isolation of bacmid DNA and analysis by PCR

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revised: 08-20-15

**Day 1: inoculate bacterial cultures**

1. Using a sterile pipette tip, inoculate a single, isolated DH10Bac *E. coli* colony into 4 ml of LB medium containing 50 g/ml kanamycin, 7 g/ml gentamicin, and 10 g/ml tetracycline.
	1. It is likely that there will be clone-to-clone variation in the expression level of your protein. Therefore, it is advisable to select several recombinant clones (white colonies) for further analysis.
	2. It is also prudent to select a non-recombinant clone (blue colony) as well. This ‘empty’ bacmid DNA will be a useful control both for PCR and for transfecting insect cells to generate a P0 viral stock.
2. Grow the cultures at 37 °C with shaking at 240 rpm for ~17 hours.

**Day 2: isolate bacmid DNA and analyze by PCR**

**Isolation of bacmid DNA**

1. Prepare a glycerol stock of each recombinant bacterial clone by mixing 0.5 ml of overnight culture with 0.5 ml of sterile 30% glycerol in a cryovial. Store glycerol stocks at -80 °C.
2. Spin down the remaining volume of cells (~3.5 ml) in a 1.5 ml microcentrifuge tube.
	1. Transfer 1.5 ml of cells to a 1.5 ml tube, spin at 10,000 rpm for 1 minute at room temperature, then aspirate the supernatant.
	2. Repeat step 2a until all of the cells have been collected.
3. Resuspend the cells in 0.3 ml of Solution I. Gently pipette the sample up and down to completely resuspend the pellet. If necessary, the sample can also be vortexed gently.
4. Add 0.3 ml of Solution II, invert to mix, and incubate at room temperature for 3 minutes. The appearance of the suspension should change from turbid to almost translucent.
	1. Do not let the incubation proceed for much longer than 3 minutes, as the bacmid DNA may become denatured.
5. Add 0.3 ml of Solution III and invert to thoroughly mix the sample. A thick white precipitate of protein and *E. coli* genomic DNA will form; place the sample on ice for 10 minutes.
6. Centrifuge the sample for 15 minutes at 15,000 rpm at room temperature.
7. Gently transfer the supernatant to a microcentrifuge tube containing 0.8 ml of isopropanol. Do not transfer any white precipitate. Invert the tube a few times to mix and place on ice for 10 minutes. Proceed directly to step 8 or store the sample at -20 °C overnight.
8. Centrifuge the sample for 15 minutes at 15,000 rpm at room temperature.
9. Carefully remove the supernatant, taking care to not disturb the pellet. Add 0.5 ml of 70% ethanol, and invert the tube several times to wash the pellet.
10. Centrifuge the sample for 5 minutes at 15,000 rpm at room temperature.
11. Remove as much of the supernatant as possible, taking care to not disturb the pellet. Air dry the pellet for 5 to 10 minutes at room temperature. Do not over-dry the pellet.
	1. Do not use a SpeedVac to dry to the DNA pellet, as this may shear the DNA.
12. Dissolve the DNA pellet in 40 l of sterile 10 mM Tris (8.0) containing 0.1 mM EDTA. To avoid shearing, do not mechanically resuspend the DNA. Allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube.
13. Store the DNA at 4 °C.
	1. Storing the purified bacmid DNA at -20 °C is not recommended as repeated freezing and thawing may shear the DNA.
	2. Bacmid DNA stored at 4 °C is stable for ~2 weeks.
14. Proceed to analyze the recombinant bacmid DNA by PCR (protocol below), or to transfect the DNA into insect cells (separate protocol available on the Sondek lab website).

**Solutions:**

Solution I: 15 mM Tris (8.0), 10 mM EDTA, 100 g/ml RNase A (filter sterilize and store at 4 °C)

Solution II: 0.2 N NaOH, 1% SDS (prepare fresh for each experiment)

Solution III: 3 M potassium acetate (5.5) (autoclave and store at room temperature)

**Analysis of bacmid DNA by PCR**

**Notes**: Recombinant bacmid is >135 kb in size. Since restriction analysis is difficult to perform with DNA of this size, PCR analysis is recommended to verify the presence of your gene of interest in the recombinant bacmid. Use of the M13 primer pair (sequences below), which hybridizes to sites flanking the mini-*att*Tn7 site within the *lac*Z-complementation region, will facilitate PCR analysis. The following guidelines assume that you are using the M13 primer pair for analysis of your bacmid DNA. In this case, following agarose gel electrophoresis, you should expect a PCR product of ~2,400 bp + the size of your insert. Bacmid DNA may also be analyzed using gene-specific primers, or a combination of an M13 primer and a gene-specific primer. In these cases, you will need to determine the expected size of the PCR product yourself. ‘Empty’ bacmid should yield a PCR product of ~300 bp.

1. For each sample, set up the following 50 l reaction in a sterile 200 l PCR tube:

|  |  |  |
| --- | --- | --- |
|  | **stock** | **add (l)** |
| 1X Pfu polymerase buffer | 10X | 5 |
| 0.2 mM dNTPs (each) | 10 mM each | 1 |
| 0.25 M M13 sense primer | 10 M | 1.25 |
| 0.25 M M13 anti-sense primer | 10 M | 1.25 |
| Pfu polymerase | - | 1 |
| recombinant bacmid DNA | - | 3 |
|  |  |  |
| sterile ddH2O | - | 37.5 |

 **Notes:** Prepare a master mix to simplify setting up multiple reactions. Prudent controls

 include: (1) no template, and (2) ‘empty’ bacmid (DNA isolated from a blue DH10Bac *E.*

 *coli* colony).

 The sequence of the M13 sense primer is: 5’-CCCAGTCACGACGTTGTAAAACG

 The sequence of the M13 anti-sense primer is: 5’-AGCGGATAACAATTTCACACAGG

1. Amplify the bacmid DNA using the following cycling parameters:

|  |  |  |  |
| --- | --- | --- | --- |
| **step** | **time** | **temperature (°C)** | **cycles** |
| initial denaturation | 1 minute | 94 | 1X |
| denaturation | 45 seconds | 94 | 35X |
| annealing | 45 seconds | 55 |
| extension | ~90 seconds/kb | 68 |
| final extension | 10 minutes | 68 | 1X |
| hold | ∞ | 4 |  |

 **Notes:** These cycling parameters have been optimized for the M13 primer pair and the Pfu

 polymerase homemade in the Sondek lab. If you are using a different polymerase, a

 unique primer pair, or have an insert that is larger than 4 kb, further optimization of the

 PCR conditions may be necessary.

1. Analyze 5 – 10 l of each reaction by agarose gel electrophoresis.