-8.1%
8	13,700	12,753	2.6%	-6.9%
9	19,100	18,563	1.6%	-2.8%
10	23,130	22,364	1.4%	-3.3%
11	27,491	26,693	1.4%	-2.9%
12	33,400	32,735	1.4%	-2.0%
13	40,800	39,926	2.5%	-2.1%

Table 4. BAC sample no. 9 was separated on four different Agilent Femto Pulse instruments with the 55 kb BAC kit, resulting in tight precision (% CV) and accuracy (% error). *n=48 over four instruments.

55 kb BAC kit BAC Sample no. 9				
Peak No.	Expected Size (bp)	Average Size (bp)*	% CV	% Error
1	75	76	2.4%	0.9%
2	200	201	1.4%	0.6%
3	400	402	1.1%	0.4%
4	600	626	2.0%	4.1%
5	800	883	5.1%	9.4%
6	1,000	1,006	5.9%	0.6%
7	1,500	1,474	5.8%	-1.8%
8	3,000	3,015	3.0%	0.5%
9	6,000	6,008	2.9%	0.1%
10	10,000	10,066	1.9%	0.7%
11	15,000	15,067	1.3%	0.5%
12	20,000	20,064	0.8%	0.3%
13	48,500	49,011	1.0%	1.0%

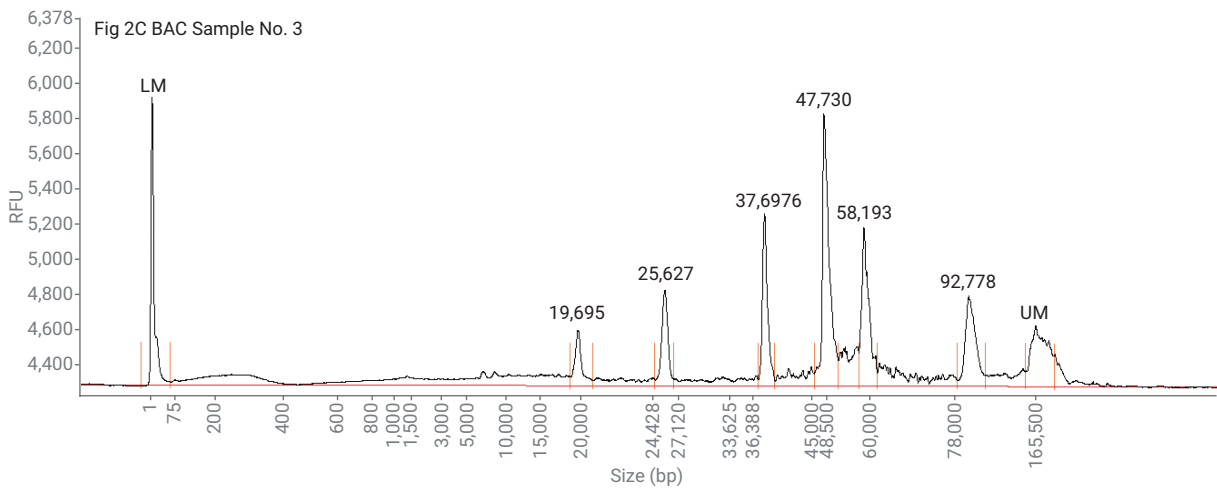
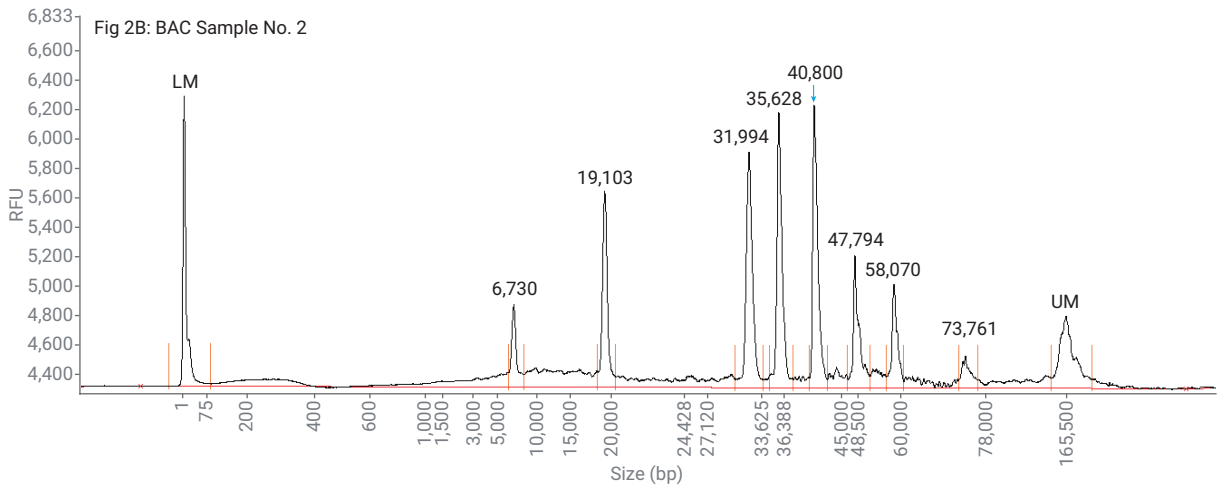
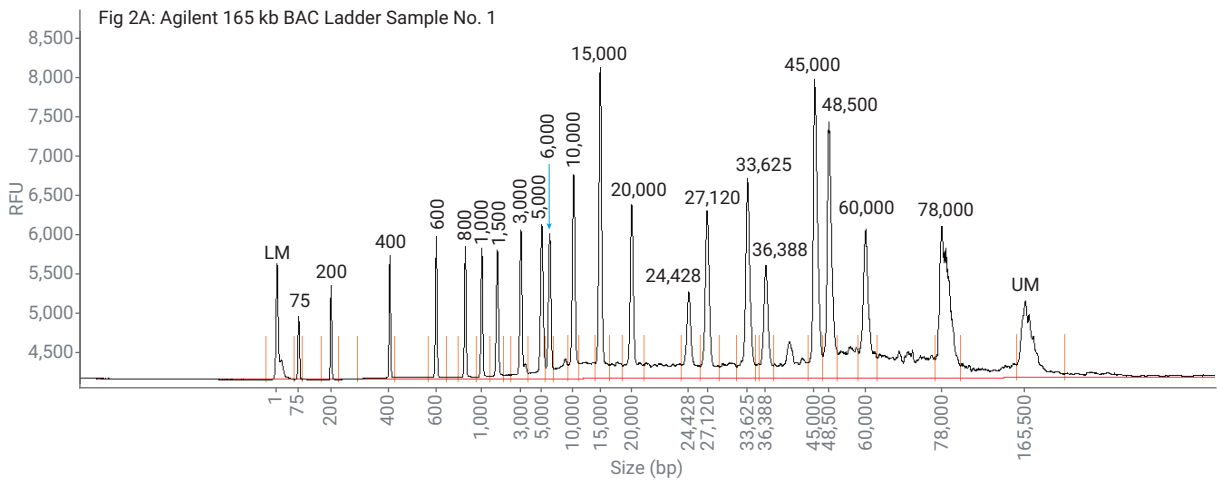


Figure 2A-C. DNA fragments separated on the Agilent Femto Pulse system with the 165 kb BAC kit. BAC sample no. 3. LM = lower marker, UM = upper marker.

Table 5. BAC sample no. 2 was separated on five different Agilent Femto Pulse instruments with the 165 kb BAC kit, resulting in tight sizing precision (% CV) and accuracy (% error). *n=60 over five instruments.

165 kb BAC Kit BAC Sample no. 2				
Peak No.	Expected Fragment Size (bp)	Average Size* (bp)	% CV	% Error
1	7,300	6,761	1.5%	-7.4%
2	20,000	19,135	0.3%	-4.3%
3	33,300	32,065	0.2%	-3.7%
4	36,300	35,691	0.4%	-1.7%
5	39,600	40,989	0.4%	3.5%
6	48,500	47,939	0.4%	-1.2%
7	55,000	58,109	0.6%	5.7%
8	79,500	73,373	0.8%	-7.7%

Table 6. BAC sample no. 3 was separated on five different Agilent Femto Pulse instruments with the 165 kb BAC Kit resulting in tight sizing precision (% CV) and accuracy (% error). *n=60 over five instruments.

165 kb BAC Kit BAC Sample no. 3				
Peak No.	Expected Fragment Size (bp)	Average Size* (bp)	% CV	% Error
1	20,000	19,485	1.3%	-2.6%
2	28,300	25,601	1.2%	-9.5%
3	37,900	37,913	1.2%	0.0%
4	48,500	47,933	1.3%	-1.2%
5	55,000	58,550	1.8%	6.5%
6	83,000	92,484	3.5%	11.4%

Pulsed Field Gel Electrophoresis

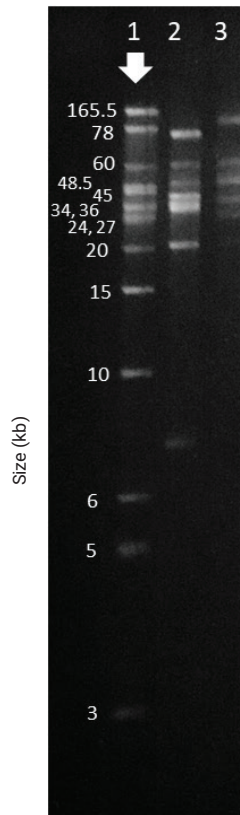


Figure 2D. PFGE separation of FP 165 kb BAC Ladder sample no. 1, BAC sample no. 2 and 3.

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 55 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. In addition, the Femto Pulse has the capability to detect and separate very small to very large fragments in the same analysis run. The Femto Pulse system offers unparalleled resolution and sensitivity compared to pulsed field gel electrophoresis in a fraction of the time.

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