HAVEY'S LAB BAC MINIPREP ISOLATION

-Marra MA, Kucaba TA, Dietrich NL, Green ED, Brownstein B, Wilson RK, McDonald KM, Hillier LW, McPherson JD, Waterston RH. High throughput fingerprint analysis of large-insert clones. Genome Res. 1997, Nov;7(11):1072-84.
-http://www.cco.caltech.edu/~schoi/BACVEC.html (D. BAC DNA Miniprep Protocol)
-observations

Solutions:

1) RESUSPENDING SOLUTION: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0 Store at 4 C
2) STOCK LYSOZYME SOLUTION: 50 mg/ml in dH_2O, aliqoted to 1 ml, store at -20°C
3) NaOH-SDS STOCK: 0.2 N NaOH, 1% SDS. Prepare fresh by mixing equal volumes of 0.4 N NaOH and 2% SDS.
4) 3M POTASSIUM ACETATE STOCK: 3M Kac, adjust the pH with acetic acid, pH 5.5

Procedure:

1) Grow overnight 5 ml culture in LB medium at 37°C containing 12.5 ug/ml of chloramphenicol
2) Centrifuge the overnight culture at full speed for 30 sec in Eppendorf microfuge several times to harvest all 5 mls of culture, at the end carefully remove all LB medium with pipet
3) Resuspend the cell pellet in 0.2 ml of resuspending solution, add 20 ul of lysozyme stock, swirl and incubate for 5 min at room temperature and 5 min on ice.
4) Add 0.4 ml of NaOH-SDS stock and invert gently. The solution should turn translucent. Incubate for 5 minutes on ice.
5) Add 0.3 ml of the potassium acetate stock and invert gently. A white precipitate should form. Freeze at -80°C for 10 - 15 min. Let thaw at room temperature.
6) Centrifuge for 10-15 minutes in a microfuge at full speed to collect the precipitate.
7) Carefully remove as much the supernatant fluid as possible (about 0.85 ml) without disturbing the pellet and transfer to a clean microfuge tube.
8) Recentrifuge for 10 minutes and remove the supernatant as above (very important step, don't omit it).
9) Add 0.6 volumes of ice cold isopropanol (usually 0.54 ml) and gently mix. Freeze at -80°C for 10-15 minutes. Warm to room temperature and centrifuge for 15 minutes in a microfuge to pellet the DNA.
10) Remove the supernatant fluid and rinse the pellet with 1 ml of ice cold 70% ethanol. Centrifuge for 2 minutes and pour off the ethanol rinse and dry, you can speed the drying by pipetting out the remaining ethanol.

11) Add 30 ul of TdE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and resuspend the pellet. Sometimes some dirt can still be visible in the tube.

NOTE:

1) For BAC end sequencing it is very important to use freshly isolated BAC DNA, the best same day, acceptable next day. Don't wait a week; usually you will get poor quality data.

2) For BAC fingerprinting you can use older BAC preps.

3) Epicentre BAC isolation kit didn't work well for me (BACMAX™ DNA Purification Kit)

4) Large quantities can be obtained by Qiagen columns; it is very long procedure, suitable for few samples.

http://www.cco.caltech.edu/~schoi/BACVEC.html

BAC Cloning Vector: pBeloBAC11

A. BAC Vector Preparation (Non-CsCL)

B. BAC Vector Preparation (CsCl)

C. Transformation

D. BAC DNA Miniprep Protocol

E. Colony Filters for Library Screening

Back to Table of Contents

pBeloBAC11 was developed by Drs. M. Simon and H. Shizuya (unpublished; Shizuya et al, 1992). pBeloBAC11 represents the second generation BAC cloning vectors, which was developed from the pBAC108L (Shizuya et al 1992) by introducing the LacZ gene to facilitate recombinant identification with blue and colorless (white) phenotypes. pBeloBAC11 is a mini-F factor based plasmid. There are three unique cloning sites: Bam HI, SphI, and Hind III, which are flanked by the T7 and SP6 promoters. These promoters can facilitate generating RNA probes for chromosome walking and DNA sequencing of the insert fragment at the vector - insert junction. The G + C rich restriction sites (Not I, Eag I, Xma I, Sma I, Bgl I, and Sfi I) can be used to excise the inserts of BAC clones. There are two selective markers for cloning purposes: LacZ gene for recombinant selection and CMR (chloramphenicol) for transformant selection. The F factor codes for genes that are essential to regulate its own replication and controls its copy number in a cell. The regulatory genes include oriS, repE, parA, and parB. The oriS and repE mediate the unidirectional replication of the F factor, and the parA and parB maintain copy number at a level of one or two per cell.

A. BAC Vector Preparation (Non-CsCL)

pBeloBAC11 is a plasmid; therefore, the procedure for pBeloBAC11DNA preparation is essentially the same as that for the conventional plasmid DNA preparation. The most important parameter is that a large volume of culture is needed to obtain enough DNA for your research because this plasmid is only present at one to two copies per cell.

1. Reagents

- TB Broth (1 liter) 12 g tryptone 24 g yeast extract 8 ml 50% glycerol Bring the volume to 900 ml with dd H2O. After autoclaving, add 100 ml of sterilized 1 M potassium phosphate. 1 M potassium phosphate (100 ml) 2.3 g KH2PO4 12.5 g K2HPO4. Bring the volume to 100 ml with dd H2O and then filter-sterilize the solution.

- Lysozyme solution (500 ml) 4.5 g glucose 10 ml 0.5 M EDTA, pH 8.0 112.5 ml 1 M Tris.HCl, pH 8.0. Bring the volume to 500 ml with dd H2O and store at 4 C. Before use, add lysozyme to 5 mg/ml.
- NaOH-SDS (sodium dodecyl sulfate) solution (200 ml) 10 ml 4 N NaOH 10 ml 20% SDS 180 ml dd H2O. Make fresh and keep at room temperature before use.
- KOAc solution (500 ml) 147 g potassium acetate 142.5 ml glacial acetic acid. Bring the volume to 500 ml with dd H2O. pH should be between 4.8 and 5.3.

2. Procedure (per liter)

a. Streak the stock cells of pBeloBAC11 in E. coli strain DH10B on an LB plate containing 12.5 ug/ml chloramphenicol and grow overnight at 37C.

b. Inoculate a single colony in 5 ml TB medium plus 12.5 ug/ml chloramphenicol and grow at 37 C for 6 - 8 hours with shaking at 250 rpm.

c. Inoculate 1 ml of the culture into 1 liter of TB 12.5 ug/ml chloramphenicol and grow at 37 C overnight with shaking at 250 rpm.

d. Harvest the cells in five 250 ml centrifuge bottles by centrifugation at 8,000 rpm (~ 10,000 g) for 10 minutes and pour off the supernatant fluid.

e. Resuspend the cell pellet in each bottle in 20 ml of lysozyme solution by repeatedly pipetting up and down and incubate on ice for 5 minutes.

f. Add 40 ml of NaOH-SDS solution to each bottle, mix gently, and incubate on ice for 5 minutes.

g. Add 30 ml of ice cold KOAc solution and mix gently. A white precipitate should form. Freeze the mixture in a - 80 C freezer until mixture is completely frozen (~ 30 minutes) and then let thaw at room temperature.

h. Centrifuge at 10,000 rpm (~ 15,000g) for 15 minutes to collect the precipitate and filter the supernatant into a clean 250 ml centrifuge bottle through 4 layers of cheese cloth.

i. Add 0.6 volume of isopropanol to the supernatant fluid, mix well, and freeze the contents as in step 7 and then warm to room temperature.

j. Centrifuge at room temperature at 10,000 rpm for 30 minutes to pellet the DNA, remove the supernatant, and rinse the pellet with 50 ml 70% ethanol.

k. Centrifuge at 10,000 rpm for 15 minutes, remove the 70% ethanol, dry, and dissolve the DNA in 1 ml TE (10 mM Tris.HCl, pH 8.0, 1 mM EDTA, pH 8.0).

l. Combine all the DNA isolated from 1 liter of culture into one clean 15 ml centrifuge tube.

m. Add DNase free RNase at a rate of 20 mg/ml and incubate at 37 C for 45 minutes to remove the RNA in the DNA.

n. Extract the DNA with an equal volume of equilibrated phenol once and 25 phenol : 24 chloroform : l isoamyl alcohol once.

o. Precipitate the DNA by adding 1/10 volume of 3M NaOAc and 2 volumes of ethanol, dry, and dissolve in 200 m1 TE (the concentration of DNA should be 0.5 - 1 mg/ml).

3. BAC Vector Linearization and Dephosphorylation

a. Set up the digestion as follows:

- H2O 59 ml
- DNA (20 mg) 20 ml
- 10 x reaction buffer 10 ml
- 40 mM spermidine 5 ml
- 10 units / ml restriction enzyme 6 ml

b. Incubate the reaction at 37 C for 2 hours.

c. Add an additional 4 ml restriction enzyme and incubate for additional 2 hours.

d. To ensure that the digestion is indeed complete, test the digestion on an 1% agarose gel and/or by transformation into E. coli using the uncut plasmid DNA as a control (circular plasmid DNA molecules should be < 20%)

. e. Extract the digest once with phenol: chloroform (1:1), precipitate, dry, and dissolve in H2O.

f. Set up the following reaction to dephosphorylate the ends of linearized vector DNA:

- H2O 33 ml
- Digested DNA (~ 20 mg) 50 ml
- 10 x CIAP buffer 10 ml
- CIAP 7 ml (0.1 units/ml)

g. Incubate at 37 C for 30 minutes and stop the reaction by adding 5 mM EDTA, pH 8.0, 0.5% SDS , and 0.1 mg/ml proteinase K and incubating at 56 C for 30 minutes.

h. Extract twice with phenol, once with phenol : chloroform : isoamy alcohol (25:24:1), precipitate with ethanol, dry, dissolve in H2O at 10 ng/ml and store in 10 ml aliquots at - 80 C.

Note: A preliminary experiment is often performed to optimize dephosphorylation conditions. A large scale of digested vector DNA is dephosphorylated under the optimal conditions.

Back to top of page

B. BAC Vector Preparation (CsCl)

1. Reagents and equipment

- HS995 cell: E. coli strain DH10B [F- mcr A Δ (mrr-hsdRMS-mcrBC) F80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 (ara,leu)7697 galU galK l- rpsL nupG] containing pBeloBAC11 (7.4 kb)
- LB broth
- Chloramphenicol stock (CM): 50 mg/ml
- LB/CM plate (30 ug/ml CM)
- Beckman (USA) SW50.1 swinging bucket rotor
- Open-top ultra-clear tube (Beckman # 344057)
- QIAGEN plasmid Maxi Kit (QIAGEN Cat# 12162, USA)
- 100 mg/ml BSA
- HK phosphatase (Epicenter, USA)

2. Procedure (4 liter)

a. Streak HS995 cells on an LB plate containing CM (30 ug/ml), X-GAL (240 ml of 20 mg/ml) and IPTG (24 ml of 200 mg/ml) and grow at 37 C overnight.

b. Inoculate a single blue colony in 4 L of LB media, prewarmed at 30 C, containing 30 ug/ml CM.

c. Grow the inoculum 20 hours at 30 C to an OD600nm between 1.0 and 1.5, and then harvest the cells in eight 500 ml centrifuge bottles by centrifugation at 6000xg for 15 minutes.

d. Remove all the traces of supernatant by inverting the centrifuge bottles for a few minutes. Isolate plasmid DNA from the cell pellet by alkaline lysis using the QIAGEN plasmid Maxi Kit according to manufacturer's specifications with modifications (Five QIAGEN tip 500s can be used for a 4 L preparation).

e. Resuspend the bacterial pellet in each bottle completely with 10 ml buffer P1.

f. Transfer the bacterial solution to eight Oakridge tubes.

h. Add 10 ml buffer P2, mix gently by inverting several times and incubate at room temperature for 5 minutes.

i. Add 10 ml prechilled buffer P3 on ice, mix immediately but gently, and incubate on ice for 20 minutes.

j. Centrifuge at 30,000xg at 4 C for 30 minutes, remove the supernatant to new Oakridge tubes and repeat this step.

k. Equilibrate five tip 500s with 10 ml buffer QBT.

l. Apply approximately 50 ml of the supernatant from step 9 to each 500 tip, and allow it to enter the resins by gravity flow.

m. Wash the tips twice each time with 30 ml buffer QC.

n. Elute DNA with 15 ml buffer QF.

o. Precipitate DNA with 0.7 volume of ice-cold isopropanol and keep at -20 C overnight.

p. Centrifuge at 30,000xg, remove the supernatant, and air-dry the pellets.

q. Resuspend the pellets in TE, and combine the DNA solution to one 15 ml tube in a final volume of 5ml. Precipitate the DNA again with 0.1 volume of 3M NaAc, pH 5.2 and two volumes of ethanol.

r. Wash the pellet carefully with ice-cold 70% ethanol, air-dry, and resuspend in 1 ml of TE.

s. Purify the plasmid DNA further by cesium chloride density gradient centrifugation in the presence of ethidium bromide.

i. Prepare a $CsCl_2$ gradient with a density of 1.59 g/ml with EtBr at 0.8 mg/ml.

- ii. Centrifuge in a Beckman (USA) SW50.1 swinging bucket rotor at 35,000 rpm for 72 hours.
- iii. After centrifugation, remove the plasmid band (lower band) from the density gradient under long wave UV light.
- iv. Extract the ethidium bromide by several extractions with ddH_2O-saturated isoamyl alcohol.

v. Dilute the DNA sample five fold with TE, and then precipitate with ethanol and centrifuge. Wash the pellet with ice-cold 70% ethanol, air-dry and resuspend in 500 ml of TE. The final yield should be approximately 50-70 mg of pBeloBAC 11 DNA from 4 L of media.

3. Vector DNA linearization and dephosphorylation

a. Set up the digestion as follows:

- pBeloBAC11 DNA (10 mg) 100 ml
- 10x reaction buffer 15 ml
- 40 mM SPD 15 ml
- Restriction enzyme (10 units/ml) 10 ml
- ddH20 10 ml

b. Incubate the reaction at 37 C for 5 h.

c. Verify the complete digestion on an agarose gel with the uncut DNA.

d. Extract the DNA with phenol followed by 2 chloroform extractions.

e. Precipitate the DNA with ethanol as above and resuspend in 100 ml of TE. Save 1 ml for a control of self-ligation test.

f. Set up the dephosphorylation reaction as follows:

- pBeloBAC11-cut DNA (10 mg) 100 ml
- 10x TA buffer 15 ml
- 50 mM $CaCl_2$ 15 ml
- HK phosphatase (1 units/ml) 10 ml

- ddH20 10 ml
- Incubate 1-2 hours at 30 C.

g. Inactivate the HK phosphatase by heating at 67 C for 30 minutes followed by the organic extractions and an ethanol precipitation as above and resuspend in TE in a final concentration of 0.1 mg/ml.

h. Assay the extent of dephosphorylation by performing a self ligation test as follows:

- Linearized vector DNA (0.1 mg) 1 ml
- 10x T4 ligase buffer 1 ml
- T4 ligase (1 unit/ml) 0.5 ml
- ddH20 7.5 ml
- Vector DNA-cut/dephosphorylated (0.1 mg) 1 ml
- 10x T4 ligase buffer 1 ml
- T4 ligase (1 unit/ml) 0.5 ml
- ddH20 7.5 ml

i. Incubate the ligation reactions overnight at 16 C, and verify the complete dephosphorylation on an agarose gel with the uncut DNA. Store one mg aliquots of the dephosphorylated pBeloBAC 11 plasmid at -80 C until needed.

Back to top of page

C. Transformation

1. Reagents

- DH10B competent cells [ElectroMAX DH10B cells (BRL, USA)] - The most widely used E. coli strain for BAC cloning is DH10B (Hanahan et al, 1991). Key features of this strain include mutations that block: 1) restriction of foreign DNA by endogenous restriction endonucleases (hsdRMS); 2) restriction of DNA containing methylated DNA (5' methyl cytosine or methyl adenine residues, and 5' hydroxymethyl cytosine) (mcrA, mcrB, mcrC, and mrr); 3) recombination (recA1).
- S.O.C. medium: 2% Bacto tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose, pH 7.0
- LB plates (150x15 mm): 12.5 ug/ml chloramphenicol, 240 ml of 20 mg/mlX-GAL, 24 ml of 200 mg/ml IPTG.
- Millipore filter unit (30,000 NMWL) (Cat # UFC3TTK00)

2. Procedure

a. After ligation, transfer the ligation mixture with a cut off pipet tip to a Millipore filter unit which is placed in a 1.5 ml microfuge tube containing approximately 1 ml of TE at 4 C to dialyze out the ligation buffer.

b. Change the dialysis solution with 0.1x TE two additional times over a 24 hour period.

c. Transform E. coli ElectroMAX DH10B cells (BRL, USA) by electroporation using the BRL Cell-Porator system with the manufacturer's transformation procedure.

d. Place micro-electroporation chambers and 0.5 ml microcentrifuges tubes on ice. Fill Cell-Porator Chamber-SafeTM with wet ice.

e. Place 1 ml S.O.C. medium in 15 ml sterilized culture tubes.

f. Add 1 ml of dialyzed ligation material to a 0.5 ml microfuge tube on ice.

g. Take out competent cells from -80 C and thaw on ice. Add 20 ml cells to each microcentrifuge tube, and gently mix them by tapping.

h. Pipet the mixture of DNA and cells into micro-electroporation chambers.

i. Electroporate with the BRL cell-Porator and Voltage Booster at the following settings:

- Voltage: 400
- Capacitance 330 uF
- Impedance Low ohms
- Charge rate: fast
- Voltage Booster resistance: 4,000 ohms

j. Remove the transformed cells from the micro-electroporation chamber and resuspend in 1 ml of SOC medium and incubate at 37 C with shaking at 225 rpm for one hour to allow expression of the CM resistant gene.

k. Plate 250 ml of the cells per LB plate (150x15 mm) containing CM, X-GAL, and IPTG.

l. Incubate for 18 to 24 h at 37 C.

Back to top of page

D. BAC DNA Miniprep Protocol

(from Silhavy - Experiments with gene fusions. Modified by S. -S. Woo and R. A. Wing)

1. Reagents

Lysozyme solution: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0 Store at 4 C. Immediately prior to use, add lysozyme to 5 mg/ml.

NaOH-SDS stock: 0.2 N NaOH, 1% SDS. Prepare fresh by mixing equal volumes of 0.4 N NaOH and 2% SDS.

Potassium acetate stock: 60 ml 5 M potassium acetate, 28.5 ml glacial acetic acid, 11.5 ml ddH2O, pH 4.8

2. Procedure

Every step including pipetting should be gentle and slow to avoid shearing the big DNA inserts.

a. Prepare a 5 ml culture of LB medium containing 12.5 ug/ml chloramphenicol.

b. Inoculate a single colony and incubate with shaking at 37 C for 18 - 20 h.

c. Centrifuge the overnight culture at 3000 rpm (approximately 1500g) at 4 C for 15 minutes using a table top centrifuge (Beckman).

d. Pour off the supernatant fluid and resuspend the cell pellet in 0.2 ml of lysozyme solution. Transfer to a 1.5 ml microfuge tube and incubate for 5 minutes at room temperature and five minutes on ice.

e. Add 0.4 ml of NaOH-SDS stock and invert gently. The solution should turn translucent. Incubate for 5 minutes on ice.

f. Add 0.3 ml of the potassium acetate stock and invert gently. A white precipitate should form. Freeze at -80 C for 10 - 15 min. Let thaw at room temperature.

g. Centrifuge for 15 minutes in a microfuge (approximately 12,000g) to collect the precipitate.

h. Carefully remove as much the supernatant fluid as possible (about 0.85 ml) without disturbing the pellet and transfer to a clean microfuge tube.

i. Recentrifuge for 5 minutes if necessary and remove the supernatant as above.

j. Add 0.6 volumes of ice cold isopropanol (usually 0.54 ml) and gently mix. Freeze at -80 C for 10-15 minutes. Warm to room temperature and centrifuge for 20 minutes in a microfuge 4 C to pellet the DNA.

k. Remove the supernatant fluid and rinse the pellet with 1 ml of ice cold 70% ethanol. Centrifuge for 2 minutes and pour off the ethanol rinse and dry the tubes upside down. Air-dry for 10-15 minutes.

l. Add 40-50 ml of TE and resuspend the pellet at 65 C for 5 minutes and centrifuge briefly.

m. Digest 10 ml of DNA with NotI to free the insert from the BAC vector.

On ice, prepare a cocktail with 20 x 9.5 ml H2O, 20 x 2.5 ml 10 x reaction buffer, 20 x 2.5 ml 40 mM spermidine, and 20 x 0.5 ml NotI. Aliquot 15 ml of the cocktail to each tube containing the 10 ml of BAC DNA. (A single restriction digestion has the following composition.) BAC DNA 10 ml 10X NotI reaction buffer 2.5 ml SPD (40mM) 2.5 ml ddH2O 9.5 ml Not I 0.5 ml Total 25 ml

[Back to top of page]

E. Colony Filters for Library Screening

1. Place nylon filters onto LB plate containing 12.5 ug/ml chloramphenicol.

2. Inoculate each filter with 96 BAC clones using a 96 well replica plating device. Repeat this step 3 additional times for a single filter if processing by hand or 16 to 36 times if using a robot (BIOMEK 2000, Beckman USA).

3. After inoculating all of the filters, incubate the plates for approximately 18 to 24 hours at 37 C.

4. One hour before processing the colonies prepare the following trays.

- 10% SDS-(1 tray)
- 0.5 N NaOH, 1.5 M NaCl-(1 tray)
- 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl, 0.001 M EDTA-(2 trays)
- 2 X SSC, 0.1 % SDS-(1 tray)
- 2 X SSC- (1 tray)
- 0.4 N NaOH-(1 tray)
- 5 X SSC, 0.1 % SDS-(1 tub)
- 2 X SSC-(1 tub)

Place 6 developing trays side by side, label in the order as above. Cut and lay Whatman filter paper in the bottom of each tray (1 sheet per tray). Pour approximately 50 ml of each solution into the appropriate tray and roll out the air bubbles with a Pasteur pipet. Pour the excess solution back into its original container for use later.

5. Carefully remove 3 filters with a flat edged forceps and place them onto the 10% SDS tray. Incubate the filters for 4 minutes.

6. After 4 minutes move the filters to the 2nd tray containing the NaOH/NaCl solution and incubate for 5 minutes.

7. Transfer the filters to the neutralizing solution (0.5 m NaOH, 1.5 m NaCl) and incubate for 5 minutes.

8. Wash the filter for 5 minutes with 2 X SSC and 1% SDS. Then, wash for 5 minutes with 2 X SSC without SDS.

9. Fix the DNA to the filter with a 20 minute incubation with 0.4 N NaOH.

10. Thoroughly wash the filters two times for 20 minutes each with a large volume of 5 X SSC + 0.1 % SDS with shaking.

11. Once the cell debris is removed, wash the filters with two 5 minutes changes of 2 X SSC. The filters are now ready for pre-hybridization.

Back to top of page

Back to Table of Contents