

Benchmarks

REFERENCES

1. **Josefsen, K., J.P. Stenvang, H. Kindmark, P.O. Berggren, T. Horn, T. Kjaer and K. Buschard.** 1996. Fluorescence-activated cell sorted rat islet cells and studies of the insulin secretory process. *J. Endocrinol.* 149:145-154.
2. **Kannourakis, G., G.R. Johnson and F. Batty.** 1988. Clonal proliferation in vitro of individual murine and human hemopoietic cells after fluorescence-activated cell sorting. *Exp. Hematol.* 16:367-370.
3. **Kitamura, T., M. Onishi, S. Kinoshita, A. Shibuya, A. Miyajima and G.P. Nolan.** 1995. Efficient screening of retroviral cDNA expression libraries. *Proc. Natl. Acad. Sci. USA* 92:9146-9150.
4. **Laiho, M., L. Ronnstrand, J. Heino, J.A. Decaprio, J.W. Ludlow, D.M. Livingston and J. Massagué.** 1991. Control of junB and extracellular matrix protein expression by transforming growth factor-beta 1 is independent of simian virus 40 T antigen-sensitive growth-sensitive growth-inhibitory events. *Mol. Cell. Biol.* 11:972-978.
5. **Ruoslahti, E. and J.C. Reed.** 1994. Anchorage dependence, integrins, and apoptosis. *Cell* 77:477-478.

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Carlos Rodriguez and Harvey F. Lodish¹

*Whitehead Institute for Biomedical Research
¹Massachusetts Institute of Technology
Cambridge, MA, USA*

Isolation of Stable Bacterial Artificial Chromosome DNA Using a Modified Alkaline Lysis Method

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The bacterial artificial chromosome (BAC) system has been developed for

Table 1. Modified Alkaline Lysis Protocol for BAC DNA Preparations

1. Inoculate an isolated colony into 3 mL of culture medium (TB or LB) containing 12.5 µg/mL chloramphenicol. The bacteria were grown overnight in a 37°C shaker incubator (250 rpm).
2. The cells (1.5 mL) were pelleted by centrifugation at full speed in a microcentrifuge for 1 min and then resuspended in 100 µL of chilled solution I (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA, pH 8.0, 2.5 mg/mL lysozyme and 100 µg/mL RNase A). Pipetting up and down is necessary to solubilize the cells. Tubes were placed on ice.
3. Add 200 µL of freshly prepared solution II (0.2 N NaOH and 1% SLS) and mix by inversion until the lysis is visible. The mixture will become clear and viscous, then return to ice.
4. Add 150 µL of solution III (5 M potassium acetate, pH 4.8), mix by inversion until a white precipitate appears, then incubate on ice for 5 min.
5. Centrifuge for 5 min at full speed in a Micromax[®] Model 851 microcentrifuge (IEC, Needham Heights, MA, USA), then transfer the supernatant to a new microtube.
6. The crude DNA is precipitated by adding 2 vol (1 mL) 95% EtOH at room temperature, mixed by inversion and kept on ice for 30 min.
7. The DNA solution is centrifuged at full speed for 5 min in a microcentrifuge.
8. The DNA pellet is washed with 500 µL 70% EtOH at room temperature, then centrifuged at full speed in a microcentrifuge for 5 min. The residual EtOH is removed by pipetting and air drying.
9. To further purify and remove contaminants, the DNA was resuspended in 100 µL of TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH7.5)/0.1% SLS containing 100 µg/mL proteinase K, followed by a 1-h incubation at 37°C. The aqueous DNA solution is extracted once with an equal volume of a phenol/chloroform mixture and once with an equal volume of chloroform.
10. The DNA is precipitated with 2 vol of 95% EtOH at room temperature, mixed by inversion and pelleted by centrifugation at full speed in a microcentrifuge for 5 min.
11. The supernatant is discarded and the pellet rinsed with 70% EtOH. After centrifugation, the EtOH is removed, and the DNA pellet is air-dried.
12. The BAC DNA pellet is resuspended in 50 µL 1× TE. The DNA concentration was estimated by fluorimetry using a Model TKO 100 DNA Mini-Fluorimeter (Hofer Pharmacia Biotech, San Francisco, CA, USA). The DNA preparation is stable for several weeks when stored at 4°C.

the cloning of large DNA inserts (3). The BAC vector can accommodate DNA inserts averaging 130 kb (range 90–300 kb). The resulting clones are rarely chimeric (2,3), which makes them an excellent source of genomic clones for the generation of contiguous physical maps. Because BACs are supercoiled plasmids, they can be isolated manually using a standard alkaline lysis protocol (1). However, efficient isolation of large plasmids using this method is difficult (4). Furthermore, when BAC DNA was isolated this way, we encountered a degradation problem, making it difficult to obtain high-mole-

cular-weight molecules after restriction digestion, even when stored for only a few days. This report describes a modification of the alkaline lysis method that allows the efficient purification of high-quality stable BAC DNA.

The alkaline lysis procedure typically relies on a differential precipitation step in which high-molecular-weight chromosomal DNA is precipitated, and the relatively small plasmids remain in the supernatant (1). However, there is an apparent loss of large plasmids (>100 kb) when potassium salt is used (4). It was then suggested that faster precipitation of lauryl sulfate with

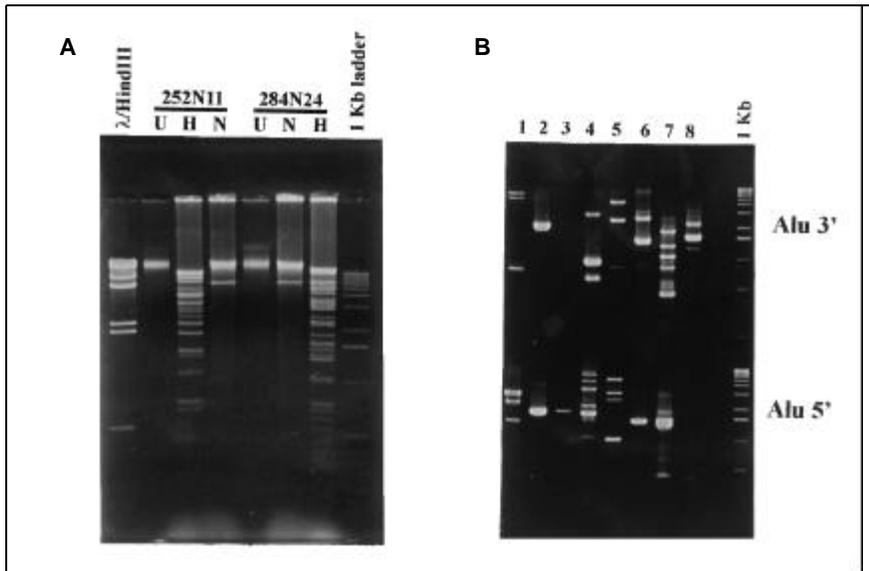


Figure 1. Characterization of BAC DNA preparations isolated by the modified alkaline lysis method. (A) Endonuclease digestion of clones 252N11 and 284N24; U: uncut; H: *Hind*III digestion; N: *Not*I digestion. (B) PCR analysis of 8 distinct BAC clones using Alu-specific primers Alu3' and Alu5'.

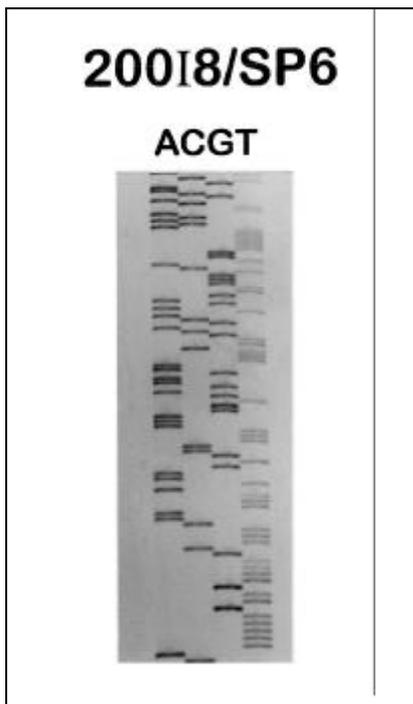


Figure 2. Direct sequencing of BAC insert ends. Sequencing reactions were done as described by the manufacturer except for the following modifications: each reaction containing ca. 500 ng of BAC DNA (clone 200I8) and 2.5 μ M of the appropriate sequencing primer (T7: 5'-TAAT-ACGACTCACTATAGGGCGA-3'; SP6: 5'-GATTACGCCAAGCTATTTAGGTGACACTATAG-AATA-3') was performed in a PCR DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) for 40–50 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 90 s. Sequence ladders generated from the SP6 end are indicated by A, C, G and T.

potassium acetate leads to the trapping of large denatured plasmids that cannot renature as fast as the small ones. On the other hand, the main advantage of potassium over sodium acetate buffer in the isolation of plasmids is that it yields plasmids with a lesser amount of RNA and chromosomal DNA contamination (4). Furthermore, the BAC DNA isolated by the standard procedure was very unstable, even for a short period of time (data not shown).

These observations were considered when we modified the original alkaline lysis protocol to isolate stable BAC DNA (Table 1). In the adapted protocol, we used sodium *N*-lauroylsarcosine (SLS) instead of sodium lauryl sulfate (sodium dodecyl sulfate; SDS) as detergent because it was reported that with SLS, no loss of large plasmids occurred in the presence of potassium salt (4). Every step was done at 4°C to minimize the nuclease-induced nicking of plasmid DNA during the isolation. Finally, a proteinase K treatment and extraction steps were both added to the procedure to reduce the degradation of the BAC DNA.

The BAC DNA from eight distinct clones (Research Genetics, Huntsville, AL, USA) was isolated from a 3.0-mL culture. The DNA isolation yields ranged from 80 ng (in LB broth) to 125 ng (in TB broth) per 1 mL culture. The

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BAC DNA was then further characterized to verify the purity and the quality of the preparation. First, little or no bacterial genomic DNA was visible on an ethidium bromide-stained agarose gel (Figure 1A). Second, the DNA was easily cut by *Hind*III and *Not*I restriction enzymes (New England Biolabs, Beverly, MA, USA) (Figure 1A). Third, the BAC clones were efficient templates for PCR amplification using Alu primers (Figure 1B). And fourth, the BAC insert ends were directly sequenced (see below). These results illustrate the high quality of the BAC DNA preparations. Furthermore, identical restriction patterns were generated every two weeks over a period of 6 months, indicating the great stability of the BAC DNA preparations (data not shown). BAC end sequences can be used to provide a source of physical markers in genome mapping and walking. Because of the presence of the T7 and SP6 promoters in the BAC vector, we have been able to directly sequence both ends of BAC inserts using the T7- and SP6-specific primers (Figure 2). The double-stranded DNA sequencing was performed by cycle sequencing (Thermo Sequenase™; Amersham Pharmacia Biotech, Oakville, ON, Canada) using an adapted protocol (see Figure 2 legend).

The procedure described here is simple and offers a productive method for isolation of high-quality and stable BAC DNA suitable for numerous applications including restriction analysis, sequencing, PCR, hybridization or fluorescence in situ hybridization.

REFERENCES

1. Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
2. Kim, U.-J., B.W. Birren, T. Slepak, V. Mancino, C. Boysen, H.-L. Kang, M.I. Simon and H. Shizuya. 1996. Construction and characterization of a human bacterial artificial chromosome library. *Genomics* 34:213-218.
3. Shizuya, H., B. Birren, U.-J. Kim, V. Mancino, T. Slepak, Y. Tachiiri and M.I. Simon. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. USA* 89:8794-8797.
4. Thomas, N.R., S. Koshy, M. Simsek and A.K. Abraham. 1988. A precaution when preparing very large plasmids by alkaline lysis procedure. *Biotechnol. Appl. Biochem.* 10:402-407.

D.S. is a scholar of the Fonds de la Recherche en Santé du Québec. This work was supported by the Medical Research Council of Canada. Address correspondence to Dr. Daniel Sinnett, Service d'Hématologie-Oncologie, Centre de Recherche, Hôpital Ste.-Justine, 3175 Côte Ste.-Catherine, Montréal, QC, H3T 1C5, Canada. Internet: sinnettd@ere.umontreal.ca.

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Daniel Sinnett^{1,2}, Chantal Richer¹ and Annie Baccichet¹

¹Hôpital Ste.-Justine

²Université de Montréal
Montréal, QC, Canada

Rapid Zymogram for Lipase

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Microbial lipases have immense use in the detergent, food, agricultural and pharmaceutical industries (5). Recently, the applications of lipases in organic synthesis have increased tremendously because of low costs, stability in organic media, wide versatility, easy use and non-requirement of added cofactors (2).

The most important and fascinating aspect of lipases is perhaps the unique physico-chemical character of the reactions they catalyze. These enzymes are perfectly water-soluble and yet act very efficiently on water-immiscible lipidic substrates that spontaneously self-organize in water as monomolecular films, bilayers, liposomes, emulsions or micelles. This catalysis occurs essentially at the lipid-water interface (8).

To study the conformation and mechanism of lipases, homogeneous preparations are required. Purification of lipases is carried out by most laboratories using conventional techniques, and the electrophoretic separation of protein in polyacrylamide gels is a common analytical technique for protein

identification (4). Detection of lipase in the native state requires an enzyme zymogram. Although native staining of lipases has been referred to by some workers, the exact protocol using Victoria blue has not been mentioned in literature. The heterogeneous state of the substrate for lipase action (triglyceride) causes difficulties during zymogram preparation. To overcome this difficulty, we have developed a rapid method for the detection of native bands by polyacrylamide gel electrophoresis (PAGE). The method involves an overlay staining technique using Victoria blue as a staining dye. The principle of the method is the hydrolysis of triglyceride (which is present in the overlay) into fatty acids that ultimately combine with the functional group of Victoria blue and result in the development of a blue zone in the overlay at places where active lipase is present.

The dye Victoria blue has been used over a long period of time for the sensitive detection of lipase in plate assay procedures (1). Activity staining of lipase on polyacrylamide gels was carried out by Gilbert et al. (3) using an agarose overlay attached to 1850-101 GelBond® Film (Amersham Pharmacia Biotech, Uppsala, Sweden) for 24 h. However, our method is more rapid (4-12 h), less cumbersome, less expensive (GelBond Film is not required) and more sensitive in terms of the quantity

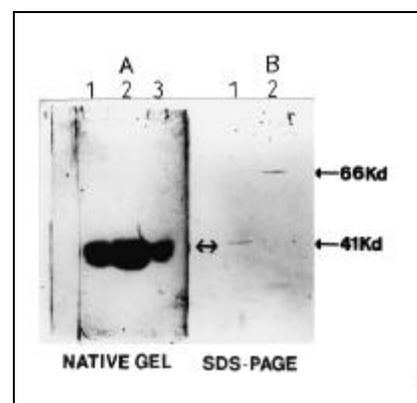


Figure 1. (A) Polyacrylamide gel (12.5%) showing active lipase from *A. terreus* stained with Victoria blue. Lane 1: 0.312 U/10 µL of purified lipase; lane 2: 0.625 U/10 µL of purified lipase; lane 3: 0.05 U/10 µL of crude culture filtrate. (B) SDS polyacrylamide gel (12.5%) showing pure lipase from *A. terreus* (41 kDa) in lane 1, along with BSA as marker (66 kDa) in lane 2. Bands are stained with Coomassie Brilliant Blue R-250.