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BAC DNA Purification for Microinjection

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Thom Saunders¹

¹University of Michigan

Transgenic Animal Mod...



Zachary Freeman

University of Michigan

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We use this protocol and it's working

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Abstract

BAC DNA has been purified successfully for microinjection by three principal methods: Anion Exchange Chromatography (Nucleobond Columns), CsCl Gradient, or Sepharose 4B-CL Chromatography. Any of these methods can be used successfully to purify BAC DNA for pronuclear microinjection when the protocols are followed carefully.

Guidelines

This method was established in the University of Michigan Transgenic Core for our users. The method is used to purify BAC DNA for microinjection into fertilized mouse eggs and BAC transgenic mouse production. The following method yields clean, non-sheared DNA. Some of the information below was taken from The Nest Group website (The Nest Group, Inc. TEL 800-347-6378, FAX 508-485-5736).

Materials

The kits are no longer available from The Nest Group, but can be purchased from Clontech (Takara Bio).

- NucleoBond BAC 100 Kit, Clontech Cat. #740579. This gives the best compromise of volume management and size (capacity). This kit contains 10 AX-500 cartridges.
- NucleoBond Buffer Set I, Clontech Cat. #740601. Without the Buffer Set I you will have to make up more of the S1, S2, S3 buffers since there is not enough in the PC kit to double the volumes of extractants of a normal plasmid, which is necessary to do BACs.
- NucleoBond Folded Filters are included in the BAC 100 kit. The filter papers will eliminate the centrifugation step after precipitation of the cellular debris and will clarify the extraction in about 10 minutes instead of a 45-minute spin. This will reduce the exposure time to nucleases as well as reduce the shearing potential of a centrifugation. This is highly recommended.

Special Conditions for Purifying BACs

- 1 When isolating BACs with the Nucleobond AX alkaline lysis-based protocol, the potential always exists for lower than expected yields to be obtained. While, in general, there are a number of factors that could adversely affect plasmid yield, in the case of low copy number plasmids, incomplete bacterial lysis is the number one culprit. The primary reason for this is that in order to get the maximum yield for a particular cartridge size, many researchers grow cultures of these plasmids that are larger than can be efficiently handled by the prescribed volumes of buffers for the cartridge. The first and most obvious solution is to increase the amounts of the buffers to be used in these cases to reduce viscosity and to promote diffusion.
- 1.1 (A) Some Golden Rules for Lysis Buffer Volumes: Use the following minimum volumes of the cell-suspension/RNase buffer S1, lysis buffer S2, and high salt co-precipitation buffer S3: Use a minimum of 4.0 ml of each buffer per 100 ml of culture regardless of which cartridge size is being used. However, if the particular cartridge requires more than this, then use the prescribed amount for the cartridge. For example, if a 100 ml BAC culture is to be processed on an AX-500 cartridge then use the prescribed 12 ml of S1, S2, and S3 for the cartridge. On the other hand, if a 500 ml BAC culture is to be processed on an AX-500 cartridge then at least 20 ml ($\{500 \text{ ml} / 100 \text{ ml}\} * 4$) of each of the three buffers should be used according to this rule to ensure proper cell lysis.
- 1.2 (B) Use of an additional volume of N5 Elution Buffer: If the elution step is repeated one additional time (see Step 8, in A Modified Alkaline Lysis Procedure for the Purification of Plasmids and Cosmids), up to 30% more DNA can be isolated. This is especially true for the AX-100 and AX-500 cartridges. Note the flow rates of Nucleobond AX cartridges are up to two times faster than for Qiagen Tip cartridges which means that clogging from higher viscosity solutions is less likely (less sensitive to cell density problems), and the amount of losses from endonucleases will be lower due to shorter contact times.
- 1.3 (C) Use of a Denaturing Elution Buffer: It has been reported in some cases that the use of a high GC content type of elution buffer helps to increase the yields of very large plasmids such as BACs regardless of their GC content. The formula for this elution buffer is as follows: 50% formamide, 1.0 M KCl, 15% EtOH & 0.1 Tris-phosphate, pH 8.5. This buffer should be heated to 60C before loading. For the recommended procedure for preparing this buffer refer to N5 Elution Buffer Preparation (see below). However, use of this formamide buffer can lead to complications in the precipitation step unless care is taken to prevent salt precipitation (room temperature propanol precipitation and centrifugation is required), formamide removal (a second propanol precipitation is required) and propanol removal (an additional ethanol precipitation is required).
- 1.4 (D) Notes Contributed by Chris Russell, RESEARCH GENETICS, INC:
crussell@resgen.com: Some BACs will yield better and more DNA when grown at room temp to 30 C rather than at 37 C. This is not generally true, but some BACs are unable to



grow vigorously at 37 C. Many BACs will yield better in LB broth than in Terrific broth or other rich broths. It may be a growth problem or a technical problem due to the presence of a higher concentration of bacterial cells. Concentration measurements by UV spectrophotometry are not very reliable and may not be meaningful, since you can have absorbing contaminants as well as significant amounts of *E. coli* DNA. Same is true for fluorometry due to the presence of *E. coli* DNA. Same goes for checking the BAC on a regular agarose gel. By pulsed-field gel electrophoresis of Not I digested BAC DNA you can evaluate the integrity, quality, and quantity of BAC DNA.

Modified N5 Elution Buffer Preparation

- 2 This modified buffer is designed to increase yields of GC-rich DNA or other 'sticky' DNA from the Nucleobond column. The buffer contains 50% formamide, 1.0 M KCl, 15% EtOH, 100 mM Tris-phosphate, pH 8.5.
- 2.1 1. Add 12.11 g Tris base to 250 ml distilled water and dissolve.
- 2.2 2. Add 74.55 g KCl to Tris solution.
- 2.3 3. Autoclave this solution.
- 2.4 4. Add 150 ml of absolute ethanol to cooled solution.
- 2.5 5. Add 500 ml of formamide to solution.
- 2.6 6. Adjust pH to 8.5 with phosphoric acid – do not overshoot pH.
- 2.7 7. Adjust final volume with sterile distilled water to 1000 ml.
- 2.8 8. Store at room temperature.

Method

- 3 Using this modified method of Birnboim and Doly, bacterial cells are lysed by a NaOH/SDS solution. Chromosomal and plasmid DNA are partially denatured under these alkaline conditions. It is important to control the duration of this denaturing step thoroughly. The following addition of potassium acetate is a crucial step as well. It results in a precipitate containing the chromosomal DNA and other cellular compounds. Plasmid DNA stays in solution and is purified to homogeneity on the corresponding NUCLEOBOND AX cartridge. For buffer volumes and operation conditions see the below. Please read the remarks before you start your preparation. Use 100-500 ml of bacterial culture (A600 approximately 1 O.D.) in conjunction with one AX-500 cartridge.
- 3.1 Cell disruption: 1. Carefully resuspend the bacterial cell pellet in buffer S1: 12 ml (see Note A above).
- 3.2 2. Add the appropriate volume of buffer S2: 12 ml (see Note A above). Mix gently by inverting the tube, and incubate at room temperature for 5 min. Do not vortex to prevent the release of chromosomal DNA from the cell debris.
- 3.3 3. Add buffer S3: 12 ml (see Note A above). Mix gently by inverting 6-8 times until a homogeneous suspension is formed. Incubate the mixture on ice for 10 minutes to begin the precipitation of SDS and cellular debris. Put the folded filter onto a 50 ml tube or equilibrated Nucleobond AX cartridge, wet it with 0.5 – 1.0 ml of water and fill it with the cooled lysate. Collect the cleared flow-through. These filters are suitable for plasmid isolation with Nucleobond AX 100 and larger cartridges, where the volume of the lysate is sufficient to effect a complete hydration and wash of the filter paper. To get the maximal recovery of DNA, rinse the filter paper with an additional 1.5 ml of water.
- 4 Equilibration: Equilibrate an AX-500 cartridge with 5 ml buffer N2.
- 5 Adsorption: Load the collected cleared flow-through from the filter paper onto an equilibrated AX-500 cartridge.
- 6 Wash: Wash cartridge with 2×12 ml buffer N3.
- 7 Elution: Elute DNA with 6 ml buffer N5 (see notes B, C, and Modified N5 Elution Buffer Preparation above). If this elution step is repeated one additional time, up to 30% more DNA can be isolated. This is especially true for the AX-100 and AX-500 cartridges.
- 8 Precipitation: Precipitate DNA by adding 0.7 volumes of room temperature iso-propanol. Centrifuge at >12000 x g at 4°C for 10-20 minutes. Wash the pellet with 70% ethanol, air dry briefly (about 5 minutes), and dissolve in microinjection buffer (see below).

- 9 Resuspend DNA: Resuspend DNA in 100 ul microinjection buffer (see below) available from the Transgenic Core.
- 10 Quantitation: Check concentration by OD 260/280. We and others have observed that there is no difference in efficiency of producing transgenic mice with circular BAC DNA or linearized BAC DNA. You may submit either form of DNA for microinjection. When you do so, also submit 10 microliters of Not I digested BAC DNA for pulsed field analysis. This will allow us to assess the purity of your DNA preparation. We will verify the concentration of your concentrated DNA sample and adjust it for microinjection to 0.5 to 1.0 ng per ul.
- 11 DNA Storage: Store resuspended DNA at 4C.
- 12 Buffer Storage: S1 – 50 mM Tris-HCl, 10 mM EDTA, 100 µg RNase A / ml, pH 8.0, 4C. S2 – 200 mM NaOH, 1% SDS, RT. S3 – 2.8 M K-Acetate, pH 5.2, 4C. N2 – 100 mM Tris, 15% ethanol & 900 mM KCl adjusted with phosphoric acid to pH 6.3, RT. N3 – 100 mM Tris, 15% ethanol & 1150 mM KCl adjusted with phosphoric acid to pH 6.3, RT. N5 – 100 mM Tris, 15% ethanol & 1000 mM KCl adjusted with phosphoric acid to pH 8.5, RT. *This salt concentration is sufficient for the elution step, because of the increased pH value of this buffer. The RNase (already heat treated and DNase-free), must be added to buffer S1 before use. S1 should be stored then at 4C. The SDS in buffer S2 will precipitate at temperatures below 20C. If this is the case, store the bottle for a few minutes at about 30C to 40C and mix well and equilibrate to room temperature before use. The SDS is removed by buffer S3 (white precipitate) and will not be loaded on the cartridge. This step is very important! Take care that the supernatant of step 5 is clear! SDS will clog the cartridge and prevent the adsorption of nucleic acids.
- 13 Preparation of Microinjection Buffer: Very Important → Microinjection Buffer. In our hands, successful BAC preparations require the polyamine microinjection buffer described below. We have stored intact BAC DNA at 4C for a year in polyamine buffer. Buffer composition is 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 30 microM spermine, 70 microM spermidine, 100 mM NaCl. This buffer is more likely to produce transgenic mice with intact, unfragmented DNA molecules. See the following references: Schedl A, Larin Z, Montoliu L, Thies E, Kelsey G, Lehrach H, Schutz G. 1993. A method for the generation of YAC transgenic mice by pronuclear microinjection. *Nucleic Acids Res* 21:4783-4787 and Montoliu L, Bock CT, Schutz G, Zentgraf H. 1995. Visualization of large DNA molecules by electron microscopy with polyamines: application to the analysis of yeast endogenous and artificial chromosomes. *J Mol Biol* 246(4):486-492. See also Lluís Montoliu's website.
- 14 1000x Polyamine Stock: 30 mM Spermine (Sigma, tetrahydrochloride, #S-1141). 70 mM Spermidine (Sigma, trihydrochloride, #S-2501). Dissolve the spermine and spermidine together in autoclaved distilled water, filter sterilize (0.2 micron filters), and store at -20



C. Since the polyamines are very hygroscopic, it is suggested that small quantities (1 gram) should be ordered and then all of it should be prepared at once.

- 15 Microinjection Buffer: For 50 ml: 10 mM Tris-HCl, pH 7.5 0.5 ml of 1 M Tris-HCl, pH 7.5 (autoclaved). 0.1 mM EDTA, pH 8.0 10 microliters of 0.5 M EDTA, pH 8.0 (autoclaved). 100 mM NaCl 1 ml of 5 M NaCl (autoclaved). 1x Polyamines 50 microliters 1000x Polyamines mix. Autoclaved H₂O up to 50 ml. NOTE: Prepare fresh and discard unused microinjection buffer.