CHEM525 Experiment 2
Cloning-1

Purpose and Theory

This week, we will be using PCR to amplify the Lactate Dehydrogenase (LDH) gene (around 1 kb in length) from *Bacillus subtilis* DNA and then prepare it to be inserted into pET28a by a restriction digest reaction with HindIII and NdeI.

We will start out with a tiny amount of *B. subtilis* chromosomal DNA. In order to produce a lot of protein and be able to get any useful results, we are obviously going to need many, many copies of the gene. Thus, this week we will amplify our tiny bit of DNA using a common biochemical method called PCR. We will then purify our LDH gene from the PCR reaction mixture and digest it for next week. Likewise, this week we will also need to the pET28a vector. We will do this by extracting and purifying it from a strain of *E. coli* that harbors the plasmid followed by a reaction with the restriction enzymes HindIII and NdeI to create an entry space for the LDH gene.

New Techniques

PCR

PCR is a way to make multiple copies of a section of DNA off of a single double-stranded template.

How it works:

When DNA is heated above a certain temperature, hydrogen bonds that hold complementary strands together break, and the DNA separates into two single strands. When the temperature is lowered again, the hydrogen bonds reform. So, say we put a little bit of our double stranded DNA to be replicated in solution with a lot of free deoxyribonucleotides (the building blocks of DNA), some DNA polymerase (the enzyme that catalyzes DNA replication), and a lot of short little strands of DNA that are complementary to the DNA template strand called primers, or oligos. There are two kinds of primers: one complementary to the DNA coding strand downstream of the gene, and one complementary to the anticoding strand upstream of the gene.

So imagine this: we take this mixture and we heat it up to break the hydrogen bonds between our strands of DNA template. Then, we slowly let the temperature cool. Since there are so many more primers in solution than template strands, chances are our primers are going to end up forming hydrogen bonds to the template before the template strands have a chance to anneal together again. Now, the DNA polymerase can come in and, starting from these primers, replicate the template DNA strands 5' to 3.' Now, there are two double stranded copies of the DNA encoding for the LDH gene. This process can be repeated multiple times, and each time we melt one DNA double strand, we get two double stranded copies back (see figure left). If we repeat this a bunch of times, we'll end up with a lot of copies of the gene!

Here's how it's useful:

Obviously, PCR is a great way to make a lot of copies of your DNA. However, it can do a lot more! PCR is also a very useful technique for introducing new sequences at the beginning and end of your gene. Recall how we design oligonucleotides to bind on either side of the gene. Well, the ENTIRE oligonucleotide does not have to match up to the template strands, just enough for it to anneal in the right place, as shown above. So, we can "program in" other desirable sequences to be attached on either side of our gene. Once the gene has replicated several times, most of the copies of the gene will have the desired sequences. Some common added sequences are restriction enzyme sites, used to incorporate the gene easily into the desired plasmid through digestion and ligation, which we'll talk more about in the following weeks. Another common sequence to add to the end of the gene is a poly-histidine tag, which is then used in column chromatography to isolate the desired protein (we'll also be talking about this a little later). We will actually use both techniques (column purification via a poly-histidine tag AND introduced restriction sites to help us get the gene into the expression vector).

Mini-prep

This is a general term used for commercially purchased kits that allow scientists to isolate and purify plasmid DNA from prokaryotes. The process involves incubation of "cell paste" (i.e. a clump of *E. coli*) with a lysis buffer (containing a detergent and a lot of NaOH) followed by a neutralization buffer. The former will burst the cells (detergent) and degrade linear DNA (NaOH) while maintaining the integrity of circular DNA. This is because circular DNA is supercoiled, which limits it's accessibility to solution conditions. The second buffer neutralized the hydroxide, resulting in a lot of cellular "gunk" to precipitate. Much of what remains is plasmid DNA and small molecules. The DNA is separated from the other solutes through interaction with a hydrophobic column a very high salt buffer. Under these conditions, the charge of the DNA is essentially neutralized by the high ionic content, allowing the DNA to interact weakly with the non-polar column. The DNA is then eluted by passing a low salt buffer or water through the column.

Digestion by Restriction Enzymes

Restriction enzymes cut DNA in certain places by recognizing specific sequences. These sequences are usually palindromes (e.g. Deny a pioneer free beer? Free beer? Free! No, I pay Ned.), in that the enzyme recognizes the same sequence on both complementary strands. Sometimes, the restriction enzymes leave what we call "sticky ends" which contain the complementary sequences able to base pair and to be ligated back together:

Safety Precautions

- Always wear glove, safety coat and goggles when in the lab.
- If a significant amount of any chemical is spilled, immediately seek the instructor for clean-up protocols.
- The lysis buffer in the Miniprep kit contains a high concentration of NaOH. Handle this with caution.

Equipment/Reagents Needed

1. Micropipettes and tips
2. TAQ 5X Master Mix (this contains DNA Polymerase, dNTP mix, and buffer).
3. DNA (Primer 1, Primer 2, Template)
4. MiniPrep Kit
5. PCR Purification Kit
6. HindIII (keep on ice)
7. NdeI (keep on ice)
8. 10X CutSmart Buffer
9. MilliQ water
10. Thermocycler
11. 37 °C water bath

Procedure

1) Perform a Polymerase Chain Reaction (PCR)
   a) Label 200 µL PCR tube with the sample name and combine the following in order:
      i. 37 µL MilliQ water
      ii. 1 µL P1 (T7 prom)
      iii. 1 µL P2 (T7 term)
      iv. 1 µL template
      v. 10 µL TAQ 5X Master Mix

   b) Place your reaction in the thermocyler. Once everyone is ready, run the following program:
      (i) 95 °C 1 minute
      (ii) 95 °C 1 minute
      (iii) 55 °C 30 seconds
      (iv) 72 °C 1 minute
      (v) Back to (ii) 30x
      (vi) 72 °C 1 minute
      (vii) Hold at 4 °C

2) Procure a tube of pET28a containing cells. Follow the MiniPrep protocol to purify. Please note that we will be eluting with 40 µL of milliQ water, NOT elution buffer.
3) Once the PCR reaction is complete, use the PCR Purification kit to purify your DNA. Elute with 50 µL of milliQ water.
4) Take a 5 µL sample of your PCR reaction and save it for next week.
5) Set up digestion reactions for each of your DNA samples. The total volume for each will be 30 µL and will contain HindIII (10X), NdeI (10X), CutSmart Buffer (10X) and the rest of the volume will be your DNA samples.
6) Place your digestion in the 37 °C bath.