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GENOMICS

Montage BAC$_{96}$ Miniprep Kit User Guide

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P36392, Rev. A, 05/04

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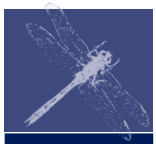


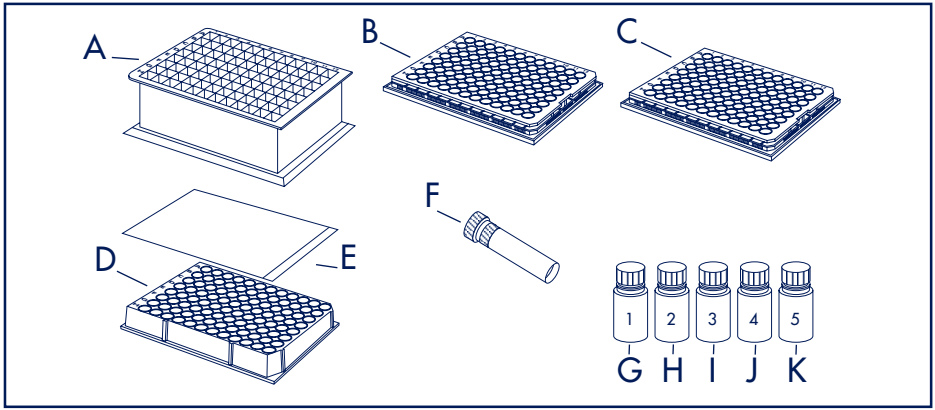
Introduction

An important step in current genome sequencing strategies is the cloning of large fragments of genomic DNA into F factor based vectors called bacterial artificial chromosomes (BACs). The cloned fragments are generally in the range of 50–300 Kb while the most commonly used BAC vectors are 7–10 Kb. Once cloned into the BAC vector, the fragments can be sequenced either directly or following PCR amplification of a specific region.

One obstacle when adapting this strategy to a high throughput format is the low copy number of these vectors. Because there are only 1–2 copies of the each BAC maintained per cell, the theoretical yield of BAC DNA from a given volume of culture is significantly lower than that for plasmid DNA. In addition, the large variability in insert size contributes proportionally to the variability in BAC DNA yield. Therefore, it is essential that the bacterial cell culturing conditions and the DNA purification procedure are optimized in order to provide sufficient high quality DNA for desired downstream applications.

The Montage BAC_{96} Miniprep Kit has been specifically designed for the rapid purification of BAC clones in a 96 well format. The kit provides all of the necessary components and reagents for the purification of high quality DNA suitable for direct BAC end sequencing. The "Sequencing of the BAC DNA" section of this manual describes optimized sequencing reaction conditions for the BAC DNA.

Kit Components



Letter	Part	Function
A	96-well culture block	Growth of host bacteria
B	MultiScreen®$_{96}$ BAC plate	Purification of BAC DNA
C	MultiScreen lysate clearing plate	Clearing of bacterial lysate
D	V-bottom storage plate	Storage of BAC DNA samples
E	Sealing tape	Sealing of BAC DNA samples
F	RNase A	Required additive for Solution 1

Kit Components, continued

Letter	Part	Function
G	Solution 1	Cell resuspension
H	Solution 2	Cell lysis
I	Solution 3	Neutralization
J	Solution 4	Wash
K	Solution 5	Resuspension/storage of BAC DNA
Not shown	Foil seal	Sealing plates during growth (must be pierced)

Additional Equipment Required

- Pipettor
- Vacuum manifold (Millipore Cat. No. MAVM 096 0R, or equivalent)
- Vacuum pump or uniform vacuum source (Millipore Cat. No. WP61 115 60, or equivalent)
- Centrifuge (for deep well culture block)
- Plate shaker
- Incubator shaker
- 96 well blocks for preculturing BAC clones (Millipore Cat. No. LSKC CB0 50)
- Foil piercing tool (e.g., Scienceware® Bel-Blotter™ with 96 23-gauge 3/4" needles)

Precautions

- MultiScreen plates are disposable, single-use-only devices.
- This kit is for research use only. Not for use in clinical applications.
- Avoid contact with Solution 2 to prevent skin irritation.
- MultiScreen plates consist of a polystyrene plate that is sealed to a polyethylene underdrain, forming 96 independent (individually sealed) wells. **Do not separate the underdrain from the MultiScreen polystyrene plate. Separation will result in plate failure and well leakage.**

Storage Conditions

The kit reagents should be stored at 15 °C to 30 °C. However, following the addition of RNase A to Solution 1, this solution must be stored at 2 °C to 8 °C and should be used within six months (not to exceed expiration date on the kit).

Procedure for BAC DNA Minipreparation

Since their development, bacterial artificial chromosomes have been used to manipulate large fragments of genomic DNA[1] 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 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[2].

Culturing the Host Bacteria

Appropriate culturing conditions are key to maximizing the yield of BAC DNA obtained when using the BAC Kit. Therefore, it is absolutely essential that the cells be grown under the following conditions:

1. Culture the bacterial host prior to purification of the BAC DNA in 2x Luria-Bertani (LB) broth. Use of other media (i.e., 2xYT or TB) is not recommended with the Montage BAC_{96} Miniprep Kit, as it will result in reduced yield and/or clogging of the purification plates.

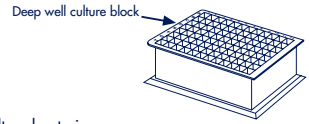
   Formulation for 1 Liter (L) of 2x LB media:

Tryptone	20g
Yeast Extract	10g
NaCl	10g

   Mix media components in a final volume of 1 liter (L) using Milli-Q® grade water and sterilize by filtration using a Stericup® filter cup or autoclaving prior to use.
2. Grow BAC cultures in 1.5 mL of 2x LB plus antibiotic (e.g., 12.5 µg/mL chloramphenicol) in the provided 96 well culturing blocks.
3. Cover the blocks with the aluminum foil tape and puncture 3–4 holes in each well to insure appropriate aeration. Secure the block in a shaking incubator and incubate at 37 °C and 320 rpm for 20–24 hours (optimal speed will vary depending on the geometry of the incubator). The final OD_{650} reading should be in the range of 3–5.

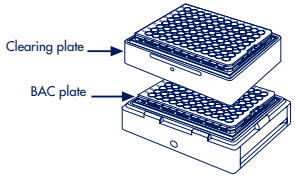
Overview of Procedure

1



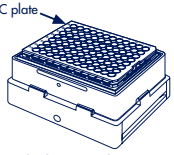
1. Culture bacteria.
2. Centrifuge.
3. Freeze cell pellets.
4. Thaw at room temperature 15 minutes.
5. Resuspend cells.
6. Lyse cells.
7. Neutralize.
8. Pipette up and down three times.

2



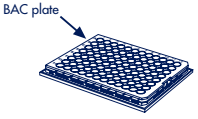
9. Place BAC plate inside manifold.
10. Transfer lysates into Clearing plate.
11. Reassemble manifold with Clearing plate on top of manifold. Apply vacuum at 8 inches Hg for 6–8 minutes or until wells are empty.

3



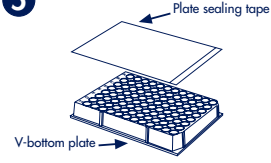
12. Discard Clearing plate. Transfer BAC plate to top of manifold. Apply vacuum at 24 inches Hg for 6–10 minutes or until wells are empty.
13. Add 200 µL of Solution 4 to each well. Apply vacuum at 24 inches Hg for 4–6 minutes or until wells are empty.

4



14. Add 35 µL of Solution 5 to each well. Shake for 10 minutes to resuspend BAC DNA.

5



15. Transfer samples directly into sequencing reactions, or into V-bottom plate for storage.

Protocol Guidelines

Before beginning the protocol for purifying the BAC DNA, review the following:

- Add RNase A (total contents of tube) to Solution 1 and mix thoroughly prior to use.
- Bring Solution 2 to room temperature before using in order to dissolve detergent that may have precipitated due to lower temperatures during shipping.
- Screw cap on Solution 2 tightly immediately after use in order to avoid destabilization that may occur upon exposure to air.

CAUTION: Avoid skin contact with Solution 2 to prevent irritation.

Note on Plate Shaker Speed

In the following protocol, all plate shaking steps after growth of the bacterial cells were performed with a Lab-Line™ titer plate shaker (Lab-Line® Instruments, Model No. 4625) at setting 6, estimated at 800 rpm. Because speed settings in different shakers may correspond to different speeds, determine which setting on your shaker offers the optimal speed before performing the protocol.

BAC DNA Purification Protocol

1. Centrifuge deep well blocks at 1500 × g for 10 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.

2. Freeze the plates containing the pellets at -20 °C for 1 hour (if desired the samples can be frozen for up 24 hours prior to processing).

   NOTE: Freezing the pellets improves resuspension and yield of BAC DNA.

3. Allow the pellets to thaw at room temperature for 15 minutes.

4. Resuspend pellets by adding 100 µL of Solution 1 (containing RNase A) to each well then mixing on a plate shaker for 5 minutes until cells are completely resuspended. If the cells are not completely resuspended, increase the shaking time as needed. Alternatively, resuspension may be achieved by vortexing or pipetting.

   NOTE: Thorough resuspension of cells is critical for successful lysis. No pellets should be visible at the bottom of the wells.

5. Add 100 µL of Solution 2 to each well without mixing or shaking. Incubate at room temperature for 5 minutes.

   NOTE: Mixing after the addition of Solution 2 may decrease yield.

6. Add 100 µL of Solution 3 to each well. Mix immediately for 2 minutes using a plate shaker.

7. Pipette the entire volume of lysate up and down three times to break up any large clumps of flocculent.

8. Place the BAC plate (labeled "BAC") inside the vacuum manifold for filtrate collection.
9. Pipette the entire lysate volume from the bottom of each deep well and dispense into the corresponding well of the lysate clearing plate (labeled "CLEARING").
10. 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/200 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.
11. 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.

12. 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.
13. Resuspend BAC DNA samples by adding 35 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.
14. Pipette retained BAC DNA from the wells of the BAC plate into the V-bottom plate for storage. The recovery 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.

Procedure for Sequencing BAC DNA

In order to simplify sample processing, we have optimized protocols for direct end sequencing[3] of BAC DNA samples resuspended in Solution 5. The sequencing protocol described below uses a fixed volume of dissolved BAC DNA (10 µL), accommodating a range of BAC DNA yields that we have observed when processing 96-clone BAC library plates (human chromosome 22 library, Invitrogen/Research Genetics). Although each BAC clone has its own optimal window for the amount of BAC template used in cycle sequencing reactions, the sample volume we prescribe has produced the highest number of Phred 20 bases[4,5] and the lowest variation in scores across a 96 well plate in titration experiments.

Reaction Set-up

Assemble a 1/4x BigDye Terminator 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
BAC DNA	10 µL
BigDye Terminator v3.1	2 µL
ABI 5X Sequencing Buffer	3 µL
10 µM Primer (10 pmoles)	1 µL
Milli-Q water	4 µL
Total volume	20 µL

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

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
4. 60 °C for 4 minutes
5. Repeat steps 2–4 for a total of 100 cycles
6. Hold at 4 °C

NOTE: The annealing temperature was optimized for use with a T7 primer (5'-TAA TAC GAC TCA CTA TAG GG-3') and the vector pBACe3.6. Optimization of annealing temperature may be required when other vectors/primer combinations are employed.

Troubleshooting

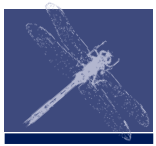
Problem	Possible Causes	Suggestions
Low BAC DNA yields	Inadequate growth conditions	Ensure that bacterial cells are grown with adequate aeration and for **at least** 20 hours.
		Check cell density. OD_{650} should be between 3 and 5.
	Use of media other than 2xLB	Use of other cell culture media will result in low yields.
	Failure to freeze bacterial pellets prior to resuspension	Freezing of cell pellets improves cell resuspension efficiency.
	Inadequate resuspension of cell pellets	Ensure that cells are completely resuspended in Solution 1 prior to addition of Solution 2.
	No antibiotic added	Ensure that antibiotics are added at to media every stage of bacterial culturing.

Troubleshooting, continued

Problem	Possible Causes	Suggestions
Low BAC DNA Yields	Over drying of BAC plate	Stop vacuum when wells appear empty.
	Failure to mix neutralized lysate	Mixing of neutralized lysate prior to filtration is essential for optimal yield of BAC DNA.
Variable BAC DNA yields	Highly variable BAC DNA inserts size (50–300 Kb) and low copy number result in variable yield of DNA mass (6-fold range)	Molar concentrations of BAC DNA will be equivalent and adequate for downstream applications.
Wells not filtering uniformly	Airlock due to bubble in the well	Agitate the lysate until bubble surfaces.
Slow filtration	Use of weak or house vacuum	Use a portable vacuum pump that produces at least 24 inches of Hg (810 millibar/610 torr.) vacuum strength.
Plates separating	No underdrain support grid	Use Millipore manifold or equivalent with support grid.

Troubleshooting, continued

Problem	Possible Causes	Suggestions
Chromosomal DNA contamination	Excessive shaking or overincubation after addition of Solution 2	Do not exceed the incubation time specified in the protocol and avoid excessive shaking or vortexing. Minimal contamination will not interfere with DNA sequencing.
No sequence from BAC clone.	Not enough BAC DNA in sequencing reaction	Ensure that enough material has been added to sequencing reaction (>100 ng).
	Use of sequencing chemistries that are not adequate for BAC end sequencing	Use BigDye terminator chemistry.
	BAC end is difficult to sequence.	Redesign primers or use alternative sequencing method for difficult regions.



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Ordering Information

This section lists catalogue numbers for the Montage BAC_{96} Miniprep Kit. See "Technical Assistance" for information about contacting Millipore. You can also buy Millipore products on-line at www.millipore.com/purecommerce.

Kits

Product	Catalogue No.	Qty/Pk
Montage BAC_{96} Miniprep Kit: plates, culture blocks, and reagents for 96 samples	LSKB 096 01	1/pk
Montage BAC_{96} Miniprep Kit: plates, culture blocks, and reagents for 4 × 96 samples	LSKB 096 04	4/pk
Montage BAC_{96} Miniprep Kit: plates, culture blocks, and reagents for 24 × 96 samples	LSKB 096 24	24/pk

Ordering Information, continued

Kit Components

Product	Catalogue No.	Qty/Pk
Solution 1, cell resuspension solution, 500 mL	LSKC RS5 00	1/pk
Solution 2, cell lysis solution, 500 mL	LSKC LS5 00	1/pk
Solution 3, neutralization solution, 500 mL	LSKN S05 00	1/pk
Solution 4, wash solution, 500 mL	LSKN F05 00	1/pk
Solution 5, storage buffer, 500 mL	LSKC TB5 00	1/pk
RNase A, 30mg (in 50% glycerol)	LSKP MRN 30	1/pk
V-bottom plates	LSKV BP1 00	100/pk
Cell culture blocks with lids, 96 wells, 2.2 mL	LSKC CB0 50	50/pk
Adhesive plate sealing tape	LSKA ST1 00	100/pk

Ordering Information, continued

Accessories

Product	Catalogue No.	Qty/Pk
Millipore vacuum manifold	MAVM 096 0R	1/pk
Stericup filter cup	SCGV U01 RE	12/pk
Chemical duty pump, 115V/60Hz	WP61 115 60	1/pk
Chemical duty pump, 220V/50Hz	WP61 220 50	1/pk
Chemical duty pump, 100V/50 or 60Hz	WP61 100 60	1/pk
Tubing, silicone, 3/16" I.D., 1.4 m	XX71 000 04	1/pk
Vacuum filtering flask, 1L	XX10 047 05	1/pk
Stopper, No. 8, perforated, silicone	XX20 047 18	5/pk
Millex®-FG_{50} filter unit	SLFG 050 10	10/pk

Technical Assistance

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