CHEM525 Experiment 2
Cloning-2

Purpose and Theory

Last week, we amplified the LDH gene by PCR and isolated the pET28a plasmid DNA from a strain of *E. coli* that harbor the plasmid. We treated both of these DNA polymers with NdeI and HindIII, endonucleases that specifically recognize and cleave the sequence shown below.

At this point, we have two complete reactions that contain DNA that we want (LDH gene in one reaction and the linearized pET28a in the other) and short pieces of DNA that we don't want. Our goal in lab this week is to separate the desired DNA fragments from the waste products and then to covalently join the plasmid DNA with the LDH gene. To accomplish the former, we will employ agarose gel electrophoresis to isolate just the desired DNA. Once purified, the two fragments will be joined using the DNA ligase enzyme.

New Techniques

Agarose Gel Electrophoresis

We use agarose gels to purify and separate the pieces of DNA that we want after performing a digest. A DNA gel, or agarose gel, is a mixture of a buffer (TBE – Tris-Boric acid with EDTA) and agarose (a polysaccharide extracted from seaweed). The TBE buffer maintains a pH of 8.3 to ensure the complete deprotonation of the phosphodiester backbone which, in turn, ensures a negatively charged polymer. The gel is cast with several wells where your DNA mixtures are placed. These wells are oriented near the negative terminal of the gel (the black electrode) and run it towards the positive end of the gel (the red electrode). How far the DNA travels depends on its size. Since we know an approximate size of our LDH gene (~1 kBa) and pET28a vector (~5 kBa), we can easily pick out the bands we want. To make it easier to see where certain sizes end up, we use a "DNA ladder" in one of our wells. This is a mixture of DNA of standard sizes that we can run in parallel to our DNA and thus estimate the size of our DNA fragments. We are able to see the DNA because Ethidium bromide is added to the gel. This molecule is planar and can readily intercalate (insert between bases in the double helix) into dsDNA. When exposed to UV light, DNA that has EB will fluoresce. The fragments that contain the correct DNA will be cut out of the gel and purified using a column purification kit similar to what we used last week.

Ligation

Treating the plasmid and insert with the same endonucleases leaves complementary sticky ends, which effectively orient the gene into the correct location of the plasmid. However, 2-4 bases pairs are not sufficient to prevent the strands from separating. To remedy this, the insert and plasmid must be covalently bound together. DNA ligase is and enzyme that catalyzes the reaction between the 3' hydroxyl group of the deoxyribose of one DNA segment and the 5' phosphate of the adjacent DNA segment. This reaction will ensure the plasmid DNA that is put into *E. coli* will contain the LDH gene.

Safety Precautions

- Ethidium bromide is a carcinogen. Always wear gloves when handling solutions containing this molecule. Make sure that gels containing ethidium bromide are put in appropriate waste container.
- Razor blades are sharp – handle with care. All razor blades should be disposed of in the sharps waste container.
- 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.
- Never look directly into UV light – it can damage your eyes! Make sure the screen is up and look through the screen at your gel.

Equipment/Reagents Needed

1. Micropipettes and tips
2. Microcentrifuge
3. UV light box
4. NanoDrop spectrophotometer
5. Tabletop vortex
6. Gel extraction/PCR purification kit.
7. 1X TBE buffer
8. Agarose
9. Electrophoresis chamber and power source.
10. 10X DNA ligase buffer
11. DNA ligase (keep on ice at all times)
12. MilliQ water

Procedure

1. Column purify your digestion reactions. Follow the instructions for the PCR purification kit. Elute with 30 μL of water.
2. Concentrate your samples to ~20 μL on the speed vac.
3. Prepare an agarose gel.
    a. Add 0.5 grams of agarose to 50 mL of 1X TBE
    b. Microwave the solution for 30 seconds or until the agarose is fully dissolved.
    c. Cool on the benchtop for 5 minutes.
    d. While cooling, prepare the gel tray according to the professor's directions
    e. Add 5 μL of ethidium bromide.
    f. Carefully transfer the solution to the prepared gel tray.
4. Prepare your DNA samples for electrophoresis by adding th appropriate volume of 6X loading dye.
5. Load you samples into the wells. Make sure to add a ladder to one of the empty wells.
6. Run the agarose gel at 110 V for 30 minutes.
7. Use the gel doc to take a picture of your gel.
8. Using the UV light box and a razor blade, carefully cut the appropriate fractions out of the gel.
9. Purify the fractions using the gel purification kit. Elute with 20 μL of milliQ H_2O.

10. Determine the concentration of your samples (NanoDrop).
11. Set up a ligation reaction :
    a. 10 ng pET28a
    b. 10 ng insert (LDH gene)
    c. 1 µL 10x buffer
    d. 1 µL DNA Ligase
    e. Add milliQ water to a total volume of 10 µL
    f. Incubate at room temperature overnight.