APh 162 – Molecular Biology

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.

Total Volume: 50 ul

Reagent:	**Amount:**
Insert Plasmid (pZS2LacUV501_Venus @ 25 ng/µl) DNA (*YFP*)*	**100 ng**
Forward Primer (10 uM)	**2.5 ul**
Reverse Primer (10 uM)	**2.5 ul**
Phusion HF Mastermix (2X)	**1X**
DDH_2O	**Calc.**

*Replace the YFP DNA with an equivalent amount of water in the no template control reaction.

You will set up <u>three</u> reactions: <u>two</u> identical reactions as described above and <u>one</u> "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 s**
2 - Denaturation	**98**	**8 s**
3 - Annealing	**62**	**10 s**
4 - Extension	**72**	**15 s**
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 µl of Buffer PBI to 100 µl 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 µl 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.