7.02 Fall 2001

RDM

Recombinant DNA methods

Agenda

DAY 4

- what results do you expect from the *ara* PCR analysis?
- computing transformation efficiency for the AG1111 cells
- isolating plasmid DNA by performing minipreps

Results of *ara* PCR analysis

• We are using agarose gel electrophoresis to look at the results of the 9 PCRs we set up and ran on Day 3. Keep in mind which experiment we're talking about when you look at these gels: this has nothing to do with the ligations and transformations we've been working on!
• All three PCRs for the wild-type strain (1-3) should show a product. You can compute the expected sizes of these products by looking up the positions where the primers hybridize. There are maps of the ara operon in appendix at the back of your lab manual.
• The first three PCRs for the mutant strain (4-6) are directly analogous to the wild-type PCRs; each of them uses a primer pair designed to amplify one of the ara genes. These PCRs will only work if the target gene is intact; if a transposon has inserted into the gene, then the PCR primers will hybridize too far apart from each other for a product to be generated.
• The last three PCRs (7-9) use a special primer that hybridizes to the beginning of the transposon, in the LacZ coding sequence. This primer points backwards (with respect to the coding sequence), and it is always paired with a forward-pointing primer that hybridizes to one of the three ara genes. A product in one of these lanes indicates that the transposon inserted into the gene whose forward primer you used in that PCR. Measurement of the product size reveals how far into the gene the transposition occurred.
• In general: for the mutant strain, PCR's 4-6 and PCR's 7-9 are correlated to each other. For instance, we expect that if we see a band in 4, we won't see one in 7, and vice versa. Instances where we see a band in both 4 or 7, or when we see a band in neither 4 nor 7, require some special explanation.

Possible anomalous results of the PCR analysis

• Band for wild-type C-For/C-Rev product is fainter than for the A and B ones. This is because the C primer pair happens to be less concentrated than the other primer pairs. This is a problem we hope to correct in the future.
• PCR #8 has a bright band, and PCR #5 has a faint one. This can result from contamination of PCR #5 by a small amount of wild-type DNA.
• PCR #6 and PCR #9 both have no bands. This can happen if the insertion into AraC was very close to the beginning. That causes the PCR product to be very small; it may run off the bottom of the gel, or it may not bind enough ethidium to be visible.
• You determine that the transposon inserted into AraA, but you see a band in PCR #8 (AraB-For/LacZ-Rev). This can happen if the transposon inserted into AraA close enough to the AraB-For primer-binding site so that the distance between the transposon and the beginning of AraB is less than 2500

base-pairs. To understand how this can happen, it is important to draw out the Ara operon and indicate where the primers bind and the exact distances between them.

Computing transformation efficiency

• Transformation efficiency is expressed as the number of transformants you get per microgram of DNA, for the particular conditions you're using. Make sure when you do this computation, you go carefully through the steps in the transformation protocol and take account of all the dilutions you made! You want to divide your colony count by the amount of DNA (in micrograms) that actually made it onto the plate, *not* the total amount of DNA you used in the transformation!

Isolating plasmid DNA using minipreps

• Plasmid DNA is small, circular, and supercoiled. Plasmid DNA, like any other DNA, is denatured by high temperature, due to disruption of the hydrogen bonds between its two strands. However, plasmid DNA is special in that when you cool it back down, it can renature very quickly because its two strands are kept in close proximity by being topologically linked.
• The miniprep protocol takes advantage of this peculiarity of plasmid DNA to isolate it away from chromosomal DNA.
• Basic idea: lyse the cells by adding detergent and lysozyme and then boiling them. This causes all the cell guts to burst out. Proteins will be denatured and aggregate together in a big blob. Chromosomal DNA, which is very sticky, will renature with partners from other cells, resulting in a huge cross-linked network of sticky DNA that will just join the protein blob.
• Plasmid DNA will snap back together immediately upon cooling, due to the special properties discussed above. Because it does not get bogged down in a sticky aggregate, it remains soluble.
• Spinning the cooled lysate results in a clear supernatant (containing plasmid DNA, RNA, and lots of salt.... how did the sale get there? Answer: EDTA) and a bloblike mess at the bottom of the tube. Many people prefer to call it "snot". You can call it what you like, but the way we deal with it is to fish it out with a sterile toothpick. This leaves the plasmid-rich supernatant.
• We add isopropanol to the supernatant. This causes the solution to become more hydrophobic, and polar molecules (like DNA) tend to want to escape with each other by precipitating out of solution. For DNA, this precipitating tendency is checked, however, by the electrostatic repulsion of their negatively-charged backbones. We overcome this obstacle by making sure there's lots of cations in the solution, in particular sodium.
• Spinning the isoproanol+solution will cause the precipitated DNA (and RNA) to pellet out, along with all the salt in it. We want to get rid of this salt so it won't inhibit restriction enzyme digests.
• To get rid of the salt, we wash the pellet with 80% ethanol, which is still polar enough to keep the DNA insoluble, but contains enough water to wash away the salt.
• Following this step, we need to get rid of the ethanol, which would also be a problem for the restriction digest. To do this, we simply put it in the warm room (37ºC) for 20 minutes or so. This process can be speeded by making sure you have taken off as much of the ethanol as possible. You can use the corner of a Kimwipe to draw off the bead of ethanol that will remain at the top of the tube after you've poured the rest off.
• Finally, the dried pellet of DNA is resuspended in TE containing RNase A to chew up the RNA. This leaves a solution of just DNA, which can be added to the analytical restriction digests you will set up.