Technical Note

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Title: Preparation and Sequencing of Fosmid DNA Templates using the Montage® BAC_{96} Miniprep Kit and Montage SEQ_{96} & SEQ_{384} Clean-up Kits and Plates

For BigDye® Terminator Chemistry using Applied Biosystems' Automated DNA Sequencers

Introduction:

Successful high throughput preparation and subsequent DNA sequencing of fosmid DNA templates present several unique technical challenges. Millipore has developed a series of protocols, which enable the purification and sequencing of fosmid DNA templates using Montage Kits. The detailed protocols provide users with methodologies for:

- The rapid purification of single-copy-per-cell fosmid DNA templates from *E. coli* using the Montage BAC_{96} Miniprep Kit
- The assembly of miniaturized BigDye Terminator v3.1 sequencing reactions for sequencing of fosmid DNA templates
- The purification of fosmid sequencing reactions using either Montage SEQ_{96} Kits or SEQ_{384} plates prior to injection onto ABI's automated DNA sequencers

When combined, these protocols provide users with a reliable, high throughput methodology to obtain high quality sequence data with long read lengths using fosmid DNA templates.

Note: These protocols were developed using ABI's BigDye Terminator Chemistry (version 3.1) followed by analysis on the ABI Prism® 3700 DNA Sequencer. Although the approach is applicable to other sequencing instruments (e.g., ABI 3100, ABI 3730) and BigDye chemistries, some additional optimization may be required to accommodate the specific requirements of these other platforms. For injection into DNA sequencers other than the 3700, ABI's default injection parameters are recommended.

I. Purification of Fosmid DNA using Montage BAC_{96} Miniprep Kit

Unlike plasmids, where copy number can be as high as several hundred per cell, Bacterial Artificial Chromosomes (BACs) and fosmids are replicated in *E. coli* hosts as single copy episomes. To insure the production of sufficient high quality fosmid DNA for sequencing applications, it is essential that the following procedure is followed precisely (*i.e.,* growth in 2xLB media).

1. Prepare fosmid precultures from colonies or glycerol stocks by inoculation into 1.0 mL aliquots of 2x LB plus antibiotic (*e.g.*, 12µg/mL chloramphenicol) in 96 well culturing blocks (2.2 mL capacity). Cover blocks with the lids provided and secure in 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).*

2. Inoculate 3 µL of fosmid precultures into 1.5 mL aliquots of 2x LB plus antibiotic (*e.g.*, 12 µg/mL chloramphenicol) in 96 well culturing blocks (2.2 mL capacity). Cover blocks with the lids provided and secure in 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 deep well blocks at 1500 × g for 5–7 minutes. After centrifugation, immediately decant culture supernatant to a container for proper disposal. Invert and tap the plates firmly on absorbent paper/pads to remove residual culture supernatant.

***NOTE**: Failure to remove media will add undesired volume to lysate.*

4. Resuspend pellets by adding 100 µL of Solution 1 (containing RNase A enzyme) to each well then mixing on a plate shaker for 3–5 minutes, or longer if necessary, until cells are completely resuspended. Alternatively, resuspension may be achieved by vortexing or pipetting.

***NOTE**: Thorough resuspension of cells is critical for successful lysis. Set the plate shaker at the highest speed possible without causing spilling, splashing, or sliding of the culture block off the shaker. Allow sufficient shaking time (3–5 minutes) to completely resuspend the cells in solution. No pellets should be visible at the bottom of the wells.*

5. Add 100 µL of Solution 2 to each well. Mix immediately with a plate shaker for 1 minute. Refer to "Notes on Plate Shaker Speed" in the Protocol Guidelines Section. Incubate for an additional 2 minutes at room temperature.

***NOTE**: Total lysis time should not exceed 5 minutes.*

6. Add 100 µL of Solution 3 to each well. Mix immediately and vigorously with a plate shaker for 2 minutes. Refer to "Notes on Plate Shaker Speed" in the Protocol Guidelines Section. At this point, the bacterial lysate is ready for transfer to the lysate clearing plate (labeled "CLEARING").

7. Place the BAC plate (labeled "BAC") inside the vacuum manifold.

8. Pipette the entire lysate volume from the bottom of each deep well, and dispense into the corresponding well of the lysate clearing plate.

9. Place the lysate clearing plate on top of the manifold. Make certain the vacuum seal is intact and adjust the vacuum to 8 inches of Hg (270 millibar – 203 torr.). Apply the vacuum, drawing the lysate through the clearing plate into the BAC plate. After filtration is complete, switch off the vacuum and discard the lysate clearing plate.

10. Place the BAC plate containing clarified lysates on top of the empty manifold. Apply vacuum at 24 inches of Hg (810 millibar – 610 torr) until wells are empty. Direct filtrate to waste. When filtration is complete, switch off vacuum.

***NOTE**: Filtration time is sample, temperature, and pressure dependent. The filters will appear shiny even after the wells are empty.*

11. Add 200 µL of Solution 4 to each well of the BAC plate. Apply vacuum at 24 inches of Hg (810 millibar – 610 torr) until wells are empty. Direct filtrate to waste. When filtration is complete, switch off vacuum.

12. Resuspend fosmid DNA samples by adding 30 µL of Solution 5 to the wells of the BAC plate. After adding Solution 5 to the wells, shake for 10 minutes on a plate shaker. Refer to "Notes on Plate Shaker Speed" in the Protocol Guidelines Section.

13. Pipette retained fosmid DNA from the wells of the BAC plate into the V-bottom plate for storage. (Recovered volume can be maximized by tilting the BAC plate before collecting the sample.) Use the sealing tape to seal wells of the V-bottom storage plate.

***NOTE**: Fosmid samples can also be transferred directly from the BAC plate into sequencing reactions. (See sequencing section below.)*

II. Sequencing Reaction Recipe:

Assemble a 1/4x BigDye Terminator[1] DNA sequencing reaction as outlined below. As with any DNA sequencing protocol, optimal primer design is essential for high quality DNA sequence results.

	1/4x Reaction
Fosmid DNA	6 µL
BigDye Terminator v3.1	2 µL
ABI 5X Sequencing Buffer	1 µL
10 µM Primer (10 pmoles)	1 µL
Total volume	10 µL

[1] Although other sequencing reaction scales may provide satisfactory sequencing results, users should balance any potential cost savings resulting from reaction miniaturization against the typically shorter read lengths and lower pass rates typically obtained with increased miniaturization.

III. Fosmid Sequencing Reaction Cycling Conditions:

Following addition of all components to the sequencing reaction, mix well and spin briefly to insure that all of the liquid is at the bottom of the wells. Appropriate sealing of the wells is essential to minimize volumetric loss during thermal cycling. Cycle reaction according to the following program (100 cycles should be used to maximize the production of sequencing products):

1. 95° C for 5 minutes
2. 95° C for 30 seconds
3. 50° C for 10 seconds[2]
4. 60° C for 4 minutes
5. Repeat steps 2-4 for a total of 100 cycles
6. Hold at 4 °C

[2] 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.

IV. Fosmid Sequencing Reaction Cleanup on SEQ_{96} or SEQ_{384}:

1. Add 15 µL Montage Wash Solution to the sequencing reaction from the previous section.
2. Mix by gently pipetting up and down 3 times.
3. Transfer the solution to the Montage 96- or 384-SEQ plate.
4. Place the SEQ plate on the vacuum manifold.
5. Set the vacuum to 23–25" Hg.
6. Apply vacuum until wells are completely empty and keep applying vacuum for 15 extra seconds (approximately 2 - 6 minutes total).

Note: For optimal results, over-drying (*i.e.*, > 1 minute after the last well is dry) should be avoided.

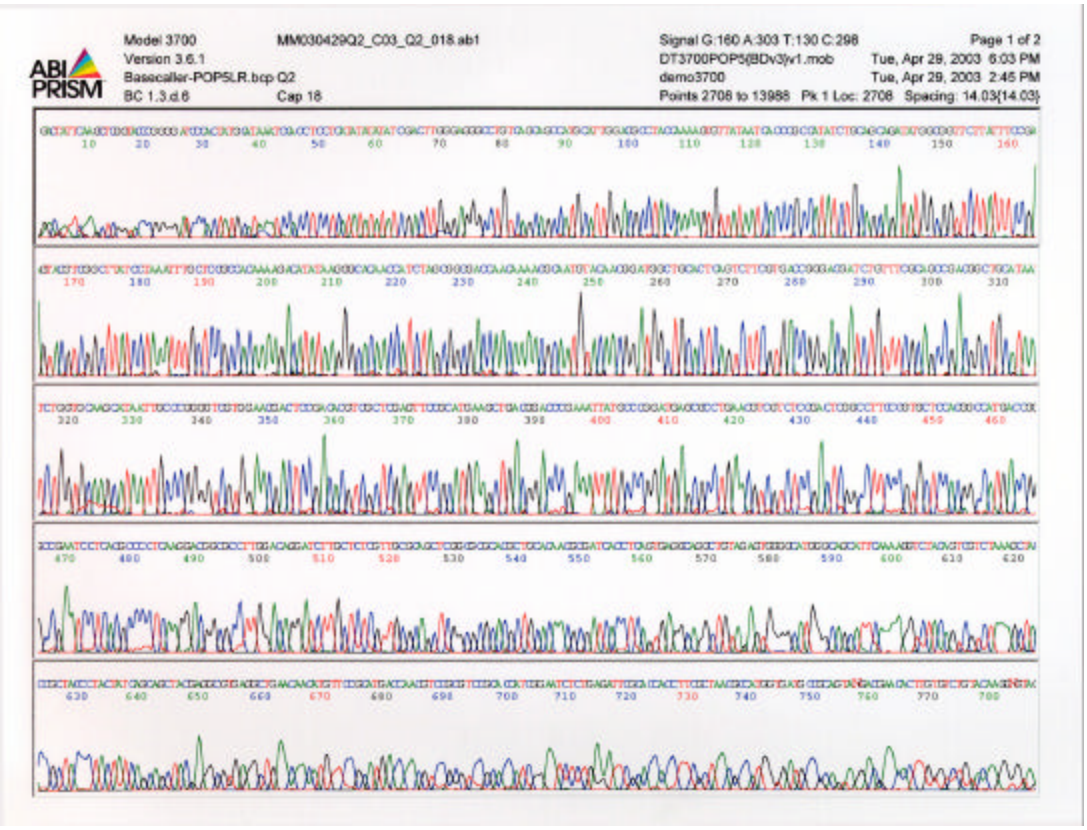
7. Shut off the vacuum source and remove the SEQ plate from the manifold.
8. Blot the excess liquid from the bottom of the SEQ plate by briefly pressing the plate on an absorbent material such as paper towels.
9. Add 25 µL of Montage Wash Solution to the bottom of each well of the SEQ plate.
10. Place the SEQ plate back on the vacuum manifold and apply vacuum until wells are completely empty. Keep applying vacuum for 15 more seconds (approximately 3 - 7 minutes total).

    Note: For optimal results, over-drying (*i.e.*, > 1 minute after the last well is dry) should be avoided.
11. Shut off the vacuum source and remove the SEQ plate from the manifold.
12. Blot excess liquid from the bottom of the plate by briefly pressing the plate on an absorbent material such as paper towels.
13. Add 15 µL of Injection Solution to the bottom of each well of the SEQ plate.
14. Resuspend the purified sequencing reaction products by gently pipetting up and down ~20-30 times, depending on the liquid handler. Alternatively, the DNA can be resuspended by shaking for 10 minutes on a microplate shaker
15. Transfer into an appropriate plate for injection.
16. Seal the plate with foil.
17. Injection for POP-5, 2kV for 15 seconds (ABI 3700).

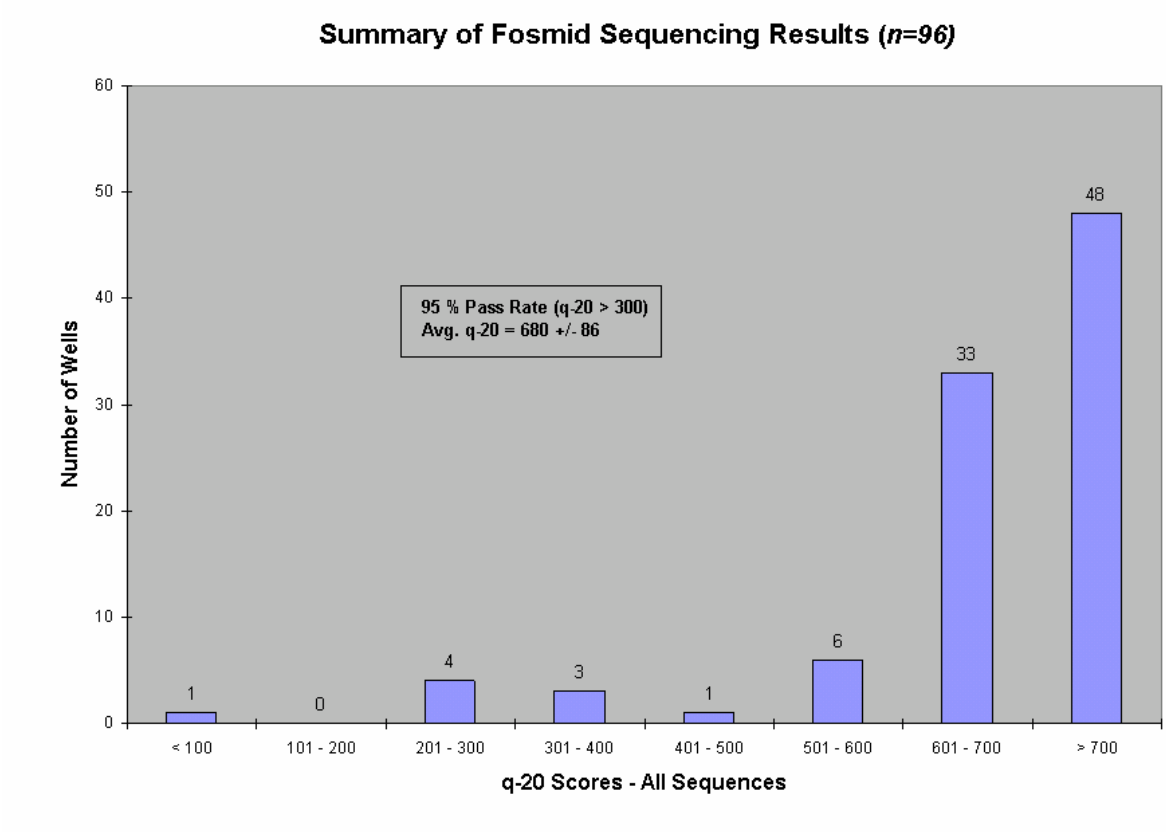
    Note: Optimization of injection conditions may be required.
18. For best results, inject the samples immediately after purification. If short-term storage is required, store the purified samples at -20^0 C.

V. Typical Data

Representative Electropherogram



Results of a ¼ x BDT v3.1 reaction, 10 mL volume, 6 mL fosmid template (q-20 = 683).



Summary:

The chart above shows the distribution of Phred-20 (q-20) Scores that were obtained with a plate of 96 different fosmid clones following the protocols described above. The vast majority (84%) of sequences displayed a q-20 score of 600 or greater, demonstrating the utility of the process.

Acknowledgement: Millipore would like to thank Genevieve Di Bartolo and the Joint Genome Institute for the use of their clones.

Ordering Information:

Montage BAC$_{96}$ Miniprep Kit:	**Millipore Catalogue Number**
1-pack	LSKB09601
4-pack	LSKB09604
24-pack	LSKB09624

Montage SEQ$_{96}$ Sequencing Reaction Cleanup Kit:	
1-pack	LSKS09601
4-pack	LSKS09604
24-pack	LSKS09624

Montage SEQ$_{384}$ Sequencing Reaction Cleanup Plates:	
10-pack	S384SEQ10
50-pack	S384SEQ50

Montage Wash Solution:	
500 mL	LSKS BW5 00

Additional equipment

MultiScreen® Vacuum Manifold	MAVM 096 0R
Tubing (for vacuum use), silicone, 3/16" I.D., (1.4 m)	X X71 000 04
Vacuum Filtering Flask, 1L	X X10 047 05
Stopper, No. 8, perforated, silicone (5pk)	XX10 047 08

Recommended equipment

Millipore Vacuum/Pressure Pump	
115 V/60 Hz	WP61 115 60
220 V/50 Hz	WP61 220 50
100 V/50 – 60 Hz	WP61 100 60
Millex® FG-50 Vacuum Pump Filter Guard (10pk)	SLFG 050 10