7.13 Experimental Microbial Genetics

Fall 2008

Bootcamp Day 6: Pooling colonies and extracting plasmid clone DNA.

Today you will collect the colonies that have grown up from yesterday's transformation, and then extract the vector DNA using the QIAprep Spin Miniprep Kit.

A. CALCULATIONS TO BUILD A COMPLETE CLONE LIBRARY

1. Remove the transformations you plated yesterday from 37°C. Calculate how many colonies you will need to extract vector from to build a genomic library using the following formula:

$$N= (\ln(1-a))/(\ln(1-(b/c)))$$

Where N=the number of colonies, a=the desired completeness of the library, b=the average fragment or insert size, c=the total genome size. In this case, we want to create a library in which the percent likelihood that a certain fragment is represented is 99%. The genome of *Pseudomonas aeruginosa* PA14 is ~6.5Mb, or 6500kb.

2. Calculate the blue to white ratio on your plates to determine the percentage of colonies on your plates that contain insert.

3. Determine how many plates from which you will have to pool colonies to build a complete library. Think: What do the blue colonies mean and how should you factor them into this equation?

B. **POOLING COLONIES**

1. Add 3mL of LB to each plate of colonies.

2. Sterilize a glass spreader by dipping it in 95% ethanol, and passing it briefly through the flame of a Bunsen burner. The ethanol will catch fire and burn off, sterilizing the spreader. Allow it to cool, but make sure not to touch the spreader to the bench top or elsewhere after this—if you do, you will need to re-sterilize it.

3. Rub the plate GENTLY with the spreader to scrape off the colonies. Be careful not to press too hard or you will dislodge chunks of agar. Pipette off the LB with colonies and collect in microcentrifuge tubes. If you need to pool more than 1000 colonies, distribute them such that there are ~1000 colonies per tube. Sterilize your spreader each time you need to put it down. **Be sure to collect the cells with the 2-4 kb inserts in different tubes from the 4-8 kb inserts!!**

C**. PLASMID DNA PURIFICATION**

The following requires the use of the QIAprep Spin Miniprep Kit. Before you start, make sure that RNase A has been added to Buffer P1 (stored at 4°C), and that ethanol has been added to Buffer PE .

1. Harvest the bacterial cells by centrifugation at > 8000 rpm (6800 x *g*) in a conventional, table-top microcentrifuge for 3 min at room temperature (15–25°C).

Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained. Pipette off any remaining medium if necessary.

2. Resuspend pelleted bacterial cells in 250 ul Buffer P1.
Ensure that RNase A has been added to Buffer P1. **No cell clumps** should be visible after resuspension of the pellet. If you have pelleted your cells in more than one microcentrifuge tube, pool them together, so that about 1000 colonies are resuspended in a TOTAL of 250uL Buffer P1.

2. Add 250 ul Buffer P2 and mix thoroughly by inverting the tube 4–6 times.
Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

3. Add 350 ul Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy.

4. Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge. A compact white pellet will form. During the spin, label an appropriate number of QIAprep spin columns on exposed purple part of the tube.

5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

6. Centrifuge for 30–60 s. Discard the flow-through.

7. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60s. Discard the flow-through.
This step is necessary to remove trace nuclease activity when using *endA*+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content.

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.

9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
Important: Residual wash buffer will not be completely removed unless the flow- through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. Place the QIAprep column in a clean, well labeled, 1.5 ml microcentrifuge tube. To elute DNA, add 50ul EB or water (30ul if higher concentration is desired) to the center of each QIAprep spin column ensuring that it covers the whole membrane (don't touch it with your tip), let stand for 1 min, and centrifuge for 1 min. Pre-warming the buffer or water will increase the yield of DNA. If you use water, make sure the pH is between pH 7.0 and 8.5.

11. Store plasmid prep at -20°C.