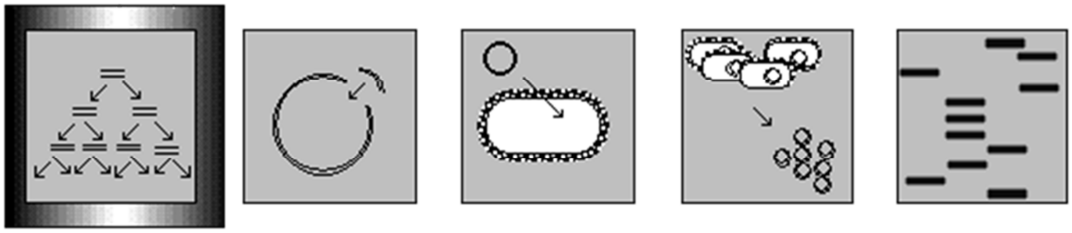
DAY 1 - AGAROSE GEL ELECTROPHORESIS AND DNA CLEANUP



Introduction

Reading: Dale et al., 3rd Ed: p33-34.

If the PCR has worked, you will have millions of copies of the fragment of DNA amplified. The fragment size is predictable from the expected distance spanned by the primers we used in the PCR (see **Exercise 1: PCR and Primer Design** for more information on primer design). So to find out whether we have successfully amplified the target sequence we want, we need to detect the presence of a characteristic size of DNA fragment in our tube. This is accomplished by **running a gel**, and is one of the most everyday actions in a molecular biology lab.

An appropriate DNA ladder (i.e. DNA fragments of known size) should always be run in parallel to enable calculation of the size of your fragments.

Method

1. Preparation of the Agarose Gel

The gel mix will be prepared for you; but the recipe is detailed in Appendix 3, should you subsequently need to make your own. The gel requires approximately an hour to set solid, so should be prepared sufficiently in advance of the time it is required. Make sure the demonstrators have added 'SYBR Safe' to the molten agarose solution. This allows fluorescent staining of your DNA, which you will see later.

- Seal the edges of a gel tray and pour in the molten agarose. If there are any air bubbles push these to the side using a pipette tip (these would distort the running of the gel), add the comb at the top of the gel, and allow to cool to room temperature.
- When the gel has set, flood the gel tank with the TAE buffer (there should be a depth of about 5 mm TAE above the gel). Once you have removed the comb, you are ready to load your samples.

2. Preparation of PCR Samples for Electrophoresis and Running the Gel

In some PCR samples it is necessary to add 'loading buffer' which contains glycerol, and dyes that separate during electrophoresis to monitor the migration of DNA. Fortunately, the Mastermix you used in the PCR already contains a blue and yellow dye (known as 'Green GoTaq® Flexi Buffer'), therefore, there is no need to add additional loading buffer. The yellow dye migrates at a rate faster than primers.

- If you have not loaded a gel before you should ask a demonstrator to show you how and you can also practise loading samples into a well using loading dye.
- You should have your two PCR samples (one containing *Drosophila* genomic DNA, and the other a negative control). Load one sample onto the gel per person – 7μl of each will be sufficient. The demonstrators will load a DNA ladder onto the gel to show you how to load successfully.
- Remember to write your name on the loading sheet so you know where your sample is on the gel.
- Finally check with a demonstrator how to place the safety lid on the gel kit and start it running at around 100 V. The gel will take approx 1 hour to run.

CAUTION: ***potentially lethal voltages. Do not handle gel kits unsupervised, and if you notice a problem in a running gel (like a leak, steam or smoke), seek help but do not touch.***

3. Storing a Sample of your PCR for Future Reference

Place 5µl of your genomic DNA PCR product into a fresh tube (labelled) and place in box at the front of the lab. This will be stored in the freezer so that you can use it as a positive control in an agarose gel on a later day of the lab.

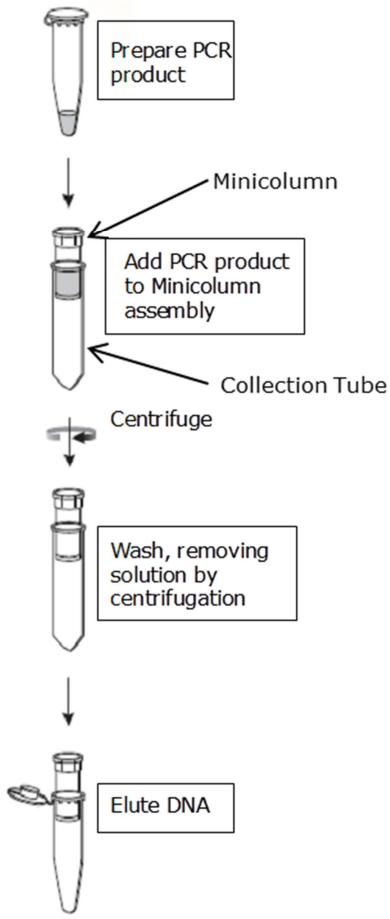
4. DNA Cleanup

We will clean up **only** the **genomic DNA** PCR product, using a commercial DNA cleanup kit. PCR products are commonly purified to remove excess nucleotides and primers. The principle is that DNA sticks strongly but reversibly to silica, which will be present in a Minicolumn. The PCR product is first mixed with a solution of guanidium isothiocyanate, which denatures and destroys any protein (including enzymes) to aid in their removal from the DNA.

1. To prepare the PCR product for the clean-up procedure, add an equal volume of Membrane Binding Solution to the remaining PCR reaction (should be about 38 µl but use a Gilson pipette to estimate the volume).

CAUTION: The guanidium isothiocyanate in the Membrane Binding Solution can denature the proteins in your skin and eyes if it comes into contact with them.

Wear gloves and goggles and work carefully! Splashes of this solution into eyes should be rinsed immediately with plenty of eyewash.



2. Clearly label the minicolumn assembly with a sample identifier and your initials.
3. Using a P200 Gilson pipette, transfer the prepared PCR product to the Minicolumn assembly and incubate for 1 minute at room temperature. Place the samples in the centrifuge and make sure these are balanced correctly in the rotor.

Ask a demonstrator if you're uncertain how to balance a centrifuge.

4. Centrifuge the Minicolumn assembly at maximum speed (13,000rpm) for 2 minutes. Remove the Minicolumn from the assembly and discard the flowthrough in the Collection tube into the disinfectant on your bench. Put the Minicolumn back into the Collection Tube.
5. Wash the column by adding 700 µl of Membrane Wash Solution to the Minicolumn. Centrifuge the Minicolumn assembly for 2 minutes at maximum speed.
6. Empty the Collection Tube as before and place the Minicolumn back in the Collection Tube. Repeat the wash with 500µl of Membrane Wash Solution and centrifuge the Minicolumn assembly for 7 minutes at maximum speed.
7. Remove the Minicolumn assembly from the centrifuge, being careful not to wet the bottom of the column with the flowthrough. Empty the Collection Tube and recentrifuge the column assembly for 2 minutes at maximum speed.

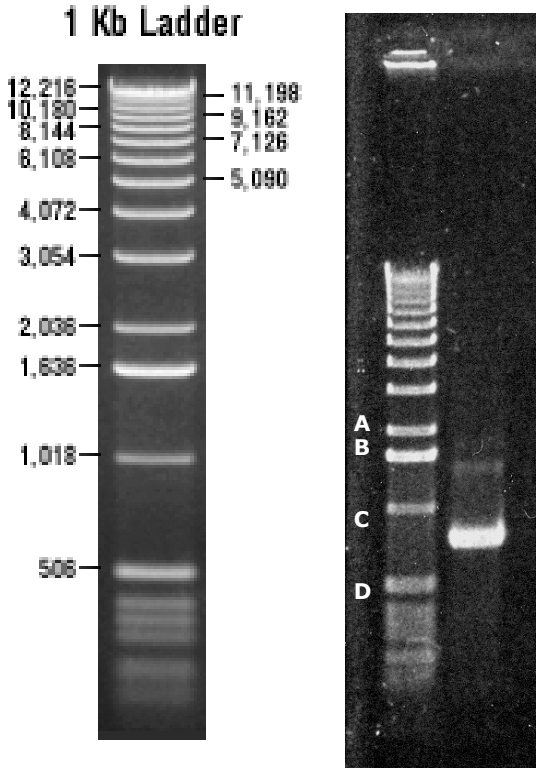
NOTE 1: The most common cause of failure in a DNA cleanup is leaving residual ethanol (contained within the Membrane Wash Solution) in the column at this stage; this may affect further stages of the practical.

NOTE 2: The second most common cause of failure results from not reading the next sentence!

8. Discard the Collection Tube and carefully **transfer** the Minicolumn to a clean 1.5ml microcentrifuge tube (**cut off the lid of the tube**). Add 40 µl* TE buffer directly to the centre of the column using a P200 Gilson (* note that the TE volume for the elution step will vary when you use this technique on later days). Incubate at room temperature for 1 minute. Put the column/tube assembly in the centrifuge and spin for 2 minutes at maximum speed. The DNA should now have **eluted** from the column into the tube.
9. Label the tube and put a lid on. This *should* contain the PCR product you will use for the rest of your labs! You should make sure your PCR product tube is fully labelled with the contents and your initials and then placed in the appropriate place to be stored until next time. Check with a demonstrator if unsure.

5. Interpreting the PCR gel

Once the gel run is complete, switch off the gel kit and carefully remove the gel tray. The demonstrators will help you get an image of the DNA on your gel using a gel imaging system. You should now able to interpret the results of your PCR.

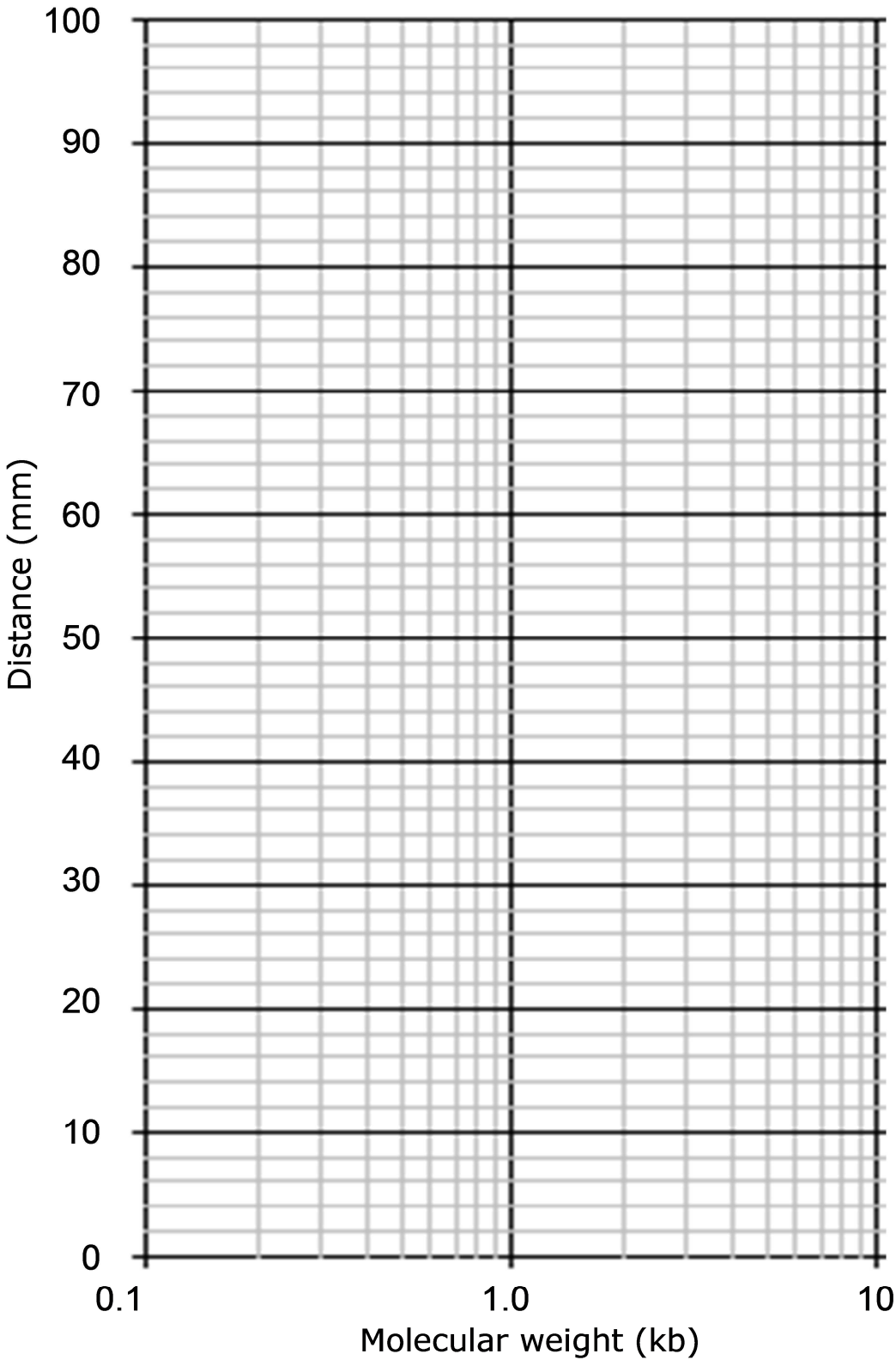


Paste your PCR gel here!

Figure 1 DNA Ladders. Here is a photograph (centre panel) of a successful PCR, showing a product from the primers and template you have used. The ladder, which you will be using today, is the Life Technologies™ 1kb DNA ladder (it's called a 1kb ladder because the high molecular weight fragments increase in size by 1kb increments, although on the 1% agarose gel the fragments >4kb are not well resolved); the fragment sizes in the 1kb ladder are shown in the left panel.

Ladder band sizes in bp (from top):
12216 / 11198 / 10180 / 9162 / 8144 / 7126 / 6108 / 5090 / 4072 / 3054 / 2036 / 1636 / 1018 / 506 / 396 / 344 / 298

- Using the log paper on the next page, plot the migration of the markers (linear, y-axis) against relative molecular weight in kb (logarithmic, x-axis). Depending upon the range of markers that you have used, you should then be able to construct a straight line fit through the data points. Finally, measure the migration of your PCR product and from the graph you have just constructed, estimate the relative molecular weight of the product in kb.


100
90
80
70
60
50
40
30
20
10
0
Distance (mm)
0.1
1.0
10
Molecular weight (kb)

Questions

Q1. What is the purpose of the DNA cleanup stage of the process?

Q2. How "pure" was your reaction? Did the PCR pull out other, spurious bands? Why might this have happened?

Q3. The fragments in the DNA ladder have the following sizes (in base pairs) but all may not be easily resolved: 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298 and some smaller pieces of DNA. Use this information to identify what size bands A-D are. Do the same with your own gel – can you identify the marker sizes?

Q4. We can "eyeball" the product and by comparison with the markers, estimate its size. How does this compare with the predicted size of the product, which you calculated in a previous question?