7.02 Fall 2001

RDM

Recombinant DNA methods

Agenda

DAY 3

- transformation
- what should be the result of each transformation?
- do Day 3.5 for other section
- PCR – show video
- application of PCR to *ara* mutants

Transformation

• Transformation is the process whereby bacterial cells are induced to take up plasmid DNA. The cells are first treated with calcium chloride to weaken their membranes and make them permeable to DNA. Cells that have been prepared in this manner are called "competent."
• Competent cells are mixed with plasmid DNA (in this case, our ligation reactions). They are gently mixed and incubated on ice to allow time for the plasmids to adhere to the outside of the cells.
• The cells are then subjected to a brief heat-shock (45 seconds at 42ºC). This causes them to take up any plasmid that adhered to them. The precise details of this process are not well understood, but the combination of calcium chloride treatment and heat shock can be assumed to both weaken the bacterial membrane and render it more permeable to plasmid DNA.
• After a brief incubation on ice to return the cells to a comfortable temperature, we give them some food in the form of SOC medium. Then we allow them to incubate one hour at 37ºC. The purpose of this incubation is to allow those cells which took up a plasmid to express the ampicilin resistance gene. They need time to synthesize the protein (called beta-lactamase) that breaks down the antibiotic. What would happen if you transfered the cells onto ampicilin plates directly after the transformation?
• After the hour incubation, the cells are diluted and plated onto agar containing ampicilin. This will allow us to select only those bacteria that took up a plasmid with the ampicilin resistance gene. In themorning, we will see colonies on the plate. Each colony of bacteria is descended from a single transformant, and all those bacteria in the colony should have the exact same plasmid in them.

What controls are we doing, and what results do we expect?

• We have already discussed the four ligations we performed, three of which are experimental controls. Each of these ligations will be used to transform bacteria. We will do two additional transformations:
• Transformation #5 will be done using pure undigested pET DNA that was never gel-purified. The purpose of this control is to make sure there is nothing in the agarose that inhibits transformation.
• Transformation #6 will be done using just ligation buffer. This should yield no colonies. If we do see colonies from this transformation, we know that the cells are amp-resistant, or the ampicilin is faulty, or the ligation mix is contaminated.

Picking colonies on Day 3.5

• When it is your section's turn to perform the Day 3.5 protocol, you will be working with the other section's six transformation plates. You will examine their plates to verify that there are colonies on plate 1 (vector + insert ligation), and that there are more colonies on plate 1 than on plates 2 and 3 put together. (Why is it important to check this?)
• If there are not enough colonies on plate 1, you can take colonies from another group.
• Inoculate six colonies from plate 1 into 2 mL of super broth containing ampicilin.
• Additionally, pick two colonies from a vector-only plate. You can choose either plate 4 or plate 5, depending on which one has better separation between the colonies.
• It is crucial that you use sterile technique during this inoculation procedure, and that you take pains not to cross-contaminate the colonies. Use a new sterile stick for each inoculation.

PCR

• We will be using the Polymerase Chain Reaction to generate many copies of a target region of DNA.
• PCR uses a polymerase called Taq, which is a thermostable DNA polymerase from the thermophilic archean *Thermus aquaticus*. It is important to use a thermostable polymerase, because PCR involves incubations at 96ºC to denature DNA. Such high temperatures would irreversibly denature an ordinary DNA polymerase. Why doesn't it denature Taq?

• A PCR reaction has the following components:
template DNA
two primers
deoxynucleotides (dNTPs)
buffer, containing salts and magnesium (a critical cofactor)
DNA polymerase (Taq)

• The steps of one cycle of PCR are as follows:
(1) Denature the template DNA by heating to near-boiling (96ºC)
(2) Cool the reaction to allow primers to anneal.
(3) Extend the annealed primers. (The temperature is raised slightly to allow the polymerase to reach maximal efficiency – about 1000 bases per min.)

• These steps are repeated over and over. If the primers are designed in such a way that they point towards each other, and the gap between them can be traversed by the polymerase in the time alloted during the extension step, then on every new cycle, new primer binding sites will be generated. During each cycle, the amount of the target DNA will be doubled.
• If you start from a single copy of the target DNA sequence, then after 30 cycles of PCR, you will have on the order of a billion copies of that target sequence.

• You only get exponential amplification if the primers are designed as discussed above. A PCR with only a single primer, or with primers pointing away from each other, only produces 1 copy per cycle. After 30 cycles, you'll have 30 copies of the target sequence, instead of a billion. The reason for this is that you're never creating any new places for primers to bind. In a successful PCR, the primters interact "synergistically," helping each other by directing the replication of each other's binding site.

• It is important to simulate several cycles of PCR on paper to convince yourself of how it works.

Application of PCR to our ara mutants

• In the GEN module, you isolated strains of *E. coli* that had mutations in their AraABCD operon. Now we'll return to those mutants and use PCR to pinpoint exactly where in the operon the mutation is. The mutation took the form of a transposon insertion. We'll be using PCR primers that are complementary to the ara genes themselves, and a primer that is complementary to the transposon, in order to amplify specific regions of DNA. Some of these PCRs will yield products, and some won't. In some cases, the absence of a PCR product will be informative.