Technical Note

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Title: **Semi-automated Method for Fosmid Mini-Preps Using Montage® BAC Miniprep$_{96}$ Kit on the Beckman Biomek® FX Nucleic Acid Preparation System**

Introduction

Genome sequencing has been accelerated forward by the use of cloning large fragments of genomic DNA into fosmids. These cloned pieces of DNA are usually up to 40 kb in size, whereas the fosmid vectors themselves are predominantly around 9 kb. Despite the challenges of low vector copy number, the need for high throughput automation of fosmid template preparation, like BAC's, is significant. Here we describe a semi-automated preparation method using the Montage BAC$_{96}$ Miniprep Kit on the Biomek FX workstation. Vacuum filtration and shaker mixing steps were performed off-line, however, these can be carried out on the FX utilizing SPE and orbital shaker ALP's, thus supporting a fully automated procedure.

The protocol was developed to maximize yields, decrease well-to-well variability, and provide high quality DNA sequence data. Culture and lysis conditions were optimized to accomplish this. The protocol, which relies primarily on vacuum filtration for sample processing, takes approximately 1 hour.

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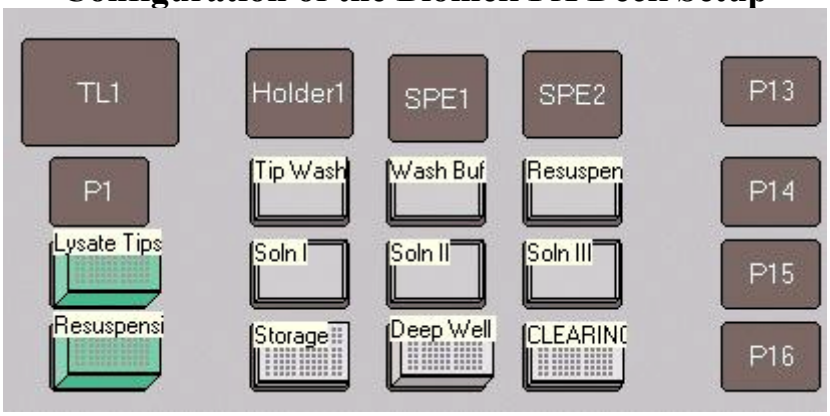
Configuration of the Biomek FX Deck Setup



Prior to starting:
Make sure deck is set up as follows:

P2: P200 Tips for alkaline lysis
P3: P200 Tips for resuspension
P4: Tip wash reservoir
P5: Solution 1 reservoir
P6: V-bottom storage plate
P7: Wash solution reservoir
P8: Solution 2 reservoir
P9: Deep well block with pelleted cells
P10: Resuspension solution reservoir
P11: Solution 3 reservoir
P12: MultiScreen$_{96}$ CLEARING or BAC plates

Procedure:

Growth and Cell Pelleting

1. Prepare fosmid precultures from colonies or glycerol stocks by inoculation into 1.0 mL aliquots of 2 x LB plus antibiotic (*e.g.*, 12µg/mL chloramphenicol) in 96 well culturing blocks (2.2 mL capacity). Cover blocks with foil sealing tape and pierce prior to securing in an incubator/shaker. Incubate at 37 °C and 320 rpm for 18 hours. Preparing precultures for seeding into cultures helps to normalize yields from well to well. This normalization of yields aids in preparation for downstream applications.

   ***NOTE**: 96 well blocks for growing 1.0 mL precultures are not included in this kit but may be ordered separately (Millipore Cat. No. LSKC CB0 50). Foil sealing tape is also not included.*

2. Inoculate 3 µL of fosmid precultures into 1.5 mL aliquots of 2 x LB plus antibiotic (*e.g.*, 12 µg/mL chloramphenicol) in 96 well culturing blocks (2.2 mL capacity). Cover blocks with foil sealing tape and pierce prior to securing in an incubator/shaker. Incubate at 37 °C at 320 rpm for 17–20 hours (OD_{650} reading should be in the range of 3.0–4.0).

3. Centrifuge at 1500xg for 5 minutes. Decant culture supernatant to a container for proper disposal.

4. Place deep well block with pellets on the Biomek FX in position P9.

Fosmid Purification

5. Start program.

6. Add 100µl of Solution 1 (P5) to the deep well block (P9) and wash tips (P4). Manually move block to shaker off-line and agitate at 800 rpm for 5 minutes.

7. Add 100µl of Solution 2 (P8) to the deep well block (P9) and wash tips (P4). Manually move block to shaker off-line and agitate at 800 rpm for 1 minute.

8. Incubate for 2 minutes at room temperature.

9. Add 100µl of Solution 3 (P11) to the deep well block (P9). Manually move block to shaker off-line and agitate at 800 rpm for 3 minutes.

10. Transfer the lysates from the deep well block (P9) to the CLEARING plate (P12). Unload lysate tips.

11. Filter CLEARING plate off-line at 8 inches Hg for approximately 10 minutes or until all wells are empty and collect the cleared lysates into the BAC plate.

12. Discard the CLEARING plate, move the BAC plate to the top of the vacuum manifold, and filter at 24 inches Hg for 8 minutes or until all wells are empty.

13. Place BAC plate in position P12, load resuspension tips (P3), and add 200µl of wash solution (P7) to the BAC plate.

14. Filter at 24 inches Hg for approximately 5 minutes or until all wells are empty.

15. Replace BAC plate to position P12 and add 30µl of resuspension solution (P10) to the BAC plate. Manually move plate to shaker off-line and agitate at 800 rpm for 10 minutes.

16. Replace BAC plate to position P12 and transfer purified fosmid samples from BAC plate to the V-bottom storage plate (P6).

Table 1a. Fosmid Yields

Average:	**0.42 µg**
Std Dev:	**0.08 µg**
CV:	**20.2%**
Min:	**0.23 µg**
Max:	**0.60 µg**

This plate represented 96 different fosmid clones which were prepared following the protocol described above. Yields were calculated using a fluorometric assay with SYBR® Green I nucleic acid gel stain (Molecular Probes, Inc). Plate average, standard deviation, and coefficient of variation are noted, as well as the minimum and maximum yields.

Table 1b. Sequencing Reaction Setup

Reaction Components	**Volumes (µl)**
BigDye™ Terminator Sequencing RR (v 3.1)	2.0
5X BigDye Buffer	1.0
Primer (10 pmoles/ul)	1.0
Fosmid DNA	6.0
Vf	10.0
Cycling conditions	
95°C	5 minutes
95°C	30 seconds
50°C	10 seconds*
60°C	4 minutes
100 cycles	

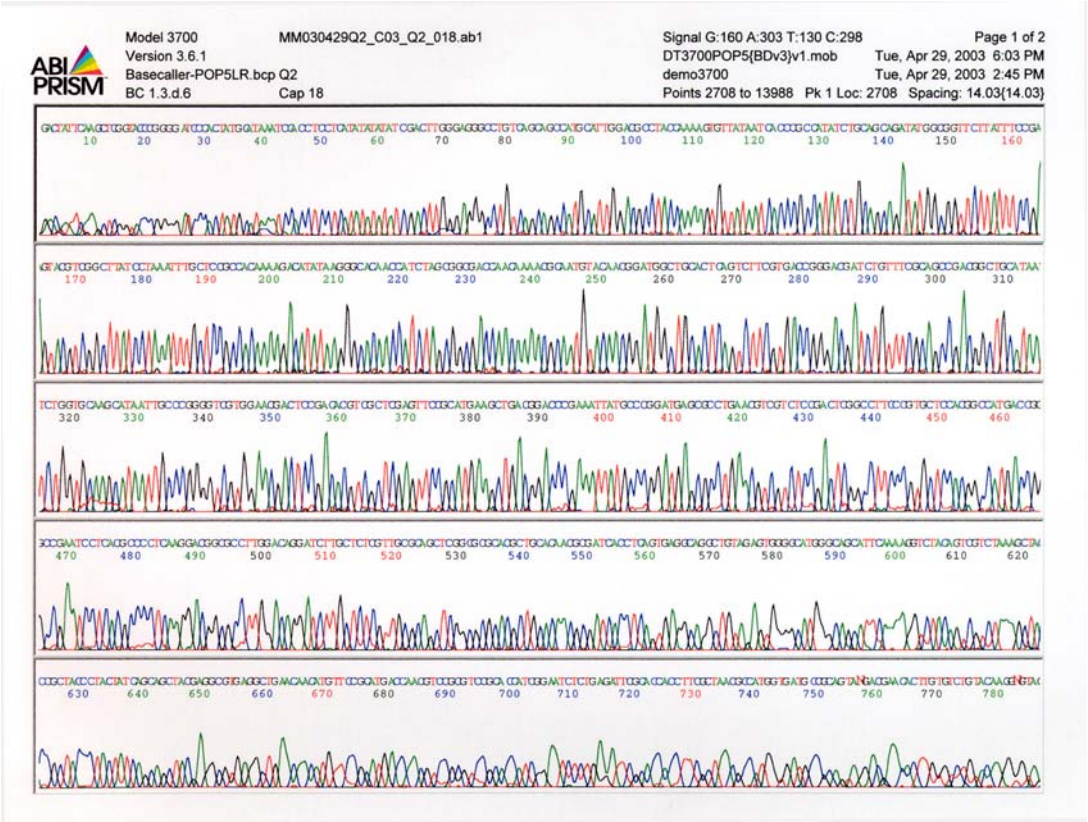
*The annealing temperature was optimized for use with a T7 primer (5'-TAA TAC GAC TCA CTA TAG GG-3') and the vector pCC1FOS™. Optimization of annealing temperature will be required when other vectors and/or primer combinations are employed.

Table 1c. Sequencing Data

Average:	**656**
Std Dev:	**133**
CV:	**20.2%**
Pass rate (>300):	**95%**
n	**96**

One plate of BigDye reactions with fosmid templates was sequenced and subsequently purified on a Montage-SEQ_{384} plate. BigDye reactions were injected at 2kV for 15 seconds and run at 6.5kV for 117 minutes. Table 1c shows the q-20 quality scores of the sequence reads determined by the software program Phred. Phred 20 scores correspond to a confidence level of 99.9% accuracy of base calling. Plate average, standard deviation, and coefficient of variation are noted, as well as the pass rate and sample number.

Figure 1. Typical Electropherogram



Montage-SEQ$_{384}$ purified ¼x BigDye Terminator sequencing reaction from a fosmid template run on an ABI 3700. Phred 20 = 683

Conclusion:

Using the Millipore Montage BAC Miniprep$_{96}$ kit for fosmids results in highly purified DNA which can be used in downstream applications such as sequencing, as was demonstrated here. The DNA yields, under these conditions, were more than adequate for obtaining superior sequence reads. The protocol is reliable and can be fully automated by incorporating a shaker ALP and performing vacuum filtration steps on the Biomek FX.

Ordering information:

Product Name	Millipore Part Number	Qty/Pack
Montage BAC MiniPrep$_{96}$ Kit	LSKB 096 01	1 X 96 well
	LSKB 096 04	4 X 96 well
	LSKB 096 24	24 X 96 well

Millipore Vacuum Manifold MAVM 096 0R

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