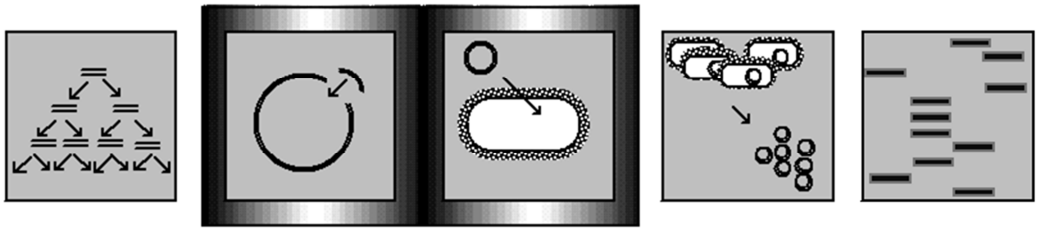
DAY 2 - CLONING A PCR PRODUCT

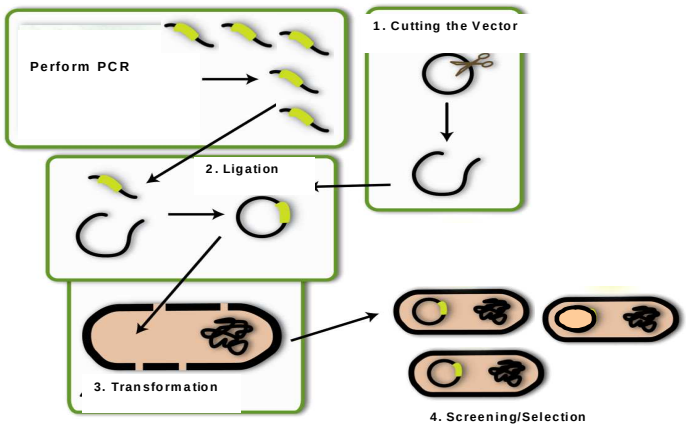


Introduction

Reading: Dale et al., 3rd Ed: p25-29.

Molecular cloning refers to the process of making multiple copies. Cloning is a commonly used method to amplify DNA fragments (such as a PCR product). You will be cloning your PCR product by **ligating** it into a plasmid vector. This process involves several steps:

1. **Breaking apart a plasmid vector** – a plasmid vector is a circular piece of DNA that has several key properties (see Appendix 4 for a diagram of the pBluescript vector you will be using in the lab).
2. **Ligation** – 'gluing' together the PCR product into the cut plasmid vector.
3. **Transformation** – inserting the newly formed pieces of DNA into bacterial cells. These transformed cells are then allowed to grow and multiply.
4. **Screening/selection** – selecting out cells that were successfully transformed with the new vector.



Although PCR is quick and straightforward, cloning PCR products can be a little problematical. Getting a PCR product to join into a plasmid vector is an inefficient and difficult process but there are several techniques that can be used to try to optimise the procedure. One of the most common methods of cloning involves the use of restriction enzymes (or to use the correct term, restriction endonucleases), which cut the DNA at a specific nucleotide sequence, known as a restriction site. In this lab, we used PCR primers that had restriction sites added near their 5' ends (a KpnI site for the forward primer, and a BamHI site for the reverse primer – see **Exercise 1: PCR and Primer Design** for more detail). The introduction of these specific changes into a PCR product during the reaction is called **PCR mutagenesis**, although the changes will be, by definition, at the ends of the PCR product. The use of different restriction sites at each end of the PCR product provides an important advantage when cloning.

The choice of restriction enzyme sites for this cloning experiment was governed by (i) cost: the enzymes should be cheap; (ii) compatibility: the sites should be present in multiple cloning sites of common vectors; (iii) ease: the enzymes should use compatible buffers, so that we can perform a double-digest; and (iv) site usage: the enzymes should not cut within the product we're trying to clone. For (iv), we can use computer programmes to search for restriction sites within a fragment of DNA of known sequence.

Restriction enzymes cut DNA at specific sites. They are derived from different bacteria (where they participate in a kind of defence system, chopping up foreign, but not "self" DNA), so they require individual reaction conditions to work optimally. The abilities to restrict **digest** DNA at **particular sites**, to **ligate** it into a **plasmid vector**, and to **transform bacteria** with it in order to amplify it, are central to **recombinant DNA technology**.

Method

1. Setting up Restriction Digests

Measure the volume of your cleaned–up PCR product by setting a Gilson P200 to the volume you think you have and sucking up the sample. If you fail to suck up the whole sample, or if you are sucking up air at the end, you need to eject the sample back into the tube, change the volume setting up or down respectively and try again.

Assemble the following components, **adding the enzymes last, on ice**:

NOTE: restriction enzymes are **thermolabile**, and **must be kept on ice** when not frozen.

Your cleaned PCR product (check what volume you have first)	up to 41 µl
10x restriction buffer	5 µl
KpnI enzyme	2 µl
BamHI enzyme	2 µl
Sterile distilled water	x µl
Final volume	**50 µl**

Seal. Mix by flicking the bottom of the tube.

NOTE: restriction enzymes, like all enzymes in molecular biology will not thank you for vortexing them. This "tube flicking" is a gentler and thorough alternative.

Label the top of the tube clearly, and transfer it to a 37°C water bath float for at least 1 hour.

2. DNA Cleanup

The reaction conditions required by DNA ligase are very precise, and differ from those needed by KpnI or BamHI. So we must clean up the DNA again.

Use *all* of your digest (~50 µl) and **remember** to add an equal volume (~50 µl) of Membrane Binding Solution. Refer back to the "DNA cleanup" section on DAY 1 for the method, but **at the last stage elute with 25 µl TE buffer**.

3. Quantification of DNA

You now have to measure the amount of DNA you have obtained following the DNA cleanup. You will use the NanoDrop spectrophotometer in the lab, which allows accurate and reliable quantitation of nucleic acids using a very small volume of your DNA sample. You will also be able to tell how pure your sample is and whether you have any contaminants present. If sample is pure (i.e. without significant amounts of contaminants such as any enzyme left from the restriction digest), you can use the spectrophotometer to measure amount of UV irradiation absorbed by the bases.

For quantifying DNA, absorbance readings should be taken at wavelengths of 260 nm and 280 nm (A_{260} and A_{280}). The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. The relationship between DNA concentration and A_{260} is as follows:

A_{260} of 1.0 for double-stranded DNA = 50 ng/µl of dsDNA

The reading at 280 nm gives the amount of protein in the sample. Pure preparations of DNA have an A_{260}/A_{280} value of 1.8 to 2.0. If there is contamination with protein, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible. So, for DNA quantification the formula becomes:

DNA concentration = A_{260} x 50 ng/µl x dilution factor (**Note**: if you have to dilute your DNA sample then the dilution factor will have to be taken into account in this calculation)

Fortunately for you, NanoDrop does all the measurements and calculations for you and all you need to do is read the final concentration!

To use NanoDrop, you will have to (with the help of a demonstrator!):

Take your DNA from the cleanup over to the NanoDrop and, using a P2 Gilson pipette, take 2 µl and place this carefully on the lower optical surface of the machine


Select the 'Measure' function and take a note of the concentration reading below:

DNA concentration =

4. Setting up a Ligation Reaction

NOTE: DNA ligase and ligase buffer are **particularly heat labile**, so should be kept on ice.

Assemble the following**, adding the enzyme last,** in 3 eppendorfs labelled with your initials:

	Tube 1 (plasmid + insert)	Tube 2 (plasmid only)	Tube 3 (ligase control)
Double Cut Unligated Plasmid (200ng/µl) (pBluescript **plasmid** precut with *Bam* HI and *Kpn*I)	200ng	200ng	-
Your *Bam* HI /*Kpn*I cut PCR product (the "insert")	100ng	-	-
Single Cut Unligated Plasmid (200ng/µl) (pBluescript plasmid precut with *Kpn*I)	-	-	200ng
5x ligase buffer	4µl	4µl	4µl
Sterile distilled water	to 19µl	to 19µl	to 19µl
T4 DNA ligase enzyme	1µl	1µl	1µl
Final volume	**20µl**	**20µl**	**20µl**

- The final volume in all tubes should be 20 µl. **Add the ligase last.**
- Incubate the ligation reaction at room temperature for at least 1 hour.

Questions

Q1. Given the relative sizes of pBluescript and the PCR product, what insert: vector molar ratio have we set up in tube 1?

Q2. What is the advantage of having different restriction sites at either end of our PCR fragment?

Q3. What is the purpose of the DNA ligase in the ligation reaction?

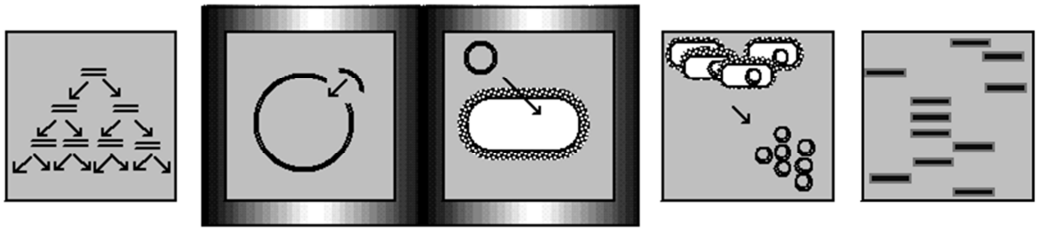
Q4. What would happen if DNA ligase was not used in the creation of the recombinant plasmid?

Q5. If you had a purified stock of DNA and you dilute 1µl of this purified DNA sample into a total volume of 50µl distilled water. You then measure the absorbance of this diluted sample in a NanoDrop at 260 and 280 nm and obtain the following readings: A_{260} = 0.550; A_{280} = 0.364.

a) What is the DNA concentration of the stock? Express your answer as µg/µl.

b) What is the A_{260}/A_{280} ratio of the purified DNA? Comment on the purity of the sample.

DAY 2 - TRANSFORMATION OF COMPETENT E.COLI CELLS



Introduction

Reading: Dale et al., 3rd Ed: p59-61.

By now, we should have ligated (or glued) our PCR product into a plasmid vector. Next we have to put it into a prepared bacterial *Escherichia coli* "**host"** strain to grow it up: this process is called **transformation**. Since both the ligation we have just done and the transformation we are about to perform are not very efficient, we will use two methods to **select** only those bacteria that contain a PCR-product containing plasmid. Selection is an important concept in this work!

NOTE: Be scrupulously clean, or some other bug may land in your cultures and contaminate them.

Method - Transformation of Competent Bacteria

NOTE: When adding DNA to the cells, make sure you have actually taken up liquid into the pipette tip and make sure that this liquid is pipetted directly into the cell suspension (not onto the side of the tube).

1. **Competent bacteria** are prepared by chilling in the presence of divalent cations such as Ca^{2+} (as in $CaCl_2$), which makes the cell membrane more permeable. You will be given -on ice- aliquots of competent *E. coli* strain DH5α which have been prepared for you and resuspended in 100mM of $CaCl_2$.

NOTE: Competent cells are very fragile. Pipette very gently; mix very gently; keep on ice and avoid temperature changes until you deliberately heat shock them.

2. Take 5 aliquots of 50 µl competent cells in screw-top vials and thaw on ice. Label as per the samples below:
3. Add to your 50 µl aliquots of competent cells:
    1. 2 µl of your "Plasmid + insert" ligation (Tube 1)
    2. 2 µl of your "Plasmid only" ligation (Tube 2)
    3. 2 µl of your "ligase control" ligation (Tube 3)
    4. 2 µl of a "teacher's ligation" of PCR product plus plasmid which is known to have worked
    5. Each pair should also set up ***one*** of the following, such that each control is carried out by your bench as a whole:
       (a) Uncut plasmid (2 µl of 10 pg/µl stock)
       (b) Cells only (without DNA)
       (c) Double cut, unligated plasmid (2 µl of pBluescript cut with BamHI & KpnI)

The significance of these important transformation controls will become clear later.

4. Tap the tube gently to mix the DNA and cells, then leave 30 min on ice.
5. Meanwhile, label 5 selective plates (L-agar which contains 50 µg/ml ampicillin, 50 µg/ml IPTG and 50 µg/ml X-gal), one for each reaction, with your initials and sample details. Also label the control, L-agar only plate, with your initials. NOTE: always label petri dishes neatly on the side of the base, not the lid.
6. Heat-shock the transformation mixtures in a 42°C water bath for EXACTLY 1 min.
7. Put the tubes straight back onto ice for 2 min.
8. Add 200 µl sterile L-Broth to each tube. Seal. Incubate at 37°C for 30 min. This is called the **expression** step.
9. Pipette 100 µl of each transformation onto the ampicillin plates and spread with a sterile plastic spreader. For the transformation of your own plasmid + insert ligation, pipette 50 µl onto the control L-agar plate (without ampicillin) and spread. **This plate represents a further control (d).**
10. Incubate all the plates upside down in the 37°C incubator overnight.

NOTE: Plates are usually incubated upside down.

Questions

Q6. What is meant by 'competent' cells?

Q7. The agar plates should be labelled on the base and NOT the lid (see step 5 in the above method). Why is this?

Q8. Why are the plates incubated upside down (see step 10 in the above method)?

Q9. Why is the expression step (see step 8 in the above method) important?

Q10. You set up a further control whereby your cells were plated out onto a plate without ampicillin (control (d) - see step 9). What do you expect to see on this plate tomorrow and why is this control important?