Procedures for the Purification of Bacterial Artificial Chromosome (BAC) DNA.

A-5

We have developed a spin column miniprep protocol for the purification of BAC DNA. No organic extractions are required. Typical yields between 0.6 and 1.0 µg are observed from an overnight 3-5 mL culture. A discussion of the data and results depicting the quality of BAC DNA purified is presented. Alternative protocols for preparation of BAC DNA from 25-250mL cultures are also presented. Observations of some factors that affect BAC yield will be discussed.

Introduction:

Current protocols for BAC isolation generally involve enzymatic steps or organic extractions and many are unique to individual laboratories. Several protocols involve modifications of existing kits (eg. Qiagen Midi) or require the use of an automated robot (Autogen 740).

Our goal was to develop a simple miniprep protocol that could yield enough BAC DNA for running one sequencing reaction as well as other biochemical characterizations (eg. restriction digest). This protocol is now commercially available in the Princeton Separations **PSI Ψ Clone BAC DNA** kit (Cat. # PP-120) for 3-5mL cultures, and in the **PSI Ψ Clone Big BAC** kit (Cat# PP-121) for 25-250mL cultures.

Materials and Methods:

A portion of a mouse BAC library was purchased from Research Genetics (Huntsville, AL) and used as a source of BAC DNA. Cultures were routinely grown in LB or Terrific Broth (Sambrook et. al, 1989) supplemented with chloramphenicol. As a control, large cultures were grown and subdivided in aliquots (3-5 mL) to allow for accurate comparisons between experimental conditions. All evaluations made within individual experiments were performed using the same culture.

BAC Yield Estimation

Yields of BAC DNA from purification were estimated using the Hoechst Assay (Molecular Probes, Eugene, OR) and read using a 96 well fluorescent microplate reader (Millipore 2350, Bedford, MA) essentially as described by Steen et. al.,1993. Commercially available Lambda or plasmid DNA was used as a measurement standard.

Sequencing

Purified BAC DNA was sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit as follows:

Template	200-400 ng	in 11.0 µL
Primer (M13 For*)	3.2 pmole	in 1.0 µL
Reaction Mix		8.0 µL

*Present in pBeloBAC11

PCR as follows:

Perkin Elmer 9600-	Step 1	98C	5 min
	Step 2	98C	30 sec
	Step 3	55C	20 sec
	Step 4	60C	4 min
	Step 5	Cycle	to Step 2 30x
	Step 6	60C	5 min
	Step 7	4C	-------

Sequencing reactions were cleaned using Centri-Sep and dried (Savant Speed-Vac). Loading buffer was added (1-3 µL) and the entire reaction was loaded in the well and sequenced using an ABI 377 sequencer.

Restriction Digests

Purified BACs were digested with Pst 1 and subjected to Agarose Gel Electrophoresis (AGE) to spot excessive chromosomal DNA and/or RNA. AGE was routinely done using 0.8% (w/v) agarose gels using 1x TAE running buffer (Sambrook et. al., 1989)

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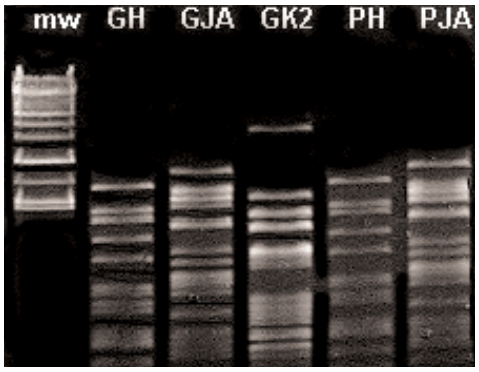
Factors Effecting BAC DNA Purity

The effect of various neutralization buffers on the final yield and purity was examined. Some protocols require phenol extraction following neutralization. In our hands, phenol extraction reduced the yield in all cases (data not shown).

Using 5M Ac(K) pH 4.8 as a neutralization buffer resulted in greater yields than 3 M KAc pH 5.5. The resulting BAC DNA was digestible with various restriction enzymes but could not successfully be sequenced even at 800 ng per reaction. Purified BAC DNA obtained using the PSI Ψ Clone BAC DNA neutralization buffer yielded sequence at template concentrations as low as 200 ng (Figure 1).

A.



B.

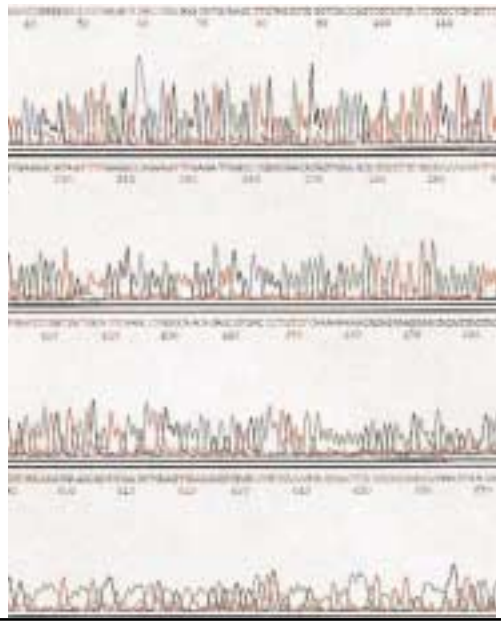


Figure 1. Quality tests of isolated Bac DNA.

A. DNA from various BAC clones was digested with Pst 1 and electrophoresed using 0.8% (w/v) agarose in 1X TAE. B. Sequence chromatograph of BAC template (200 ng) sequenced as described above. The sequence is readable beyond 600 bases.

Factors Effecting BAC DNA Yield

The stringent copy number of BACs (~ 1 copy/cell) limits the users ability to influence yield. We examined some growth conditions to see the effect on yield. Adding chloramphenicol (Cm) to broth medium at 20 µg/mL instead of 12.5 µg/mL increased yields (although occasional rearrangements were seen using Cm at 20 µg/mL). These results are listed in Table 4. The reason for this increase is unclear.

Table 4. Comparison of yields (µg) with different BAC cultures grown in 5 mL TB with Cm at either 12.5 or 20 µg/mL.

BAC	Cm 12.5	Cm 20	% increase
Hed	0.12 µg	1.28 µg	975.6
K2	0.375	0.60	57.9
JA	0.289	0.505	75.0

Growing culture in Terrific broth (TB) instead of LB appeared to increase yields due to the increased culture density. These results are listed in Table 5.

Table 5. Comparison of yields (µg) with different BAC cultures grown in 5 mL TB (Cm 20 µg/mL) or LB (Cm 20 µg/mL).

BAC	LB	TB	% increase
P[1]	0.982 µg	1.73 µg	76.5
O3	0.446	0.83	86.0
N3	0.522	1.55	197.0
Hed[1]	0.842	0.975	15.7

[1] There was apparently significant effect with this BAC culture.

The best yields were generally seen when a single colony was inoculated from a plate not more than 2 days old (Table 6).

Table 6. Yields of several BAC DNAs compared from cultures (5 mL TB, Cm 20 µg/mL) inoculated from single colonies on agar plates of different ages.

BAC	Overnight	1-week	% difference
P1	0.823 µg	0.611 µg	+25.7
JA	0.900	0.505	+44.0
K2	0.656	0.592	+10.0

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Cultures inoculated from overnight plates (or plates not more than 2 days old) appear to reach culture densities OD_{600} of ~6.0 within 16-18 h. Cultures one week old generally gave equivalent yields when allowed to grow to the same density (~24h). We have noticed that cultures greater than 3 weeks old would generally have reduced yields of 25-50% (data not shown). Occasionally inserts would be lost after continuous subculture (2-3 mos.) or if a colony was picked from a plate greater than 4 weeks old.

Very low yields (<50ng/prep) are often due to a recombination event resulting in insert loss (especially when working with a clone that normally gives higher yields). In such an instance restriction digestion and electrophoresis should be used to check BAC size. Note that because BACs have stringent replication origins (and a fixed low copy number) the bigger the BAC molecule the greater the yield will be. Recombination will result in a smaller BAC molecule and therefore a lower yield.

User losses can occur at the isopropanol precipitation step and 70% ethanol wash step since the pellets (≈1µg DNA) are difficult to see. Use a consistent method of orienting microcentrifuge tubes in the centrifuge rotor. The location of the pellet will be known even if it isn't visible and can be avoided when removing supernatants with a pipet.

Chromosomal DNA Contamination

Excessive chromosomal DNA in the final prep can be the result of poor lysis conditions or having a culture density that is too high. The conditions for this kit were worked out using 3-5mL overnight cultures with an OD_{600} of about 6.0. If culture densities are higher (>8.0 to 10.0) poor lysis will result and chromosomal DNA or RNA may be found in the final prep.

Vortexing or mixing too vigorously at the lysis step may also result in chromosomal DNA in the final step. Gentle inversion is all that is necessary. The incubation times following addition of the lysis and neutralization buffers are important in order to properly precipitate the chromosomal and cellular debris. Otherwise the result will be a thick blob of material that will be extrememly difficult to centrifuge. It is also important to note that centrifugation conditions for the precipitation have been worked out at 20,000 x g for 10 minutes. Centrifugation at a lower g force will require longer spin times to completely pellet the debris

Conclusions:

Our BAC miniprep protocol yields DNA sufficient for at least one (generally two) sequencing reactions and biochemical analysis (e.g. PCR or restriction digestion). Up to 1.0 µg BAC DNA can be purified from a 5 mL Terrific Broth culture. The BAC DNA is pure enough for sequencing of as little as 200 ng template. The BAC DNA also appears stable at 4C for at least 10 days and indefinitely at -20C.

References:

Sambrook, J., E.F. Fritsch, and T. Maniatis. *Molecular Cloning-A Laboratory Manual*. Cold Spring Harbor Press, 1989.

Sinnett, D., C. Richer, and A. Baccichet. *BioTechniques* 24:752-754, 1998.

Steen, R.G., C.G. MacDonald, A.L. Weaver and A.M. Pitt. *BioTechniques* 15:932-933, 1993.

Protocol for 3-5mL culture media

using the **PSI Ψ Clone BAC DNA** kit (Cat# PP-120).

We recommend growing cultures for 20-24 hr in Terrific Broth containing 20 µg/mL chloramphenicol. This should result in an OD_{600} of 4-6.

1. Add 0.5mL Resuspension Buffer (1) to RNase A tube. Mix gently. Transfer 0.25mL of this RNase A solution to remaining Resuspension Buffer (1). Mix well.
2. Centrifuge a 3-5 mL overnight culture. Pour off the culture broth.
3. Resuspend the cell pellet by gently pipetting in 0.5 mL Resuspension Buffer (1) with RNAse A. Transfer to a sterile 1.5 mL microcentrifuge tube.
4. Add 0.5 mL Lysis Buffer (2) and mix by **gentle** inversion (note: lysate may not clear). Incubate at room temperature for 10 min.
5. Add 0.5 mL Neutralization Buffer (3) and mix by **gentle** inversion until a thick white precipitate forms. Chill in an ice bath for 10 min.
6. Centrifuge (20,000 x g) for 10 min, collect the supernatant (≈1.4 mL) and transfer to a sterile test tube (e.g. 15 mL Falcon tube or equivalent).
7. **Dilute the cleared lysate with 1.0 mL sterile water.** Mix by pipetting.
8. Add 0.5 mL Equilibration Buffer (4) to the BAC column, vortex, and allow the column to drain by gravity.
9. Apply the dilute lysate (≈2.4 mL; will take two applications) to the column and drain by gravity. The PSI Pressure Tip may be used to initiate flow of lysate.
10. Wash BAC column with 2 x 1.0 mL Wash Buffer (5) and drain by gravity. Centrifuge at 750 x g for 2 min to dry the column.
11. Add 50-100 µL Elution Buffer (6) to the top of the resin and incubate 2 min at room temp. Centrifuge at 750 x g for 2 minutes to collect the eluent.
12. Precipitate the BAC DNA by adding 1 vol. of isopropyl alcohol and mixing. Centrifuge for 30 min at 20,000 x g to pellet the DNA. Remove the supernatant. Wash the pellet with 200µL 70% EtOH. Centrifuge at 20,000 x g for 5 min. Remove excess EtOH and air dry.
13. Resuspend in 20µL TE or other low salt buffer.

Protocol for 25-250mL culture media

using **PSI Ψ Clone Big BAC DNA** kit (Cat# PP-121)

This protocol is designed to isolate template quality BAC DNA from cultures of 30mL LB (containing 12.5 ug/mL chloramphenicol) with a culture density as measured by OD_{600} between 4.0 to 6.0. Typical yields are between 5 to 7 ug DNA per prep.

Use a scaling factor of 10 to adjust for variable culture volumes. For example: a 30mL overnight culture with an OD_{600} of 4.0 would be centrifuged and resuspended in 3mL Resuspension buffer (with 100 ug/mL RNAse A) then 3mL Lysis buffer, followed by 3mL neutralization buffer.

1. Remove 1mL Resuspension Buffer and add it to the RNAse A tube to dissolve the RNAse A, add this back to the Resuspension Buffer and mix.
2. Add 3mL Resuspension Buffer to the cell pellet and resuspend by gentle pipetting.
3. Add 3mL Lysis Buffer and mix by **gentle** inversion. Allow the lysis to continue for **20 minutes** at room temperature.
4. Add 3mL Neutralization Buffer and mix by **gentle** inversion until a thick white precipitate forms and incubate on ice for **20 minutes.**
5. Centrifuge the precipitate at 25,000 x g (using an SS-34 rotor or equivalent) for 30 minutes to clarify the lysate. Pour off the lysate into a clean tube; if lysate is not clear, invert to mix and centrifuge again.
6. Add 3mL BAC Binding Resin to the clarified lysate and invert several times. Incubate at room temp for 10 min (inverting every 2 minutes to mix). Then pour the solution into the column barrel provided and allow it to drain by gravity.
7. Add 3 x 5 mL Wash Buffer allowing to drain by gravity, then place the column in an empty 50 mL conical tube (Falcon Tube or equivalent) and remove excess wash buffer by centrifugation at 750 x g for 2 minutes in a bench top centrifuge. Discard the wash.
8. Add 1mL elution buffer to the column and incubate for 2 min, centrifuge as above (preferably in a new tube). Then repeat the elution once again.
9. Precipitate the DNA by adding 1 volume isopropanol and mixing. Centrifuge at 20,000 x g for 30 minutes. Remove supernatant. Wash the DNA by adding 2mL 70% ethanol and mixing. Centrifuge at 20,000 x g for 5 minutes. Remove the excess 70% ethanol and allow the pellet to air dry for about 5 minutes.
10. Resuspend in a suitable volume of TE or other low salt buffer of choice.

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