Automating Montage BAC_{96} DNA Miniprep on Perkin-Elmer's Evolution P3 Platform

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Abstract

Bacterial Artificial Chromosome (BAC) libraries are critical tools for both physical mapping and the finishing process of genome sequencing. In BAC-end sequencing, the ends of thousands of BAC clones are sequenced and the data is assembled along with a number of other markers to generate a scaffold of the genome. BAC-ends are also useful for filling gaps and bridging contigs in the final stage of assembly and completion of the genome sequence. One obstacle to adapting this strategy to a production sequencing environment is the low copy number of these vectors. Since the F factor is generally maintained at only 1- 2 copies per cell, the BAC DNA yield obtained from a given volume of culture is significantly lower than that for plasmid DNA. Therefore, it has been necessary to modify the growth and DNA purification protocols, as well as sequencing reaction conditions, in order to maximize yields and obtain high quality DNA sequence data from these BAC samples. Besides the challenge of obtaining adequate yields for BAC-end sequencing applications, it is also difficult to produce enough BAC clones to support a production sequencing environment. We demonstrate here an automated solution for the processing of BAC DNA minipreps and subsequent end sequencing on a 96-tip Evolution™ P^3 Precision Pipetting Platform (PerkinElmer, Inc.) which enables easy setup using PerkinElmer's WinPREP® software and provides high-throughput processing for confident results. Purification of BAC clones and sequencing cleanup occur in a 96-well format using Millipore's Montage® BAC_{96} miniprep kits and SEQ_{96} sequence reaction cleanup platforms which provide DNA of sufficient quantity and purity for direct BAC-end sequencing demonstrated on an ABI 3700 capillary sequencer.

Overview

Bacterial artificial chromosomes have been designed to manipulate large fragments of genomic DNA and are the vehicles of choice for the cloning and sequencing of large regions of genomic DNA. Like plasmid DNA, BAC DNA is replicated in an *E. coli* host. However, the low copy number of the BAC vectors (1–2 copies per cell) greatly reduces the yield per unit of culture volume. The procedure we have developed for BAC DNA purification is designed to optimize DNA yields, and includes a modified version of a common alkaline lysis method used for isolation of plasmid DNA from bacteria.

A single BAC clone, 435G12, from a chromosome 22 library (Research Genetics, Inc.) was used instead of a library plate in order to eliminate variability that is intrinsic to BAC clones, and better measure variation in yields due to the miniprep system. The vector used for construction of the chromosome 22 library is pBeloBAC11 and the host strain is DH10B. The E. coli host cells were grown in 1.5 mL aliquots of 2X LB media containing aqueous chloramphenicol (Sigma) for 21 - 23 hours. Cells were pelleted via centrifugation at 1500 xg for 5 minutes.

The minipreps were processed using a semi-automated method for the purification of BAC DNA in a 96-well format. The protocol does not require any centrifugation following cell pelleting of the host bacteria. Alkaline lysis steps are performed in the same 96-well culture block used for cultivation of the BAC clones. Following lysis, clarification of the bacterial lysates and purification of the BAC DNA are performed using vacuum driven filtration. The BAC DNA is retained by the size-exclusion membrane in the second filter plate while proteins and contaminants are washed through and removed. The BAC DNA is then resuspended in solution on top of the membrane and recovered by manual pipetting. The entire purification procedure, starting with cell pellets, can be performed in approximately 1 hour using one vacuum manifold setup. Throughput can be increased using multiple manifolds. The yields are sufficient for sequencing from both BAC ends, restriction digest analysis, PCR, or DNA fingerprinting.

Method

Resuspension
- 100 µL/well of Solution 1
- Shake for 5 minutes
- *Incubate for 30 minutes to improve BAC yields

Lysis
- 100 µL/well of Solution 2
- Shake for 1 minute
- Incubate for 2 minutes

Neutralization
- 100 µL/well of Solution 3
- Shake for 3 minutes

Lysate Transfer and Clearing
- 300 µL/well transferred to CLEARING plate
- Vacuum filter for 10 minutes at 8 in Hg (750 mbar)
- Discard CLEARING plate and move BAC plate to manifold top
- Vacuum filter for 8 minutes at 24 in Hg (250 mbar)

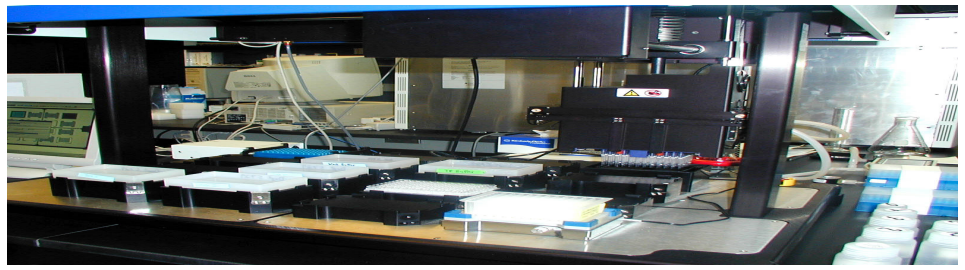
Wash Step
- 200 µL/well of Solution 4
- Vacuum filter for 4 minutes at 24 in Hg (250 mbar)

Resuspend DNA
- 40 µL/well of Solution 5
- Shake for 10 minutes
- **Recover purified DNA samples manually from BAC plate

*This optional step helps increase BAC yields.

**Manual transfer ensures maximum volume recovery from the wells of the BAC plate.

Deck Layout and WinPREP 2.1 on the Evolution P^3

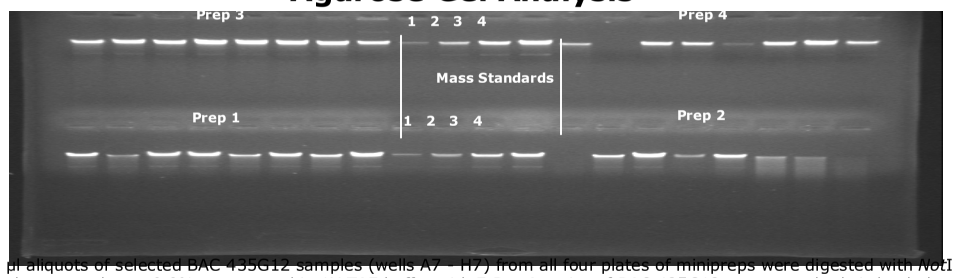


BAC DNA Yields

	Prep #1 Yield (ug)	Prep #2 Yield (ug)	Prep #3 Yield (ug)	Prep #4 Yield (ug)
Average	0.92	1.15	1.13	0.92
Standard Deviation	0.49	0.58	0.56	0.53
Coefficient of Variation	53.04%	50.65%	49.21%	57.64%
Minimum	0.02	0.09	0.09	0.01
Maximum	1.81	2.06	1.90	2.07
Sample Size	96	96	96	93

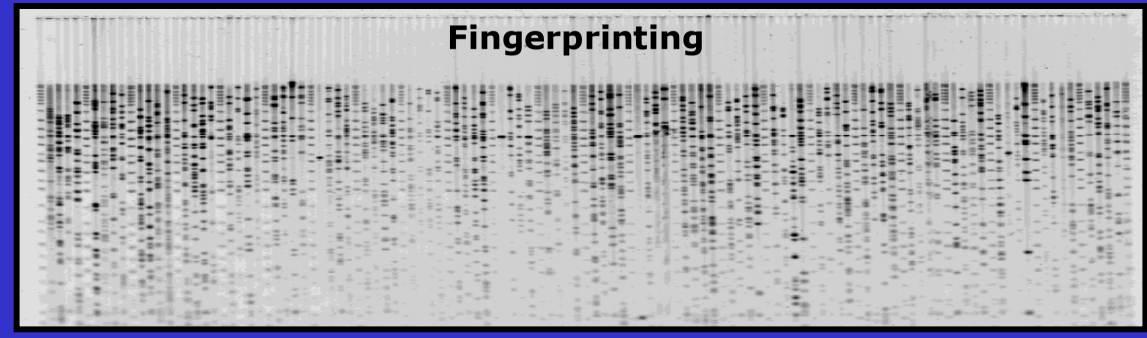
Yields were measured with a SYBR Green I nucleic acid gel stain luorometric assay from Molecular Probes, Inc.

Agarose Gel Analysis



8 µl aliquots of selected BAC 435G12 samples (wells A7 – H7) from all four plates of minipreps were digested with NotI and separated on a 0.8% agarose gel in 1X TAE buffer with EtBr. A series of BAC 435G12 mass standards is loaded between the sample sets
(Stds: 1 = 50 ng, 2 = 100 ng, 3 = 200 ng, 4 = 300 ng).

Fingerprinting



Direct Sequencing of BAC DNA Samples with BigDye™ Terminator Chemistry

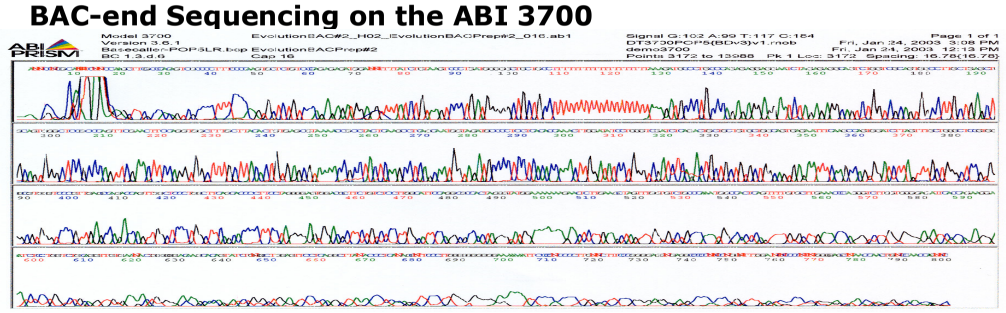
1. Transfer 10 µL of BAC DNA samples to a thermal cycling plate and set up sequencing reactions as follows:

BAC DNA	10 µL
BigDye™ terminator mix	3 µL
5X buffer (400mM Tris-HCl, pH 9.0, 10mM $MgCl_2$)	3 µL
Primer (10µM)	1 µL
H_2O	13 µL
Total volume	30 µL

2. Following addition of all components to cycle sequencing reactions, mix well and spin briefly. Cycle reactions according to the following program:

a. 95°C for 5 minutes
b. 95°C for 30 seconds
c. 50°C – 55°C for 10 seconds
d. 60°C for 4 minutes
e. Repeat Steps b-d for 75 cycles
f. Hold at 4°C

BAC-end Sequencing on the ABI 3700



Representative electropherogram of a sequencing reaction from a BAC 435G12 sample template. Samples were sequenced using BigDye Terminator chemistry version 3.1 (Applied Biosystems). The sequencing reactions were purified using the Montage-SEQ_{96} Sequencing Cleanup Kit on the Evolution P^3 prior to injection onto the ABI PRISM 3700. Injection parameters: 2kV, 25 seconds. Run parameters: 6.5kV, 117 minutes.

PHRED 20 Data

Run #	Avg. Phred 20 Score	Pass Rate (>100 Phred 20 bases)	CV	n
1	358	90%	25%	86
2	363	95%	26%	91

Conclusions

- **Montage kit is adaptable to Perkin-Elmer Evolution P^3 with:**
  - **Processing time of approximately 1 hour for 96 samples using one vacuum manifold setup (excluding the optional Solution 1 incubation step)**
  - **Minimal manual intervention**
- **BAC DNA generated with the Montage BAC_{96} kit provides**
  - **Superior sequence reads**
  - **Suitable template for fingerprinting**

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