A Workflow for Obtaining High Quality Sequencing Data from Bacterial Artificial Chromosome (BAC) DNA

Abstract

Bacterial artificial chromosome (BAC) DNA is difficult to use as a sequencing template, because of its large size (usually >100,000 bp). A modified sequencing protocol has been demonstrated for BAC DNA using the BigDye® Terminator Cycle Sequencing Kit. Additionally, implementation of the BigDye® XTerminator™ Purification Kit for reaction cleanup resulted in increased signal intensity and overall reading quality of the sequence data comparable to that from plasmid templates.

Introduction

Eliane Escher and colleagues operate a sequencing facility at the Institute of Molecular Biology, University of Zurich. This group routinely analyzes 1,500 to 2,000 sequencing reactions each month that use plasmids, PCR products, and bacterial artificial chromosomes (BACs) as templates for cycle sequencing, using the Applied Biosystems GeneAmp® PCR System 9700 (thermal cycling) and 3730 DNA Analyzer (capillary electrophoresis).

BAC DNA is more difficult to sequence than plasmids or PCR products. Its large size makes it prone to formation of secondary structure and necessitates using microgram quantities of highly purified DNA to generate sufficient signal for accurate base calling and maximal read length. The high volume of work done at Ms. Escher's facility demands employment of cost- and time-efficient sequencing methods to enable routine integration of BAC sample analysis into their schedule (Figure 1). This application note describes their use of the BigDye® Terminator v3.1 Cycle Sequencing Kit [1] and BigDye® XTerminator™ Purification Kit to obtain BAC DNA sequence information with reading quality comparable to that from plasmid templates.

Sequencing Reaction Optimized for Large Templates

Purification of BAC DNA. BAC DNA was purified from bacterial cultures (2 mL overnight cultures) using a modified alkaline lysis procedure and anion-exchange resin [2–4]. Poor template quality is the most common cause of sequencing problems and can lead to weak signal, noisy data, overlapping peaks, or no usable data. For BAC DNA, 1–3 µg template per reaction is typically recommended. For the experiments presented here, 3–5 µg template was used.

Cycle Sequencing. BAC DNA was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit. The initial protocol used by Ms. Escher's group, which included standard thermal cycling conditions that were developed for plasmid and PCR

1. Set Up Reaction	2. Run Sequencing Reaction	3. Clean Up Reaction	4. Read Sequence
BigDye® Terminator v3.1 Cycle Sequencing Kit	GeneAmp® PCR System 9700 Veriti™ Thermal Cyclers	BigDye® XTerminator™ Purification Kit	**Instruments** 3130/3130*xl* Genetic Analyzers 3730/3730*xl* Genetic Analyzers **Software** Sequencing Analysis Software with KB™ Basecaller Software
The BigDye® Terminator v3.1 Cycle Sequencing Kit is designed for high quality, long reads using robust, flexible chemistry. This kit is compatible with AT- and CG-rich regions and a variety of templates, including plasmid, single-stranded, BAC, cosmid, fosmid, bacterial genomic, and lambda DNA, as well as PCR and rolling circle-amplified products.	The GeneAmp® PCR System 9700 and Veriti™ Thermal Cyclers can be used for cycle sequencing reactions. Both instruments are available in 60-, 96-, and 384-well format. The Veriti 96-Well Thermal Cyclers are compatible with fast or standard PCR cycling protocols and include the VeriFlex™ Block, with 6 independent temperature blocks to provide precise control over PCR optimization. The GeneAmp PCR System 9700 accommodates samples up to 0.5 mL.	The BigDye® XTerminator™ Purification Kit provides a simple purification method for DNA sequencing reactions and increases signal intensity. Cleanup is complete in ~40 minutes and requires less than 10 minutes of labor. More effective than ethanol precipitation and less expensive than column purification and other commercial kits, the BigDye XTerminator Purification Kit removes dye blobs by capturing unincorporated dye terminators, dNTPs, and salts that can interfere with base calling and electrokinetic sample injection.	Applied Biosystems offers a full range of multicapillary systems that support a complete range of genetic analysis projects (sequencing, resequencing, microsatellite analysis, AFLP, LOH, SNP screening, and validation). Sequencing Analysis Software with KB™ Basecaller Software enables you to basecall, trim, display, edit, and print data from our entire line of capillary DNA sequencing instruments for data analysis and quality control.

Figure 1. Overview of the Cycle Sequencing Workflow for BAC DNA.

*This protocol is a modification of the recommended Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit Protocol. The results illustrated here are those of the contributing laboratory, and optimization may be required for best results in your laboratory setting. Please be advised that Applied Biosystems supports the performance of BigDye Terminator Cycle Sequencing Kits when Applied Biosystems protocols, reagent formulations, and kit storage recommendations are followed.

products [5], was modified to include thermal cycling conditions optimized for large DNA templates (Figure 2, Panel A). A Sephadex-based purification method (Figure 2, Panel B) was used to clean up the reaction before analysis by capillary electrophoresis on an Applied Biosystems 3730 DNA Analyzer. Plasmid templates were analyzed in parallel as controls.

BAC DNA cycle sequencing reactions using the initial protocol yielded low-signal products that were accompanied by a significant quantity of unincorporated dye terminators ("dye blobs"), with raw fluorescence data mostly at baseline (Figure 3, top panels). Analysis of 6 independent samples using the Sequence Analysis Software with KB™ Basecaller resulted in a sample score of 3.17, indicating low quality of the analyzed data [Figure 4, row 2; mean average signal intensity value = 97; length of read (LOR) = 36 bases].

A modified cycle sequencing protocol, which included a longer initial denaturation step and an increased number of sequencing cycles (Figure 2, Panel A), generated data with distinct peaks, but a significant number of large unincorporated dye terminator peaks were still evident (Figure 3, bottom panels). The analyzed data from 11 independent samples showed improved results (sample score: 29.17; mean average signal intensity value:151; LOR: 796 bases; Figure 4, row 3) compared to the initial protocol.

BigDye® Terminator v3.1 Cycle Sequencing Kit	
Modified Protocol	
Reaction (10 µL Reaction Volume)	
DNA template	3–5 µg BAC DNA
Primer	10–15 pmol
BigDye® Terminator Ready Reaction Mix	1.0 µL
5X Sequencing Buffer	1.5 µL
Cycling Conditions [5]	
Denaturation	5 min, 95°C
	50 cycles:
Denaturation	30 sec, 95°C
Annealing	10 sec, 52°C
Elongation	4 min, 60°C
Thermal Cycler	96-Well GeneAmp® PCR System 9700[A]

A Also compatible with the Applied Biosystems Veriti™ 96-Well Thermal Cycler

Panel A. Cycle Sequencing Protocol

	Reaction Cleanup	
	Sephadex G-50 Column MultiScreen HTS Filter Plates	BigDye® XTerminator™ Purification Kit
Protocol	Centrifuge 5 min, 910 x g	Add 45 µL SAM Solution Add 10 µL BigDye XTerminator Solution[B] Vortex 1800 rpm[C], 30 min, room temp Centrifuge 2 min, 13,000 x g
Capillary Electrophoresis	Applied Biosystems 3730 DNA Analyzer[D] 48 capillaries 36 cm capillary array length POP-7 polymer Standard Sequence module[E] with 8 sec injection time and 1 kV voltage Sequence Analysis Software with KB™ Basecaller[F]	

B Use wide-bore (>1 mm) pipette tips
C For experiments described here, 1600 rpm was used. The vortexing step is critical to achieve optimum results. View the Product Description page for a list of recommended vortexers/adaptors at www.appliedbiosystems.com/gameoverblobs
D Compatible with other Applied Biosystems DNA Sequencers, including the 3130-series sequencers
E Special run modules for use with the Big Dye XTerminator Purification Kit and Data Collection Software are available at www2.appliedbiosystems.com/support/software/ [6].
F Compatible with Variant Reporter™ Software for Resequencing

Panel B. Sequencing Reaction Cleanup and Analysis Protocols

Figure 2. Summary of Protocol Modifications. Panel A: BAC DNA was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the 96-well GeneAmp® PCR System. The standard protocol [5], developed for plasmids/PCR products, was modified as follows: the initial denaturation time was increased to allow the large template to denature more completely, and the cycle number was increased to adjust for the lower copy number of template. Results are shown in Figure 3. **Panel B:** Summary of the two sequencing reaction cleanup methods that were compared. Purified cycle sequencing products were analyzed by capillary electrophoresis on the Applied Biosystems 3730 DNA Analyzer. Results are displayed in Figure 5.

Powerful Reaction Cleanup Technology

The BigDye® XTerminator™ Purification Kit, which desalts and removes unincorporated nucleotides from sequencing products, results in increased signal intensity of the extension product peaks (data not shown). The experiments by Ms. Escher's group show that these improvements are critical for consistent, high quality sequencing data from BAC DNA.

The modified cycle sequencing protocol was used with BAC DNA samples, followed by either the Sephadex-based method or the BigDye XTerminator Purification Kit for reaction cleanup before capillary electrophoresis (Figure 2, Panel B).

As expected, the raw fluorescence signal data was much higher in sequenced BAC samples purified with the BigDye XTerminator Purification Kit compared to those purified with the Sephadex-based method (data not shown). In addition, the dye blobs seen in samples purified with the Sephadex-based method (Figure 5, top panels) were absent in BigDye XTerminator Purification Kit-treated samples (Figure 5, bottom panel), demonstrating that the BigDye XTerminator Purification Kit efficiently removes unincorporated dye-labeled nucleotides. Analysis of 8 independent samples purified with the BigDye XTerminator Purification Kit yielded quality parameters that were equivalent to or exceeded those observed with plasmid control

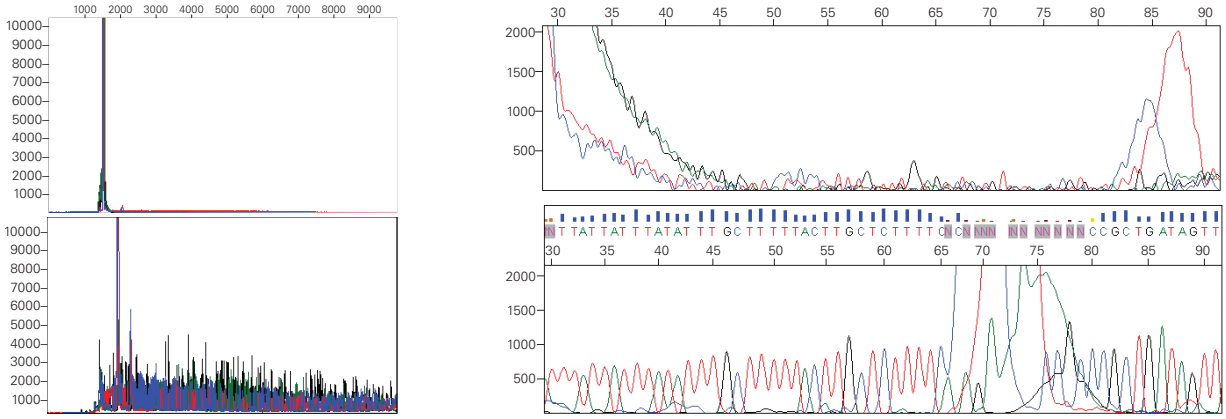
Useful Definitions

Clear Range is the part of the sequence that has the highest quality, that is, with the fewest errors and ambiguities, and with confident basecalls and good spacing.

Quality Value (QV) is an established metric for determining quality sequencing data. QV>20, which typically is considered acceptable, means the probability that the base was miscalled is no greater than 1%.

Sample Score is the average QV in the clear range or in the entire read when no clear range is determined. It is a useful metric to determine the quality of the data.

templates (mean average signal intensity value:1835; sample score: 27.13; LOR: 788 bases; Figure 4, row 4). The LORs of BigDye XTerminator Purification Kit-treated and Sephadex-purified samples were similar, however, the average signal intensity value was more than 10-fold higher for the BigDye XTerminator-purified samples (Figure 4, rows 3 and 4).



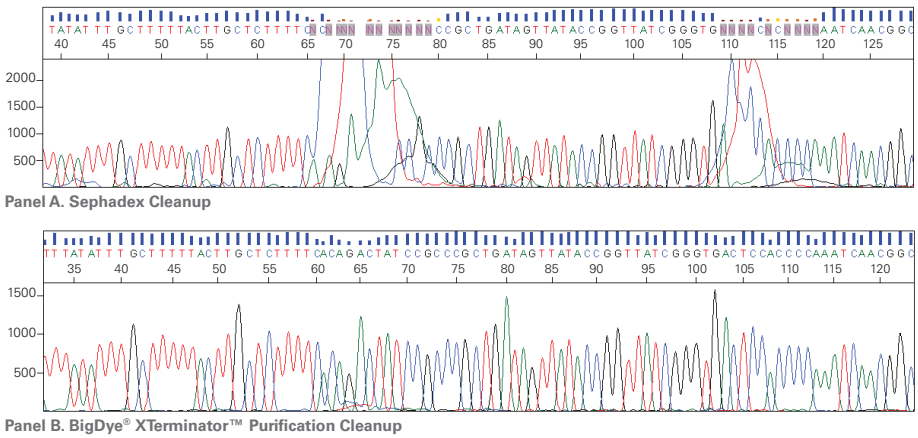
Panel A. Raw Fluorescence Data

Panel B. KB™ Basecaller Data

Figure 3. Extended Denaturation and Cycling Enables Cycle Sequencing of BAC DNA. Two cycle sequencing protocols were used to sequence BAC DNA with the BigDye® Terminator v3.1 Cycle Sequencing Kit, followed by Sephadex-based reaction cleanup. The results from the initial protocol, which included standard thermal cycling conditions developed for plasmid and PCR products [5], are shown on the top of each panel, and the results from using the modified protocol descibed in Figure 2, Panel A, are shown on the bottom of each panel. **Panel A:** Raw data from capillary electrophoresis on the Applied Biosystems 3730 DNA Analyzer. **Panel B:** Analyzed data using the KB™ Basecaller software. Blue bars indicate the QV value of the corresponding bases. No basecalling was possible from the data in the top panel. (Data courtesy of E Escher, Institute of Molecular Biology, University of Zurich, Switzerland.)

Protocol			N	BigDye® Mix (µL)	QV>20	Sample Score	LOR	Mean Average Signal Intensity
Sample	Cycling	Cleanup						
Plasmid	Standard (30 cycles)	Sephadex	42	0.5	645.86	34.79	704	551
BAC DNA	Standard (30 cycles)	Sephadex	6	0.7	17.83	3.17	36	97
BAC DNA	Modified (50 cycles)	Sephadex	11	1.0	658.36	29.27	796	151
BAC DNA	Modified (50 cycles)	BigDye® XTerminator Purification Kit	8	1.0	652.75	27.13	788	1835

Figure 4. Workflow for BAC DNA Templates: Modified Thermal Cycling Conditions and BigDye® XTerminator™ Purification Kit Cleanup Generate Results Similar to or Better than That Seen with Plasmid Templates. Data analysed using KB™ Basecaller software. Row 1 summarizes data from control cycle sequencing reactions using plasmid templates. N: number of independent samples; QV>20: average number of bases with QV>20; LOR: length of read, length of sequence with average QV ≥20 for 20 base increments; Mean Average Signal Intensity: mean of the average signal intensity for N samples. Note: See footnote on page 1 for limitations to Applied Biosystems support of modifications to recommended protocols. (Data courtesy of E Escher, Institute of Molecular Biology, University of Zurich, Switzerland.)



Panel A. Sephadex Cleanup

Panel B. BigDye® XTerminator™ Purification Cleanup

Figure 5. Workflow for BAC DNA Templates: Modified Thermal Cycling Conditions and BigDye® XTerminator™ Purification Kit Cleanup Produces High Quality Sequence Data from BAC DNA. BAC DNA was sequenced with the modified protocol using the BigDye® Terminator v3.1 Cycle Sequencing Kit. Sephadex (Panel A) or the BigDye® XTerminator™ Purification Kit (Panel B) was used to clean up the sequencing reactions. Data analyzed using the KB™ Basecaller software. Blue bars indicate the QV value of the corresponding bases. (Data courtesy of E Escher, Institute of Molecular Biology, University of Zurich, Switzerland.)

Conclusions

Ms. Escher's results demonstrate that using the BigDye Terminator v3.1 Cycle Sequencing Kit and a slightly modified protocol, which included a longer initial denaturation step and an increased number of sequencing cycles, enabled sequencing of BAC DNA. In addition, reaction cleanup using the BigDye XTerminator Purification Kit increased the signal intensity of BAC sequence data by more than 10-fold, compared to Sephadex-based cleanup. Use of the BigDye XTerminator Purification Kit consistently eliminates "dye blobs."

The workflow described here yields BAC sequencing data whose quality meets or exceeds that of plasmid sequencing, measured by mean signal intensity value, sample score, and length of read (Figure 4). The challenges of sequencing large BAC DNA are met by using the BigDye Terminator v3.1 Cycle Sequencing Kit and BigDye XTerminator Purification Kit.

Scientific Contributors

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REFERENCES

1. Rosenblum BB, Lee LG, Spurgeon SL, Khan SH, Menchen SM, Heiner CR, and Chen SM (1997) New dye-labeled terminators for improved DNA sequencing patterns, *Nucleic Acids Res* **25:**4500–4504.
2. Marra M, Weinstock LA, Mardis ER (1996) End sequence determination from large insert clones using energy transfer fluorescent primers, *Genome Res* **6:**1118–1122.
3. Zhao S, Shatsman S, Ayodeji B, Geer K, Tsegaye G, Krol M, Gebregeorgis E, Shvartsbeyn A, Russell D, Overton L, Jiang L, et al. (2001) Mouse BAC ends quality assessment and sequence analyses, *Genome Res* **11(10):**1736–1745. Erratum in: *Genome Res* **11(11):**1968.
4. Venken KJT, He Y, Hoskins RA, Bellen HJ (2006) P[acman]: A BAC Transgenic platform for targeted insertion of large DNA fragments in D. melanogaster, *Science* **314:**1747–1751.
5. Applied Biosystems (2002) BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol, Rev A. Search for P/N 4337035 at www.appliedbiosystems.com.
6. Applied Biosystems (2006) BigDye® XTerminator™ Purification Kit Protocol Summary. Search for P/N 107PR04-01 at www.appliedbiosystems.com.

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Description	Quantity	Part Number
BigDye® Terminator v3.1 Cycle Sequencing Kit	24 rxns	4337454
	100 rxns	4337455
	5000 rxns	4337457
	1000 rxns	4337456
	25000 rxns	4337458
BigDye® XTerminator™ Purification Kit	1 kit: 2 mL (~100, 20 µL rxns)	4376486
	1 kit: 20 mL (~1000, 20 µL rxns)	4376487
	1 kit: 50 mL (~2500, 20 µL rxns)	4376484
	1 kit: 800 mL	4376485

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