Application Note

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Bacterial Artificial Chromosome Analysis on the Agilent Femto Pulse System

Authors

Abigail Gagliardi and Felipe Cabrera,
Regeneron Pharmaceuticals, Inc.

Whitney Pike,
Agilent Technologies, Inc.

Abstract

The Agilent Femto Pulse system with the Agilent 165 kb BAC kit can be utilized to provide reliable and quick analyses of digested bacterial artificial chromosome (BAC) fragments. Conventional methods for analysis of these large DNA fragments require ample amounts of sample and lengthy run times. The Femto Pulse provides fast analysis of BACs with high sensitivity and resolution using highly optimized pulsed-field capillary electrophoresis methods. Here, the Femto Pulse was successfully employed as a screening tool for BAC quality by providing faster visualization and sizing of digested BAC DNA fragments.

Introduction

BAC constructs are commonly used as stable and versatile tools for molecular biology research by hosting large DNA fragments in order to manipulate genes of interest and their regulatory elements. BACs can be used to influence gene expression in specific cell types and genetic disease models. Successful downstream results rely on proper insertion of the gene[1,2]. Thus, verification of successful insertion of the gene of interest into a BAC is an essential quality control (QC) checkpoint for all BAC applications.

Since BACs can host large DNA inserts (100 to 300 kb), quality assessment is often performed using pulsed field gel electrophoresis (PFGE) to resolve larger sized fragments. However, the separation time for these gels is extensive, often 8 to 16 hours, causing severe workflow bottlenecks. In addition, PFGE analysis requires ample volume and time-consuming staining and destaining protocols for imaging the gel. Alternately, the Femto Pulse system offers automated pulsed field electrophoresis with the ability to run up to 12 samples in parallel, with minimal sample input requirements. The 165 kb BAC kit for the Femto Pulse provides high resolution and sensitivity for visualizing and sizing high-molecular weight DNA fragments such as digested BAC samples up to 165 kb in less than 3 hours (Table 1)[3,4].

As an example, the workflow designed by Regeneron Pharmaceuticals, Inc. demonstrates how unmodified genomic fragments, or BACs, undergo recombination through the insertion or deletion of genetic material to construct a final targeting vector for downstream use (Figure 1). This application note highlights Regeneron Pharmaceuticals' use of the Femto Pulse system as a screening tool for fast and accurate QC of their BACs and vectors, and compares their results to conventional PFGE. For the purposes of this screening, restriction digest of the BAC and vector samples results in fragments of various sizes, which can be detected via electrophoresis. If the BAC recombination was successful, the fragment sizes of the vector in relation to the original BAC are observed to change accordingly. Analysis of the digested BAC and vector samples using the Femto Pulse enabled Regeneron Pharmaceuticals to save precious time in their workflow and provided them with better separation resolution of the BAC fragments and higher sensitivity than PFGE.

Table 1. Comparison of PFGE and the Agilent Femto Pulse system.

Attribute	PFGE*	Agilent Femto Pulse system
Samples per Run	1 to 14	1 to 11
Sample Amount	Nanograms	Picograms
Separation Time	10 to 24 hours	3 hours
Total Time (Inc. Gel Prep., Separation, and Staining)	12 to 26 hours	3 hours
Size Range	Up to 10 Mb	75 bp to 165 kb
Result Format	Agarose gel image; requires manual annotation and documentation	Digital gel image and electropherogram; automated sizing analysis

**Attributes based on Bio-Rad Chef Mapper XA system*

Experimental

Sample preparation

Digested BAC and vector DNA samples were analyzed using both pulsed-field gel electrophoresis (PFGE) and automated pulsed-field capillary electrophoresis on the Agilent Femto Pulse system for comparison. A concentration of 200 to 400 ng of BAC DNA was digested with various restriction enzymes (New England Biolabs) and incubated for 3 hours at appropriate temperatures in a 20 µL total volume. After incubation, the digestion reactions were heat inactivated. The samples were then separated by the Femto Pulse system with the Agilent 165 kb BAC kit (p/n FP-1004-0275) and by PFGE on the Bio-Rad CHEF Mapper XA (p/n 1703670) and CHEF-DR III (p/n 1703700) systems.

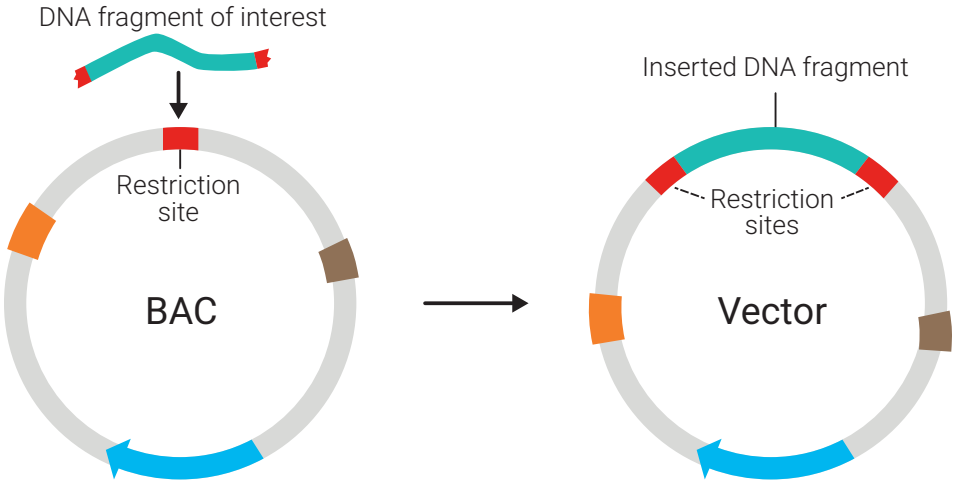

Figure 1. Schematic of the workflow utilized by Regeneron Pharmaceuticals, Inc. Unmodified bacterial artificial chromosomes (BACs) undergo recombination via the insertion or deletion of genetic material, generating a construct or final targeting vector. If recombination is successful, the size of the vector will differ from that of the original BAC.

Femto Pulse separation

The digested samples were prepared for analysis according to the *Agilent 165 kb BAC Kit Quick Guide for the Femto Pulse System*[5]. Salts present in the samples were diluted out following the additional sample preparation instructions in Appendix A of the quick guide[5]. Briefly, the digestion reaction was diluted 10X using Dilution Buffer 0.25x TE (p/n DNF-498). A 2 µL volume of the diluted sample was then mixed with 18 µL of the gDNA Diluent Marker Solution (p/n FP-8001) using a wide bore pipette. To account for any potential effects of the sample matrix on the mobility of the DNA fragments and achieve the most reliable sizing analysis, the 165 kb BAC Ladder working solution was prepared according to the specifications for a 10X sample predilution. The samples and ladder were run in parallel on the Femto Pulse system.

PFGE separation

Two µL of prepared 10X loading dye was added to 18 µL of the remaining sample from the restriction digest and loaded on a PFGE gel using a method to allow for separation of fragments between 2 to 200 kb for 10.5 hours on the Bio-Rad CHEF Mapper XA or CHEF-DR III PFGE systems. A 1% PFGE-grade agarose gel (SeaKem Gold Agarose, Lonza p/n 50150) was prepared, cooled, and cast into the casting stand from Bio-Rad and loaded with 20 µL of the sample prepared with loading dye.

Commercially available DNA markers, 1 kb DNA Extension Ladder (Invitrogen p/n 15517-022) or Quick-Load 1 kb Extend DNA Ladder (New England Biolabs p/n N3239S) and the MidRange I PFG Marker (New England Biolabs p/n N3551) were also loaded to allow for size analysis. The agarose gel was post-stained with a 10X Gel Red solution (Biotium p/n 41003-1) and then imaged using an AlphaImager HP to capture the gel image exposed with UV at 365 nm. The resulting gel image was used for comparison to the data obtained from the Femto Pulse.

Results and Discussion

BAC workflow

Using the workflow developed by Regeneron Pharmaceuticals, BACs are genetically modified to generate vectors that can be used in various downstream applications. Regeneron Pharmaceuticals utilizes the Femto Pulse as a screening tool to QC vectors to compare to the original BAC clone and ensure that the recombination has occurred as expected. Screening is done by performing a restriction digest with different enzymes, which should result in the BAC and vector being digested into linearized fragments of different sizes. The approximate size of the fragments can be visualized via gel electrophoresis and compared to the expected sequence size. If the recombination is successful, the fragment sizes of the vector will differ from those of the original BAC.

For example, shown in Figure 2 is a BAC and a vector digested with a PvuI restriction enzyme. The BAC and vector digests were analyzed with PFGE and on the Femto Pulse for comparison. Figure 2A displays an image of the PFGE gel for the digested samples. To determine approximate sizing, two ladders were analyzed alongside the samples, each with different sizing ranges. The size of the sample was approximated by visually aligning the fragment with the ladder peaks. As shown in the expected sizes in Table 2, the BAC sample is expected to show four fragments—98, 32, 6.2, and 2.7 kb. The vector should display three fragments—101, 32, and 2.7 kb. The absence of the 6.2 kb fragment, and the slight size shift of the 98 kb fragment in the BAC to 101 kb in the vector, is indicative of successful recombination. The fragments are clearly evident on the PFGE gel image, but the size shift is not visible and accurate sizing is not possible.

Alternately, analysis with the Femto Pulse generates both a digital gel image and an electropherogram for easy visualization of the samples. Figures 2B and C show the same BAC and vector samples analyzed on the Femto Pulse, with each of the fragments clearly pictured in each image. The Femto Pulse provides a size for each fragment, allowing for direct comparison to the expected size, as shown in Table 2. Sizing on the Femto Pulse is made possible by the addition of a lower and upper marker to the samples, allowing for alignment to the ladder. As the upper marker is 165 kb in size, larger samples may merge with the marker peak. For most accurate screening, Regeneron Pharmaceuticals thus recommends choosing restriction enzymes that will result in smaller fragments.

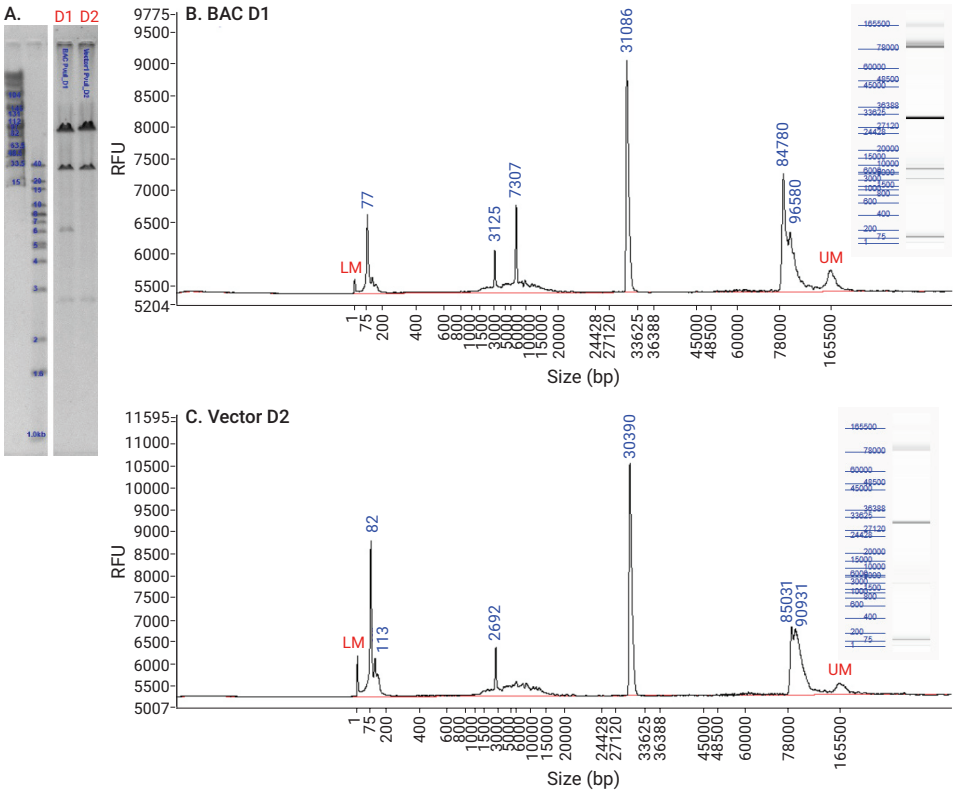

Figure 2. Analysis of BAC samples on the Agilent Femto Pulse system aligns with observations made by PFGE. A) PFGE gel image of the BAC (lane D1) and vector (lane D2). Sizing was estimated by visualization of two ladders run concurrently. Femto Pulse electropherogram and digital gel image of the B) BAC and C) vector.

Table 2. Comparison of the expected BAC and vector sizes reported by the Agilent Femto Pulse system with the Agilent 165 kb BAC kit. (Note: the largest fragment splits into two partially resolved peaks. For sizing, the peaks were analyzed together using the smear analysis function in Agilent ProSize data analysis software.)

BAC Expected Size (bp)	BAC D1 Femto Pulse Size (bp)	Vector Expected Size (bp)	Vector D2 Femto Pulse Size (bp)
98,186	93,008	101,249	93,968
32,257	31,086	32,257	30,390
6,165	7,307	2,655	2,692
2,655	3,125		

For the example shown in Figure 2, the size shift of the largest fragment was not visible by traditional PFGE but is detected with the higher sensitivity provided by the Femto Pulse. While both the PFGE gel and the Femto Pulse data indicated that the vector passed the QC screening, the Femto Pulse was able to obtain this data in 3 hours, instead of the overnight run generally required for PFGE analysis, and provided higher sensitivity and thus more confidence in the BAC recombination application.

Femto Pulse sensitivity

The Femto Pulse offers higher sensitivity in the smaller size range than PFGE. An inherent issue with PFGE, the long separation method utilized to parse out the larger fragments in a sample, often causes any smaller fragments to run off the gel. In the example shown in Table 3, digestion of both the BAC and vector samples are predicted to result in four fragments. The two fragments with expected sizes below 500 bp cannot be visualized on the PFGE gel image (Figure 3A) but are clearly evident on the Femto Pulse results (Figure 3B to D).

The larger fragments in this sample are easily visualized with both the PFGE and Femto Pulse. However, digestion of the vector with NotI (Figure 3C) shows the presence of an additional insert at 6.7 kb with the Femto Pulse, which was not evident in the PFGE image. Further, a double digestion of the same sample (Figure 3D) unexpectedly resulted in two extra peaks, one at 4.9 kb that was also seen on the PFGE image, and one at 1.7 kb, only seen on the Femto Pulse. With PFGE alone, the BAC and vector B6 would have been considered successful by disregarding the smaller fragments and relying solely on the larger bands that were visualized. However, the presence of the extra inserts seen in the vectors using the Femto Pulse allowed Regeneron Pharmaceuticals to fail this sample and QC another construct before moving on with their workflow, saving them precious time and reagents.

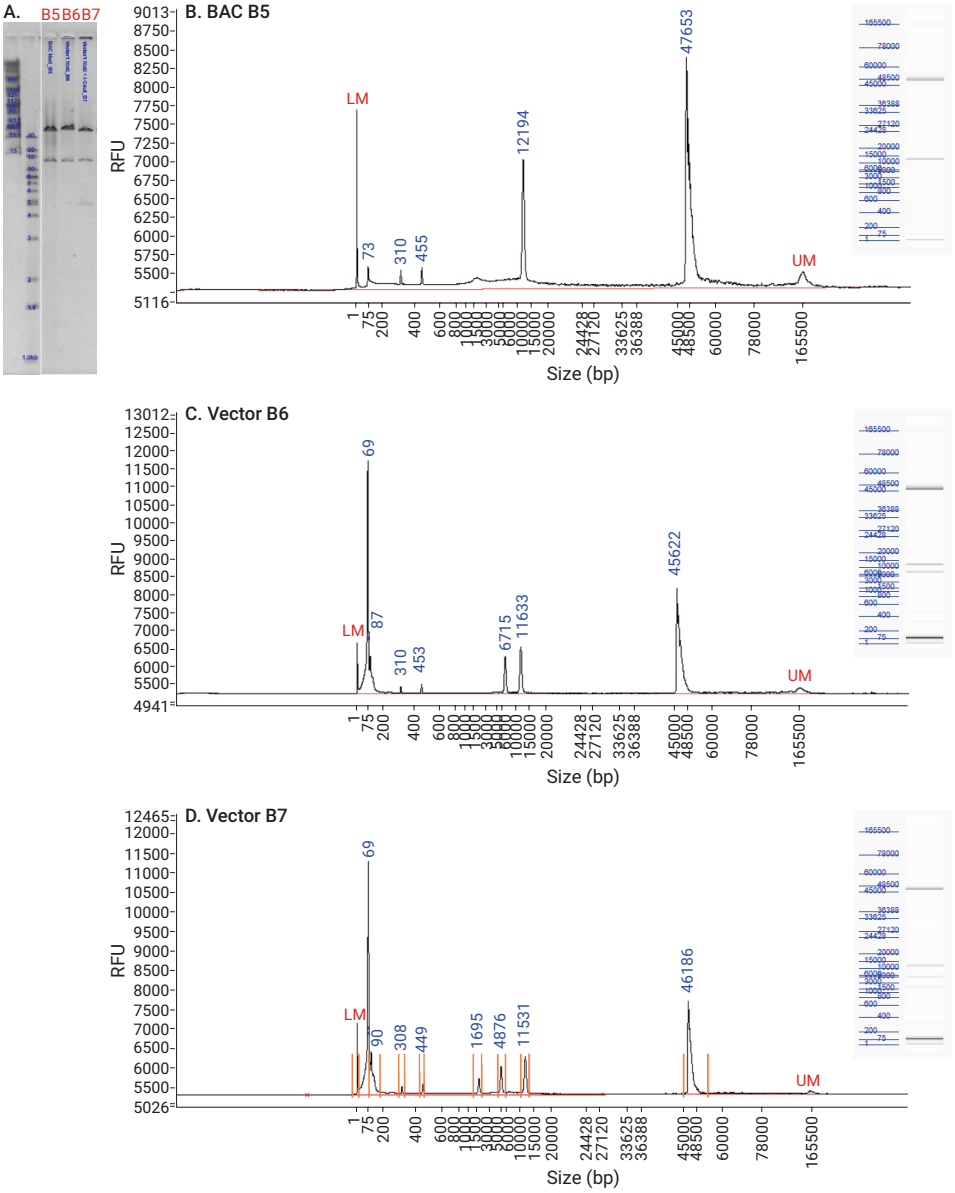

Figure 3. The Agilent Femto Pulse system displays higher sensitivity than PFGE. A) PFGE gel image of BAC and vector linearized fragments. The size of the BAC sample was deemed successful, despite the absence of the expected 457 and 325 bp fragments, as these smaller bands are known to run off the gel with the extended separation method. B) The Femto Pulse results displayed all four expected fragments for the BAC sample. C) Vector B6 was considered to be an unsuccessful recombination, due to the insertion of a fragment at 6.7 kb with NotI digestion seen with the Femto Pulse that was not evident in the PFGE (A, lane B6). D) The same sample was thus further tested using a double digestion to confirm size after recombination. The Femto Pulse showed an insertion at 1.7 kb (not seen on PFGE) and 4.9 kb, which was unexpected but correlated with the PFGE results. The vector failed screening because it did not match the predicted sizing.

Table 3. Comparison of the expected BAC and vector sizes to the sizes reported by the Agilent Femto Pulse with the Agilent 165 kb BAC kit.

BAC Expected Size (bp)	BAC B5 Femto Pulse Size (bp) (Pass)	Vector (B6, B7) Expected Size (bp)	Vector B6 Femto Pulse Size (bp) (Fail)	Vector B7 Femto Pulse Size (bp) (Fail)
47,537	47,653	51,534	45,622	46,186
11,472	12,194	11,472	11,633	11,531
457	455		6,715	4,876
325	310			1,695
		457	453	449
		325	310	308

Conclusion

This application note demonstrates the ability of the Agilent Femto Pulse system to provide sizing and electropherogram profiles that are very comparable to those of PFGE. Further, the design of the Femto Pulse system allows for highly sensitive detection for conservation of samples, fast separations to save user time, and detection of small fragments that are often lost in PFGE analysis. Thus, the automated electrophoresis system provides better objective sample insights that may be critical in the decision-making process for downstream applications. The Femto Pulse can be successfully employed as a quality control tool in BAC screening workflows for verification of gene insertion by sizing digested BAC fragments.

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