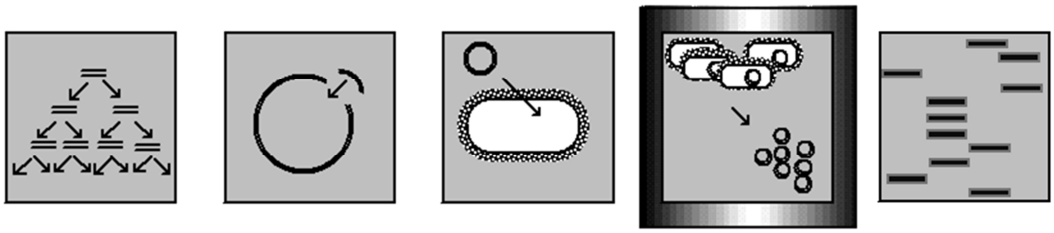
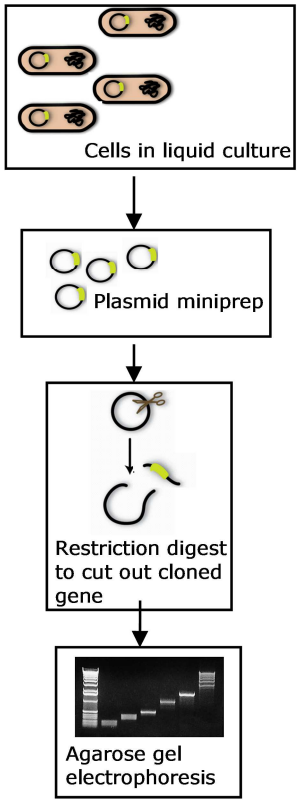
DAY 4 – PLASMID MINIPREP



Introduction



We hope that the plasmid isolated from the white colony will be a recombinant plasmid which carries our PCR product. There are several ways to check this, for example, by PCR, restriction digest or sequencing. We will be doing a restriction digest to determine if the plasmid carries the *Nrg* gene amplified DNA. First, we will harvest plasmid DNA from the bacteria grown overnight then use restriction enzymes (BamHI and KpnI) to cut out the cloned PCR product from the plasmid (this should only happen in bacteria from the white colony). Finally, we will run the samples on an agarose gel to check the size of the resulting DNA. This should allow us to see if the "white" plasmid does indeed contain an insert of about the correct (ie PCR product) size.

We start by harvesting the cells grown in liquid culture by spinning them down to a pellet, then extracting the plasmid. There are several ways of doing this, the simplest is to resuspend the pellet, and lyse the cells in a mixture of alkali and detergent. When the alkali is then neutralised, almost everything *except* the plasmid is precipitated down (unlike the proteins and chromosomal DNA, plasmid DNA can renature under these conditions).

These experiments are performed on various scales, and are named on the basis of the volume of the "overnight". Usually, a set of plasmid minipreps is produced from 5–10 ml overnight cultures, and the plasmids are digested or sequenced to establish which contain the insert of interest. If more DNA is required a midiprep (about 50 ml) or a maxiprep (about 400–1000 ml) can also be prepared.

1. Plasmid extraction

A brief outline of the procedure shown here:

Remember that you are working with **recombinant bacteria**

Spin down cells. Resuspend in buffer

↓

Lyse cells with detergent. Neutralise to precipitate proteins & genomic DNA

↓

Spin and save supernatant. Collect plasmid on a Minicolumn. Wash with alcohol.

↓

Elute DNA with aqueous buffer

Now collect your overnight cultures and mix briefly to resuspend the bacteria; each person should do one miniprep, noting which is which! If your overnights failed, borrow someone else's - there will be spare liquid.

1. Your aim is to produce a pellet corresponding to 4.5 ml culture in an eppendorf tube that only holds 1.5 ml. For each culture:
   - Put 1.5 ml (2 x 750 µl) culture in a labelled eppendorf. Centrifuge at 10,000rpm for 2 min. Decant the liquid supernatant into disinfectant.
   - Add a further 1.5 ml of the same culture to the pellet in the same tube (be careful not to mix up the "blue" and "white" cultures), spin down cells as above and remove supernatant by pouring or with a pipette.
   - Repeat the above step. You should now have a single cell pellet from 4.5 ml of bacterial culture.

Get rid of **all** the supernatant after the last spin (any medium remaining can be briefly spun to the bottom of the tube and removed using a P200 Gilson). *Any contaminants could inhibit restriction enzymes later on.*

2. Add 250 µl Cell Resuspension solution. Mix thoroughly by pipetting up and down with a P200 (plus yellow tip). *Any lumps of cells that remain will not lyse in the next step, reducing your yield.*
3. Add 250 µl Cell Lysis solution. Invert tube four times to mix; don't vortex. *This lyses the cells in a mixture of alkali and detergent.* Liquid should go visibly "gloopy" as the chromosomal DNA is released. Incubate at room temperature for about 2 min until the cell suspension clears.
4. Add 10 µl Alkaline Protease Solution and mix by inverting the tube 4 times. Incubate at room temperature for 5 min. Alkaline protease inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA. **Do not exceed 5 minutes of incubation with this enzyme, as nicking of plasmid DNA may occur!**
   From now on, be careful and don't treat the DNA violently. The separation of chromosomal DNA from plasmid is based on size, and if you shear the chromosomal DNA into smaller pieces, it will copurify with the plasmid.
5. Add 350 µl ice-cold Neutralisation solution. Invert the tube sharply four times to mix thoroughly. You may see a white flocculent (flaky) precipitate. *The rapid neutralization causes the protein and chromosomal DNA to precipitate, the plasmid however is able to renature under these conditions.*
6. Spin down at maximum speed for 10 minutes.

Plasmid DNA will now be purified from the bacterial lysate using microcentrifugation to force the cleared lysate through a Minicolumn and the plasmid DNA, which has bound to the column, will be washed.

7. Label the Minicolumn with your initials. Transfer about 800 µl of the cleared lysate to the Minicolumn. *Take care not to transfer any of the white precipitate; this would reduce the purity of the product.*
8. Centrifuge the supernatent at maximum speed for 1 minute. Remove the Minicolumn from the tube and discard the flowthrough from the Collection Tube. Reinsert the Minicolumn into the Collection Tube.
9. Add 750 µl of Column Wash Solution to the Minicolumn. Centrifuge for 1 minute as before. Remove the Minicolumn from the tube and discard the flowthrough. Reinsert the Minicolumn into the Collection Tube.
10. Repeat step 9 using 250 µl of Column Wash solution and centrifuge this time for 2 minutes.
11. Place the Minicolumn into a labelled 1.5ml eppendorf (remove the lid), being careful not to transfer any of the Column Wash Solution.
12. Elute the DNA by adding **40 µl TE buffer** directly into the column using a P200 Gilson pipette, then spinning for 1 minute. After eluting the DNA, discard the Minicolumn.
    Again, note that residual alcohol in the column is the main reason for poor yield.
13. When finished, place a lid on the tube and **label it carefully** with your initials and "blue plasmid" or "white plasmid" and keep on ice. You are now ready to set up your restriction digests.

2. Digestion (cutting) of your plasmid DNA with KpnI and BamHI

You should now have plasmid DNA purified from (i) a white colony and (ii) a blue colony. You will now digest a sample of each plasmid with BamHI and KpnI. In **fresh eppendorfs**, set up the following reaction, **one digest for each of your plasmids**, as follows (40 µl final volume):-

- 22 µl water
- 4 µl 10x restriction buffer
- 10 µl of your plasmid DNA preparation, then add
- 2 µl KpnI
- 2 µl BamHI

Flick the tube and spin down briefly in the microfuge. Incubate ≥ 1 hour at 37°C. **This will be your "cut" plasmid**. Meanwhile**,** store the remainder of your plasmid preparation on ice **(this is your "uncut" plasmid).**

3. Agarose gel electrophoresis

Prepare your samples for gel electrophoresis as follows:

- To the tube containing your 5 µl sample saved from the original PCR, add 15 µl water plus 6 µl loading dye.
- For each "uncut" plasmid, pipette 3 µl into a small eppendorf and add 17 µl water and 6 µl loading dye.
- For each "cut" (ie restriction digested) plasmid, pipette 20 µl into a small eppendorf and add 6 µl loading dye.

On a 1% agarose gel in TAE buffer, run pairs of "uncut" and "cut" samples of each of your plasmids and the PCR sample (with the **PCR next to the "white cut"** sample). **Load the whole volume for each sample**. Your gel should also have a **DNA ladder**.

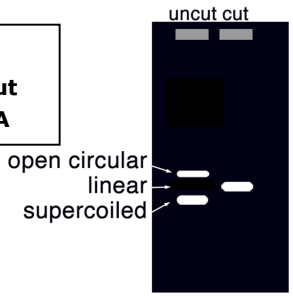
More than one group will be using the gel. Make sure there is at least one well left empty between your samples and those belonging to the next group. Mark on the sheet provided which samples are in which lanes.

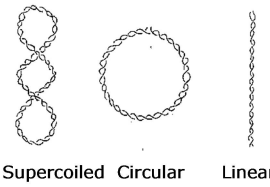
Run then photograph your gel.

4. Interpreting Your Gel

Interpreting the gels showing plasmid DNA is not always straightforward and requires practice. In order to interpret your results, first study the following and read the notes to help you understand.

Representation of an Agarose Gel showing Cut and Uncut Plasmid DNA





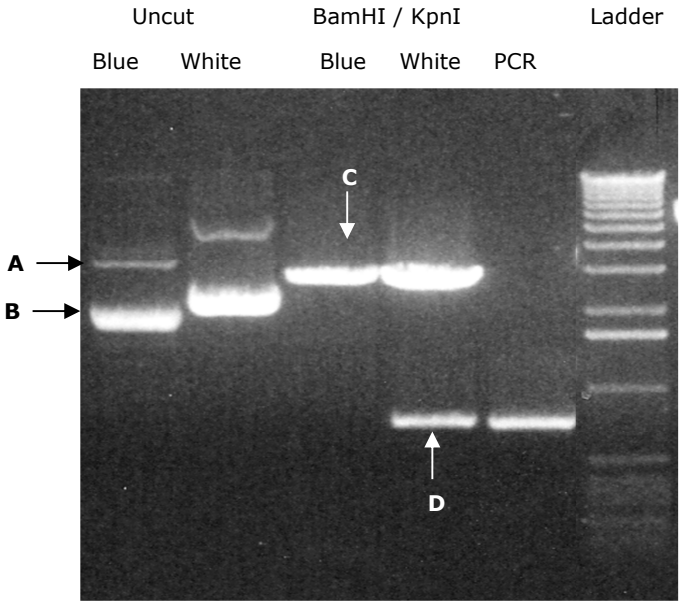
Representation of Plasmid DNA Conformations

(a) **Movement of DNA fragments.** Pieces of DNA, for example, DNA fragments cut by restriction enzymes or PCR products (like the gel you ran on Day 1), are **linear** pieces of DNA. They separate in an agarose gel according to size. The smaller pieces travel the fastest and farthest through the gel matrix. In the 'cut' lane of the above representation of a gel, this shows plasmid DNA that has been cut open with a restriction enzyme and is therefore now in a linear form. It has run as a single band on the gel.

(b) **Conformations of uncut plasmid DNA**. Unlike linear DNA, uncut plasmid DNA has several distinct conformations that can be identified when the uncut plasmid is electrophoresed in an agarose gel.

- **Supercoiled DNA** is the fastest conformation of the uncut plasmid. It can wind up into a compact structure. Impact taking a circle of string and rolling it around in your hands until it forms a little ball. Because of its compact shape, supercoiled DNA is the fastest moving conformation in the gel.
- **Circle DNA** is also called relaxed DNA. This has a more like a floppy circular conformation and therefore is the slowest moving conformation in an agarose gel.

Now, have a look at your own gel and also a similar one shown below, then answer the questions.



The gel opposite shows a typical result. Samples are run against a DNA ladder (remember, this contains **linear DNA fragments of known sizes**).

The first two lanes are uncut plasmids from a blue colony and a white colony, respectively.

The second two lanes show BamHI+KpnI digested plasmids from the blue and white colonies. The white colony plasmid is seen to have an insert of about the same size as the original PCR product in lane 5.

Questions

Answer the following questions using both the gel photograph shown on the previous page and your own gel photograph.

Q2. Why can we not calculate accurate sizes for the **uncut** plasmids from this gel?

Q3. On the gel photograph, why does the "cut" pBluescript plasmid in lane 3 (labelled 'C') run more slowly than the major band of "uncut" pBluescript plasmid in lane 1 (labelled 'B')?

Q4. Why are there multiple bands in the "uncut" pBluescript lane (labelled 'A' and 'B')?

Q5. Following on from the above question, why do we not see multiple bands in the "cut" pBluescript lane?

Q6. Is there evidence for any contamination with *E. coli* chromosomal DNA or with RNA? Where would you expect these nucleic acids to appear on the gel?

Q7. How does the size of the insert (labelled 'D') compare with that of the original PCR product on your gel photo?