Recitation Notes RDM Day 2-Spring 2005
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Where you are on the overview - On day 1, you performed the restriction digests on the 2 plasmids (in order to move the *gfp* gene from pUGFP to pET).

Today, three things being done on subcloning project:
1. phosphatase vector
2. gel purification/isolation
3. ligation to create new plasmid.

Today, we also begin the PCR project, by inoculating the mutant for O/N culture.

1. CIP (calf intestinal phosphatase) treatment of vector

What does CIP do? It is a phosphatase, so it cuts phosphates off molecules.

5'-G-OH P-AATTC-3' ⟹ 5'-G-OH HO-AATTC-3'
3'-CTTAA-P OH-G-5' 3'-CTTAA-OH OH-G-5'

Why do we use it? What do we use it for?
- We phosphatase the vector, so that the vector cannot self-ligate (if small amount of vector gets cut only by one enzyme, not both).

Why do you NOT want to phosphatase the insert?
- Because then the ligase will not be able to create the phosphodiester bond to ligate the vector and insert together.

*We've used alkaline phosphatase conjugated antibodies before (PBC) and will again (DEV).

2. Gel purification/isolation

Today, you will run your digest reactions on a low melt gel.

Why low melt?
- Because the low melting temperature of the agarose allows you to perform the ligation reactions with the DNA still in the agarose gel.

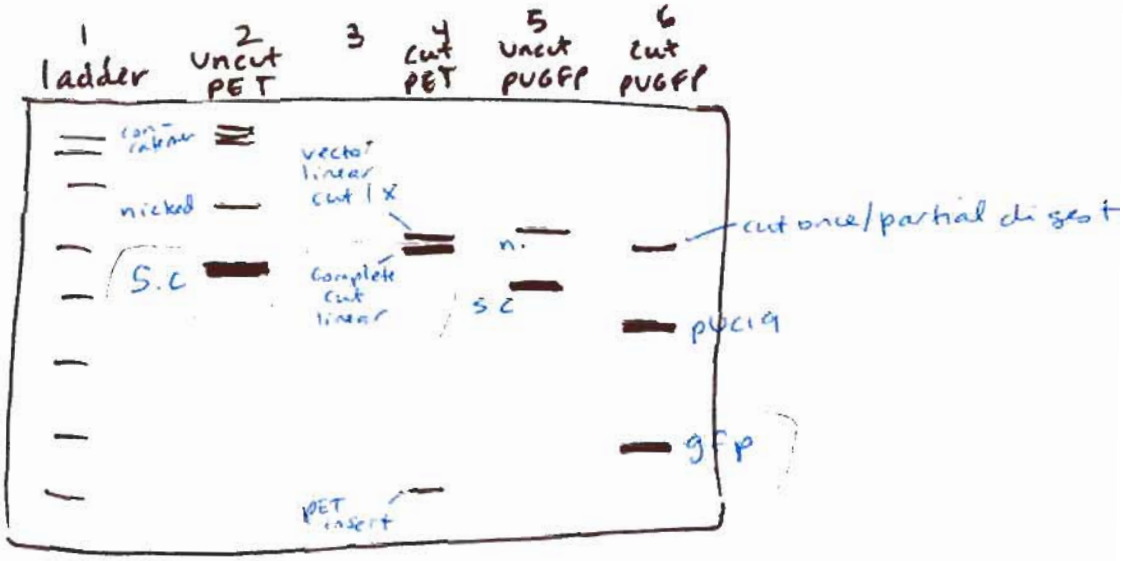
Low melt gels are very fragile!! Cut bands with new razor blade for each band.

What size bands do you expect to see in the gel? What is each band? Which bands do you want to cut out? (GFP insert = 1 kb, pET vector = 4 kb, uncut pET = bright supercoiled band)

(see diagram on next page)

Day 2 Notes continued

What low-melt gel should look like



cut these out

3. Ligation

Components of reaction mixture:

1. Ligation Buffer:
    a. Tris (buffer)
    b. DTT (reducing agent; keeps proteins in native conformation)
    c. $MgCl_2$ (cofactor for many DNA enzymes)
    d. ATP (energy used to form phosphodiester bond)
    e. BSA (takes up space in tube so DNA will find each other easier)
2. Ligase
3. Vector DNA (pET vector)
4. Insert DNA (*gfp* gene)

Keep enzymes on ice!

Is the orientation of insertion of the *gfp* gene important?

- Yes, because the 5' end of the gene should be following the promoter element in the vector. That is why the experiment was designed using 2 different restriction enzymes (one on each end of the insert/vector) than only 1.

possible other products (other than vector + insert), how we try to minimize these;

make sure gel is melted before adding ligase!

Ligation reactions set up:

1. digested pET + GFP insert
2. digested pET only (control for background, phosphatasing)
3. GFP only (control for gel isolation)
4. uncut pET (control for transformation)

4. **Inoculate Ara$^-$ mutant** – provides source for DNA to do PCR analysis. What will this experiment tell us? (Where your insertion is in your *ara* mutant) Don't forget to use sterile technique to inoculate!