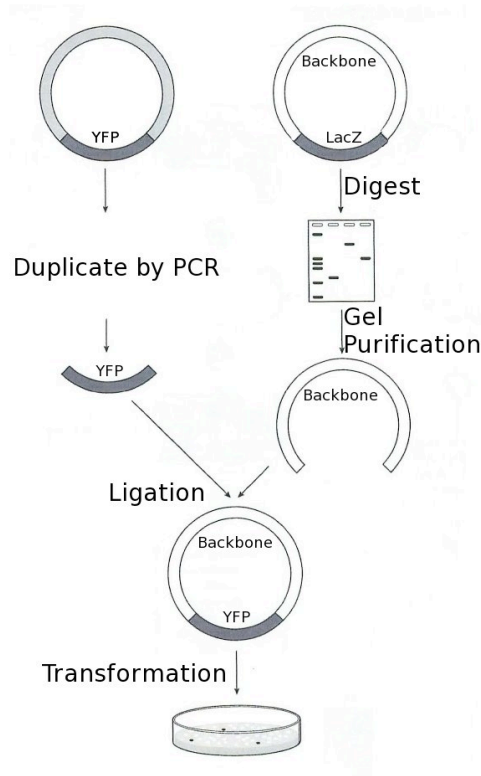


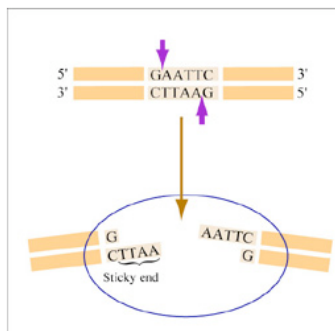
Aph 162 – Molecular Biology

Introduction:

In the next weeks you will transform *E. coli* to glow yellow by inserting a plasmid with the gene for YFP (yellow fluorescent protein). To do this, we will perform four steps: prepare the insert by copying the YFP insert by PCR, remove the LacZ gene from the backbone with restriction enzyme digestion, ligate the YFP gene in its place on the backbone and transform *E. coli* via electroporation to express the genes on the plasmid. (To make it a little easier, we have purified digested backbone from a gel for you.)



The keys to this “swap” are restriction enzymes. These enzymes cut DNA based on a recognition sequence, a region of DNA with a particular pattern of bases. For example, EcoRI cuts DNA like so:



This results in two “sticky ends” that can then match to DNA of the correct sequence. We will digest the backbone plasmid using two of these restriction enzymes, KpnI and HindIII, to separate the LacZ gene from the rest of the backbone. Using two different enzymes with different recognition sites and different “sticky ends” allows us to insert the YFP gene in the correct orientation. Along the way, we will explore PCR and how it duplicates DNA and how gel electrophoresis moves fragments of DNA in an agarose gel.

Day 1

PCR (Polymerase Chain Reaction) of the Insert

In this step we will amplify the insert DNA and prepare it for restriction digest. See the included 'Phusion HF Mastermix' handouts for more details.

Reagent:	Reaction (make 2):	Control:
Insert Plasmid (pZS2LacUV501_Venus DNA (YFP))	10 ng	Calc DDH ₂ O
Forward Primer (10 uM)	2.5 µl	2.5 µl
Reverse Primer (10 uM)	2.5 µl	2.5 µl
Phusion HF Mastermix (2X)	1X	1X
DDH ₂ O	Calc.	Calc.
Total Volume	50µl	50µl

Note: In many of the protocols to come, **Reagents** will be listed as 2X or 5X or 10X, etc, meaning that (unless otherwise directed) they should be added to relevant solutions such that the *final* concentration of the reagent is 1X.

You will set up a total of three reactions: two identical reactions as described above and one “no template control.” The no template control reaction is the same as the normal reaction, but leaves out the YFP template (sterile water is used instead). Why do you think this is an important control to perform?

Procedure:

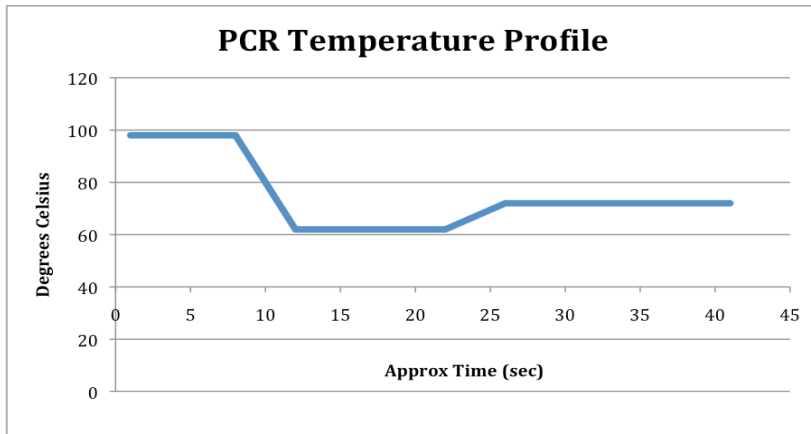
1. In *thin-walled* PCR tubes, mix the DNA, primers, mastermix and water together. Briefly centrifuge this tube to pull all liquid to the bottom. We will call this tube the 'PCR reaction.' Label your tubes for the three reactions as clearly as possible!
2. Hopefully the idea of thermally cycling during PCR is conceptually clear, now we must program the actual device to perform this task – your TA will help you with this.

Step:	Temp (C):	Time:
1 - Initial DNA Denaturation	98°	45 sec
2 - Denaturation	98°	8 sec
3 - Annealing	62°	10 sec
4 - Extension	72°	15 sec
5 - Final Extension	72°	5 min
6 - Hold	4°	Indef.

Steps 2 – 4 will repeat for a total of 35 times.

How much total amplification is that? What limits maximum amplification?

3. You will run one of the reactions and the no template control reaction on an agarose gel during the next day of molecular biology. The third reaction will be purified, as described below.



PCR purification of the insert

The PCR reactions you performed now contain the amplified insert DNA that you want to restriction digest in the next step of the subcloning—however, they also contain a lot of other things (e.g., free nucleotides) that decrease the efficiency of restriction enzymes. Because of this, you will need to purify the amplified DNA using a simple procedure and the QIAquick PCR purification kit from Qiagen. You will only be purifying one of the reactions (do not purify the no template control!).

1. Add 5 volumes of Buffer PBI to 1 volume of the PCR sample and mix. *For example*, add 500 μ l of Buffer PBI to 100 μ l PCR sample.
2. To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 s at maximum speed.
3. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 60 s at maximum speed.
5. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the empty column for an additional 1 min at maximum speed.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

6. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
4. To elute DNA, carefully add 35 μ l Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge the column for 1 min at maximum speed.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. Ask your TA to check that you have done this properly before your final spin.

NanoDrop

As a final step, you will use the NanoDrop spectrophotometer to determine the concentration of your purified, PCR amplified DNA.

Please see the TA demo.