BACKGROUND TO THE LAB

Drosophila melanogaster, otherwise known as the fruit fly, provides an excellent model organism for studying behaviour. The flies demonstrate complex behaviours, particularly during courtship and mating. Males perform an intricate series of behaviours, including species-specific songs generated by wing vibrations, in order to attract a mate. Virgin females are generally receptive to males, but after mating the females become unreceptive and fend off males who court them. Several mutations have been isolated that affect the courtship behaviour of males and/or females, for example, chaste, dissatisfaction, paralytic and spinster. In this practical you will attempt to discover the molecular basis for an X-linked mutation, icebox, which was first characterized as causing homozygous females to be unreceptive to the advances of males. Males who carry the mutation take longer to complete courtship. By genetic mapping the mutation was localized to a specific region of the Drosophila X chromosome. This chromosomal region contained several genes, including the neuroglian gene (Nrg), which encodes a cell adhesion molecule that plays a role in brain development; thus Nrg was a **candidate gene** for the icebox mutation. To test whether the icebox mutation was indeed an **allele** of Nrg, genetic tests were carried out between icebox and known alleles of Nrg. In other words, if female flies carry the icebox mutation on one X chromosome and an Nrg mutation on the other X chromosome, are they normal in phenotype (which would suggest that icebox is in a different gene from Nrg) or mutant in phenotype (telling us that icebox is an allele of the Nrg gene)? The studies indicated that icebox was indeed an allele of Nrg.

Q1. Why is *Drosophila melanogaster* used as a model organism?

Q2. What is meant by the term 'allele' of a gene?

Q3. What is the difference between the 'phenotype' and 'genotype' of an organism?

The next question is to discover what the exact DNA change is in the *icebox* mutant, and to try to establish how this mutation results in the observed phenotype. Various tools can be used to identify which region of a gene sequence carries a mutation, for example, SSCP (single strand conformation polymorphism) and DGGE (denaturing gradient gel electrophoresis). Analysis of *icebox* DNA indicated one or more differences in the sequence encoding the start of exon 3. This is the region of *Nrg* that you are going to clone and sequence from *icebox* mutant flies, to try to characterize the mutation that leads to the *icebox* phenotype.

If all goes well, you will actually analyse on the last day, the sequence of the **same** piece of DNA you amplified on the first day. However, it would be **really exceptional** for something like this to run without a hitch in the real world (ask one of the demonstrators!), so we will have the relevant materials ready to "fix" you up, if your particular experiment fails at any time. Good luck!

Additional Resources

KMC O'Dell (2003) The voyeurs' guide to *Drosophila melanogaster* courtship. Behavioural Processes **64(2):** 211-223

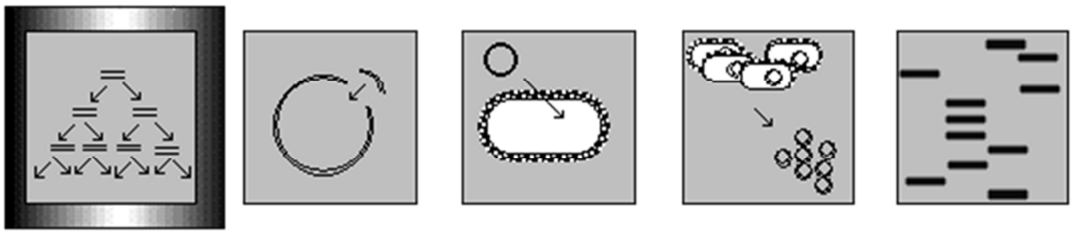
M Hortsch (1996) The L1 family of neural cell adhesion molecules: old proteins performing new tricks. Neuron **17:**587-593

From Genes to Genomes: Concepts and Applications of DNA Technology. Dale, Von Schantz and Plant. Wiley-Blackwell (2012).

An Introduction to Genetic Engineering. Nicholl. Cambridge Press University (2008)

Please note that the above books can be borrowed from Lab 624 in the Boyd Orr overnight.

DAY 1 - PCR



Introduction

Reading: Dale et al., 3rd Ed: p109–119

In this lab, you will set up and analyse a **Polymerase Chain Reaction (PCR).** PCR, invented in 1984, has become essential to almost every aspect of molecular research and clinical diagnosis. The *Drosophila* genome project, like that for humans, is completed and we therefore have access to the complete sequence of the *Nrg* gene from normal flies. We have designed primers for you that will anneal to the sequence either side of our target region and amplify specifically this DNA. You will have a go at designing PCR primers while your reactions are in the PCR machine.

To perform PCR, the following are placed in a plastic eppendorf tube:

- a DNA template (which can be either genomic or cDNA, derived from RNA)
- a FORWARD primer, which complements the reverse strand
- a REVERSE primer, which complements the forward strand
- excess nucleotide monomers ("dNTPs")
- a suitable buffer
- *Taq* DNA polymerase

The ingredients are mixed and placed in a computer-controlled heating block. The reaction runs over approximately 3 hours.

Today, PCR is so widely performed that the basic reagents can be purchased ready-mixed. Your PCR "Master Mix" already contains the dNTPs, buffer and *Taq* polymerase, and so only requires primers and template to be added. It also contains a green dye as a visual aid. You will set up a PCR, and while this is running, you will carry out an exercise on primer design. When the PCR has finished, you will run the products out on a gel, quantify the product and purify the DNA. If the reactions succeed we will save the DNA for next time, when we will attempt to clone it into the plasmid cloning vector pBluescript.

Method

1. Setting up the PCR

Before starting, make sure you know how to dispense liquids **accurately** with a Gilson pipette (Appendix 2)! It is important that you work cleanly, or you will end up doing PCR with *human* genomic DNA as your template!

Each pair should set up **two** PCRs. Make sure you use thin-walled PCR tubes for this:

Tube 1: *Drosophila* genomic DNA template

Tube 2: No DNA negative control (adjust the final volume with **sterile** distilled water as required)

The ingredients and volumes are listed in the table below:

PCR Recipe:		Tube 1	Tube 2
	PCR Master Mix (dNTPs + *Taq* pol + buffer + dye)	25 µl	25 µl
	Template (*Drosophila* genomic DNA)	2 µl	
	Forward primer (10nM)	2 µl	2 µl
	Reverse primer (10nM)	2 µl	2 µl
	Sterile distilled water	19 µl	21 µl
	Final volume	**50 µl**	**50 µl**

Close the tubes, and write your initials on the top of each using a marker pen. Make sure you also distinguish which contains the template and which is the control. Mix the contents by flicking the tube and shake down or spin **very briefly at low speed** (less than 4K for a few seconds only) to collect the contents to the bottom of the tubes. Put the tubes into the PCR machine when you are ready.

2. Running the PCR

Load the tubes into the PCR machine. Your demonstrator will start the reaction, which will last around 3 hours. You can watch the progress of the temperature cycling on the display panel of the PCR machine.

For this reaction, we will use the following programme:

Start		95°C	5 min	to ensure the genomic DNA is fully denatured
Then 5 X	denaturing	95°C	1 min	these 5 cycles have an extended denaturing as the original
	annealing	60°C	30 sec	genomic DNA will take a long time to denature after each
	extension	72°C	1 min	synthesis step
Followed by 25 X	denaturing	95°C	30 sec	in these 25 cycles the denaturing time has been reduced
	annealing	60°C	30 sec	as by now "short", easily denatured PCR products will
	extension	72°C	1 min	represent significant amounts of the available template
And finally		72°C	5 min	to ensure that all products are completely extended

3. Primer Design Exercise

While you are waiting for the PCR to finish, answer the questions at the end of this section and then work through '**Exercise 1: PCR and Primer Design** '. You may find videos within the Molecular Methods app helpful in understanding primer design - http://molecularmethods.clinmed.gla.ac.uk/.

4. After the PCR

We will now:-

A. Run some of the PCR product and the control reaction on an agarose gel to check the success of the PCR.

B. Save 5µl of the PCR product for running on a gel on a later day of the lab.

C. Purify the remaining PCR product from the other reagents, using a commercial DNA clean–up kit.

Each of these steps is described in the next section.

Questions

Q4. The template used in your PCR reaction can either be genomic DNA (gDNA) or complementary DNA (cDNA). What is the difference between these?

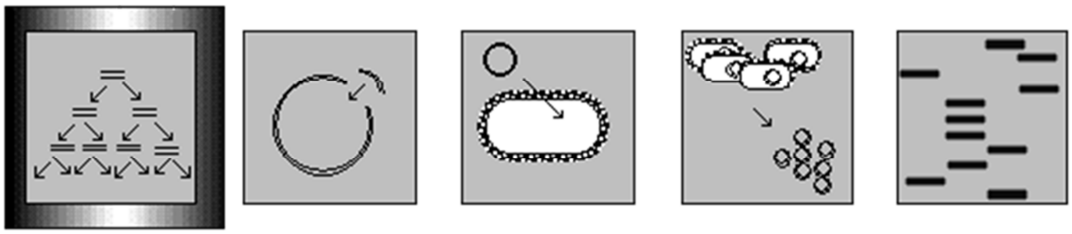
Q5. What does *Taq* DNA polymerase do in a PCR?

Q6. Thin-walled tubes cost ten times as much as conventional eppendorfs. Why do you think they are important for PCR?

Q7. What is the function of the heated lid in the PCR machine?

Q8. Use the sequence and primer information of the gene (see **Exercise 1: PCR and Primer Design**, Figure 2) to calculate the expected size of the PCR fragment.

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 predicted 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

* READ APPENDIX 3 BEFORE DOING THIS PART OF THE LAB.*

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.

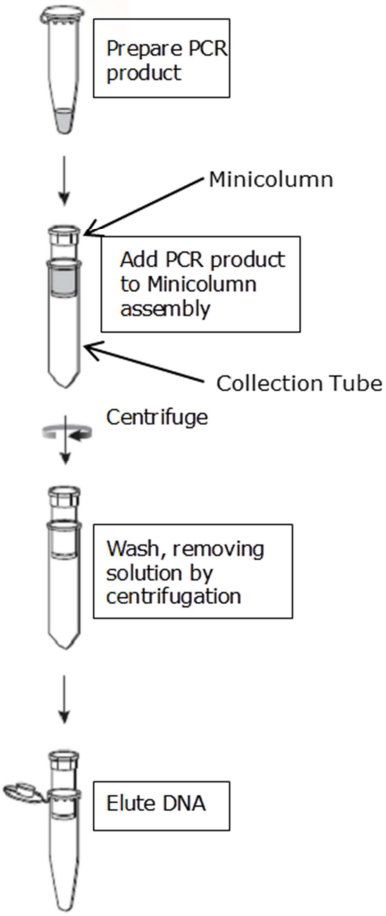
4. DNA Cleanup

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.

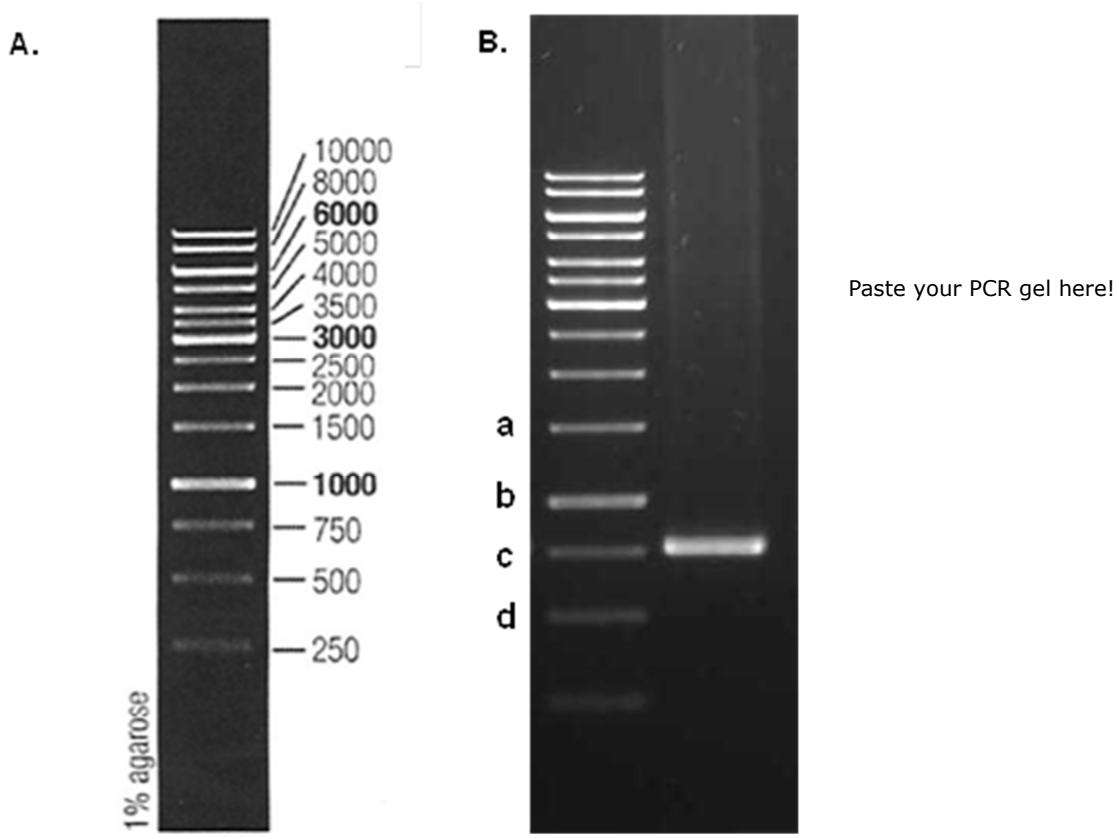
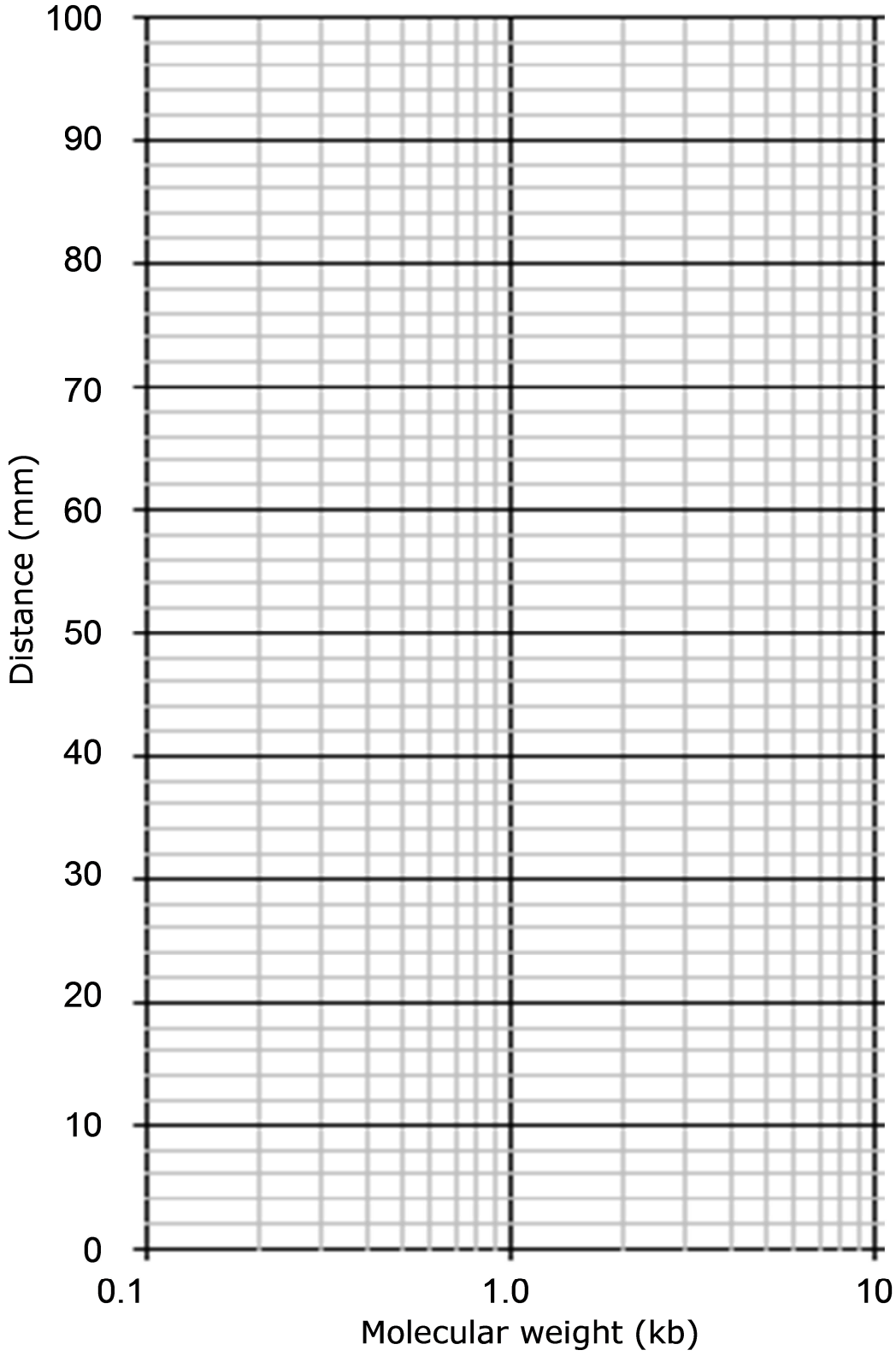


Figure 1. DNA Ladder. (A) The DNA ladder which you are using is the Thermo Scientific 1kb DNA ladder (it is called a 1kb ladder because the high molecular weight fragments increase in size by 1kb increments). The DNA fragment sizes are indicated in the panel. (B) This shows a successful amplification of Drosophila Nrg PCR product from the primers and template you have used.

- 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

Q9. What is the purpose of the DNA cleanup stage of the process?

Q10. How "pure" was your reaction? Did the PCR pull out other, spurious bands? Why might this have happened?

Q11. The fragments in the DNA ladder are indicated in Figure 1A. Use this to identify what size bands a-d are in Figure 1B. Do the same with your own gel – can you identify the marker sizes?

Q12. 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?