BNS2002 Spider ID practicals

Practical 1
26th September 1400-1700 (Group 1)
or 27th September 0900-1200 (Group 2)

Practical 2
28th September 1000-1300 (Group 1)
or 29th September 0900-1200 (Group 2)

Practical 3
5th October 0900-1200 (Group 1)
or 6th October 1000-1300 (Group 2)

Please make sure you come to your timetabled session. There will also be computer-based practical sessions after these lab-based ones – check your timetable for info.

Part 1. Pipetting practise

Rationale:

Proficient pipetting is probably the most crucial part of laboratory work.

- Pipettes are delicate pieces of equipment with high accuracy, which can easily be knocked off their calibration.
- Pipettes can also be the main source of laboratory errors/contamination, and thus can cause a lot of problems.
- Mistakes during pipetting may cause your experiments to fail or to be irreproducible.
- Pipettes are a standard bit of laboratory kit, and familiarity with them is great for your employability.

Purpose:

The purpose of this guide is to train you in the basic techniques of pipetting using a variable automatic micropipette.

Choosing the correct micropipette

Each micropipette has a range of volumes for which it is accurate and reliable. The range should never be exceeded. The range of variable volume pipettes you may encounter include:

	Pipette	Volume(s)	Uses
Small	P2	0.2-2µl	Measurement and transfer of micro-volumes, PCR, DNA sequencing, enzyme-assays
	P10	1-10µl	
Medium	P20	2-20µl	Measurement and transfer of general aqueous solutions, acids and bases
	P100	10-100µl	
	P200	20-200µl	
	P1000	100-1000µl	
Large	P5000	1-5ml	Measurement and transfer of large volumes.
	P10ml	1-10ml	

Setting the desired volume

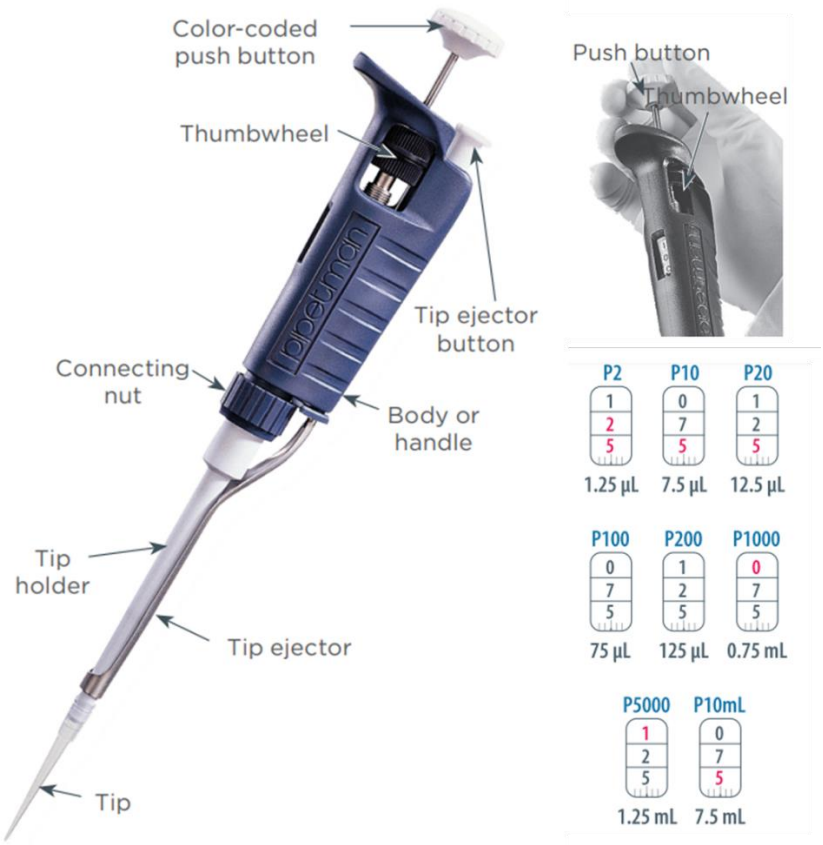
The volume of liquid to be aspirated is indicated in the front display window of the pipette. The numbers are typically coloured black or red to indicate the position of the decimal point. The volume is set by turning the thumbwheel.

To obtain maximum accuracy when setting the volume, proceed as follows:

1. Turn the thumbwheel or the push-button slowly to set volume required
2. Next turn the thumbwheel approximately 1/3 of a turn ‘past’ this volume.
3. Next, slowly turn the thumbwheel back to reach the required volume setting, making sure not to overshoot the required volume mark.

Using this procedure for every setting/resetting ensures a more ‘precise repeatable’ setting of your pipette.

CAUTION: Do not rotate the volume in the display beyond the minimum or maximum level the pipette can deliver. In the best case, this will lead to reduced accuracy and in the worst case could damage the pipette

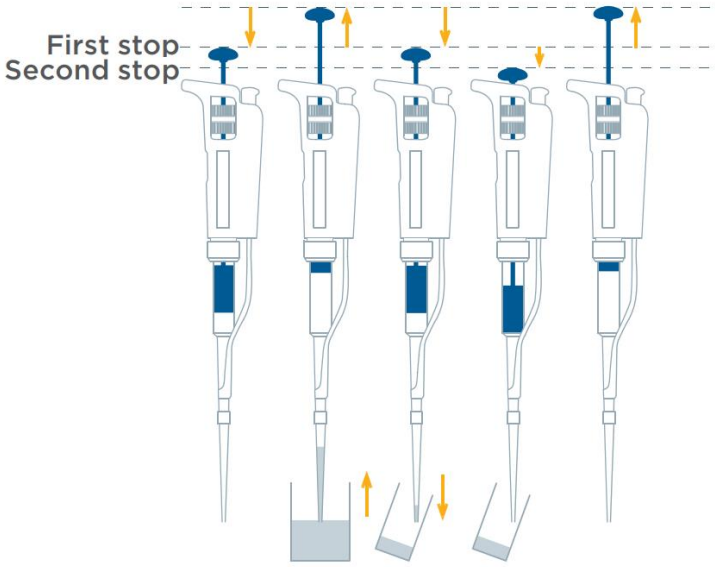


Fitting a tip

To 'correctly' fit a tip onto a pipette place the tip of the pipette, firmly but gently, into the top of a tip. Then use a slight twisting motion to ensure you have a firm and airtight seal. DO NOT 'bang' the pipette into the tips. This may not result in forming a good seal, leading to inaccuracy in aspiration, and may damage very fine tips. When you have finished fitting a tip, close the lid of the tip box – this lessens the chance of contamination.

Aspirating and Dispensing liquid

1. BEFORE immersing the tip into the liquid, you must push out the same amount of air out by pressing the push button to the first stop slowly and smoothly.
2. While still holding the push button down, hold the pipette VERTICALLY, immerse the tip into the liquid, and hold it at a constant depth below the surface. Slowly release the push button so that the pipette will draw the liquid into the tip. As the depth of liquid lowers, so must the tip of the pipette. Once the plunger button has completed been released, hold the pipette in the liquid for one additional second to complete aspiration. Remove the pipette tip from the solution and continue to hold the pipette vertically. You may wipe any droplets from the outside of the tip using the ‘side’ on the liquid container. Take care not to touch the tip’s orifice.
3. To deliver the sample, place the tip against the inside wall of the recipient vessel at a slight angle (100-400). Press the push button slowly and smoothly to the first stop. Wait at least one second as it dispenses.
4. Press the push button further to the second stop to expel any residual liquid from the tip. DO NOT release the button until the tip is out of the vessel to which the liquid was delivered. Keep the press button pushed down as you withdraw the tip from the vessel so as not to re-aspirate any of the transferred liquid.
5. 5. Release the push button slowly and smoothly.



Tip removal

The tip may be ejected from the tip holder by pressing the tip ejector button while holding the pipette tip over a waste container. Tip changes are required only if aspirating a different liquid, sample or reagent. If contamination of the tip is a concern, then a tip change is always appropriate.

Exercise 1

The purpose of this exercise is to give you an opportunity to use a few different sizes of pipettes, and to try to refine your accuracy and precision.

'Blank' the balance with a weigh boat. Using the same weigh boat and in replicates of 5, measure and weigh out the following volumes, remembering to tare (zero) the balance between each replicate. Remember that 1ml of water weighs 1g.

	Volume			
Replicate	1000µl	500µl	250µl	100µl
1				
2				
3				
4				
5				
Mean				
Median				
SD				

Post-class exercise:

Calculate the mean, median, and standard deviation for each volume in Excel or SPSS. How accurate were you? Were you more accurate at higher or lower volumes?

Part 2. The Bradford Assay

The purpose of this exercise is to give you some more opportunities to practice your pipetting, and a chance to get some experience of more techniques and equipment.

The Bradford assay is a simple and accurate procedure for determining the concentration of solubilised protein. It involves the addition of an acidic dye (Coomassie Brilliant Blue G-250) to a protein solution and subsequent measurement at 595nm with a spectrophotometer. The dye turns blue when bound to protein and has a preference for three amino acids (tryptophan, phenylalanine, and tyrosine). Using known concentrations of proteins we can produce a standard curve that allows us to deduce the concentration of unknown proteins. Here you'll practice your pipetting to make a standard curve for bovine serum albumin (BSA).

Spectrophotometer use:

- Place the unit on a clean flat surface which is free from drafts and vibrations.
- The sample chamber lid must be fully closed during measurement and before any readings are recorded.
- All sample containers must be handled with care, by the top, bottom and non-optical surfaces only. Any finger marks evident must be removed.
- Samples and standards should not be stored in open cuvettes or sample containers as evaporation will change the value and lead to staining of the walls which may be irreversible.
- Samples should be allowed to equilibrate to ambient temperature before measurement.
- Cuvettes and sample holders must be filled to a minimum level which covers the light path (all Jenway spectrophotometers have a beam height of 15mm).
- The instrument must be calibrated to zero absorbance/100% transmittance prior to taking readings.

Exercise 2

Calibrating the spectrophotometer

All measurements require calibration to a blank, for maximum accuracy this should be prepared with care using the same deionised water or solvent used for dissolving or diluting the sample.

1. The calibration must be performed at the same wavelength at which the sample will be measured.
2. Turn on the power supply and the unit and allow to warm up for 10 minutes.
3. To select the absorbance measuring mode, use the LEFT or RIGHT arrow keys to move the cursor to the "ABS" indicator on the display window.
4. Adjust the wavelength to 595nm using the UP and DOWN arrow keys.
5. Insert a cuvette containing the blank (clear) solution into the sample chamber and close the instrument lid. Press the CAL key. The instrument will perform a zero % transmission calibration followed by a 0.000 Absorbance calibration. Once the calibration has been performed a sample can be measured.

Bradford Assay Method

To prepare your standard curves, take 5 tubes and label them BSA1 to BSA5.

1. Prepare dilutions of the BSA solution in the tubes as indicated in Table 1 below.
2. Add 500µl Bradford Solution to each tube and mix thoroughly but carefully with the vortex mixer.
3. Incubate for 5 minutes, then transfer the solution to a 3ml cuvette
4. Set the wavelength of the photometer to 595nm, calibrate using Tube 1 mixture.
5. Take the absorbance at 595nm for all samples and record your data in Table 1.

Table 1. Protein standard dilutions and results.

Tube	BSA (µl)	PBS (µl)	Bradford Solution (µl)	Protein dilution factor	Protein Concentration (µg/µl)	BSA absorption @ 595nm (au)
1	0	2500	500			
2	5	2495	500			
3	15	2485	500			
4	25	2475	500			
5	35	2465	500			

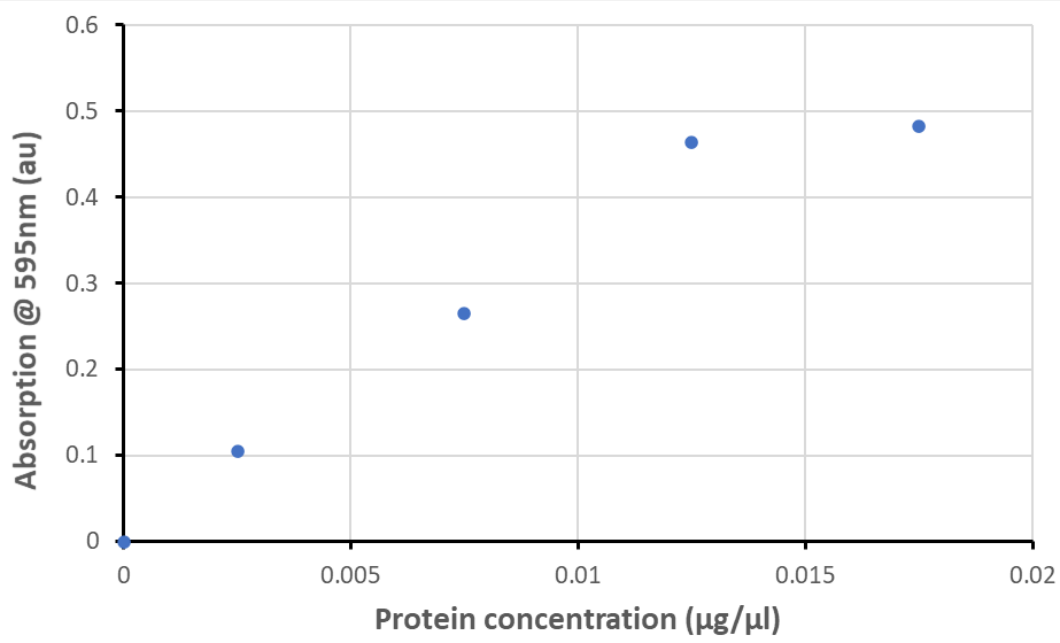
Notes:

- The initial concentration of the standard solution is 1.5µg/µl.
- Dilution factor = Final volume (V_2) ÷ Initial volume (V_1)
  e.g. if you take 100µl of solution A and mix it with 200µl of solution B

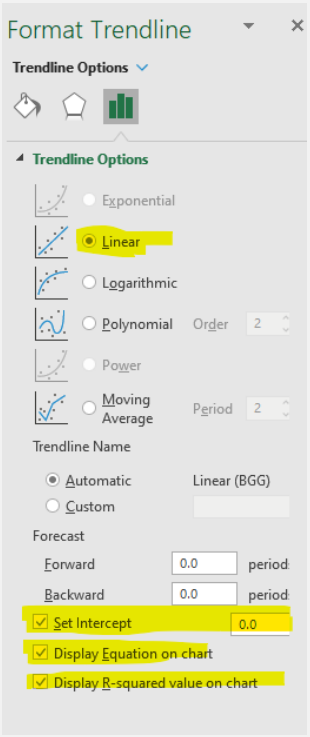
  Dilution Factor = (200+100) ÷100
  = 300 ÷100
  = **3**

  Therefore you would have performed a 1:3 dilution of solution A

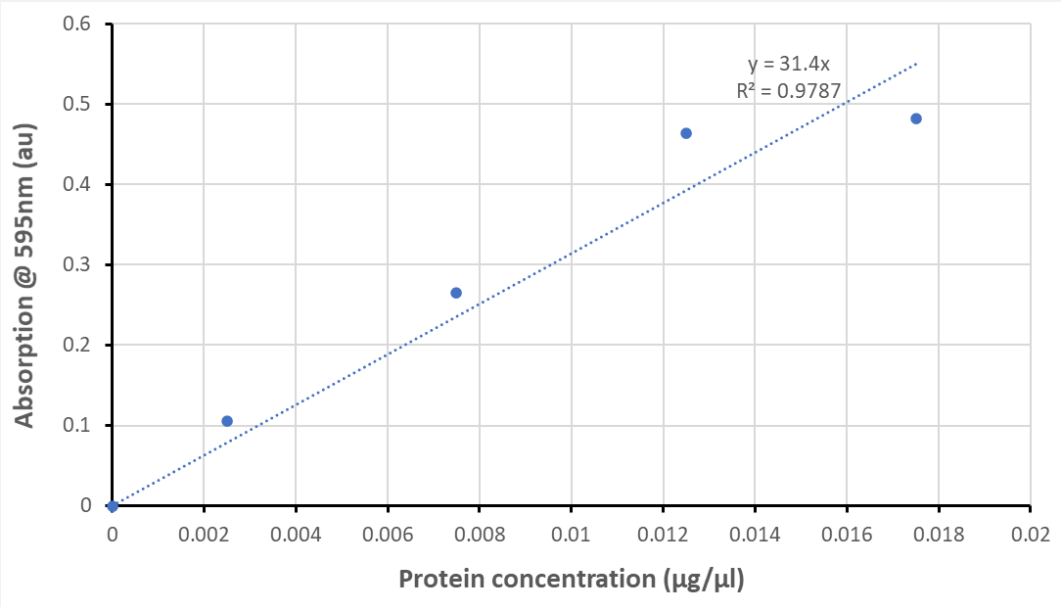
Post-class exercise:
Make a scatterplot with absorption on the Y axis and protein concentration on the X axis. Don't forget to include units. I've used Excel, but you're welcome to use SPSS or R if you prefer.



Right click on one of the data points, and select 'Add trendline' to add a linear trendline. Set the intersect at 0 so your trendline passes through 0, and select the options to add the equation and R^2 values to the chart.



Your graph should now look something like this (remember you can reposition and resize things to make them easier for the reader to see):



The R^2 value tells us how good a fit the line is to our data. It's basically a percentage scale, so higher values are what we're after (that's very simplistic, but will do us for now). Mine are coming in at around 98%, which isn't too bad. How good a fit is your trendline? If your pipetting and experimental technique have been accurate and precise, you should get a pretty good fit.

Part 3. DNA extraction and PCR

Rationale:

Identifying species can be difficult. In this practical, you will see how DNA, which is present in all cells and tissues in all life stages can be used to identify species, especially where we have agreed upon standard sequences for comparison and where extensive databases exist for comparison.

Purpose:

This practical will give you hands on experience of standard molecular biology techniques, and an opportunity to generate data for computational analysis.

We'll be using spin column-based techniques to extract DNA from a preserved arthropod specimen. The first (lysis) step involves a solution of chaotropic salt and detergents that destabilises hydrogen bonds and other intramolecular interactions. This denatures proteins (including those that would degrade DNA) and solubilises lipids in cell membranes, so the DNA is released from the cells in the sample. We often add a proteinase to help degrade proteins more quickly, especially if tissues have a tough proteinaceous components. The salts, together with ethanol, help DNA to bind to a silica membrane, which is then washed to remove proteins and polysaccharides, and finally to remove the salts. Some pigments may remain on the column, but these tend not to interfere with downstream procedures. Residual ethanol can interfere with things like PCR though, so it's important to remove this before releasing the DNA from the silica membrane in the elution step.

You've been provided with a spider leg, preserved in ethanol. Using just a leg like this means we don't have to kill spiders to ID them. We use ethanol as a preservative, as it kills microorganisms that would otherwise degrade the specimen, removes water from the tissues (slowing down enzymatic processes), and it denatures DNA and DNA-degrading enzymes.

You'll be extracting DNA with Qiagen DNeasy blood and tissue kit, using the supplementary protocol for insect samples. Make sure your tubes are clearly labelled, as you'll need to be able to keep track of them through the protocol.

DNA extraction

Step	What to do	Tick when done
1	Use forceps to transfer your spider leg to a fresh tube, trying to take as little ethanol with it as possible	
2	Add 180µl of buffer ATL to the leg sample	
3	Use a micropestle to grind the tissue into smaller pieces, or at least break it up a bit	
4	Add 20µl of Proteinase K to your sample	
5	Incubate at 56°C for ten minutes. Mix by occasional inversion or gentle vortexing during the incubation.	
6	During the incubation step, mix 200µl of buffer AL with 200µl of 100% ethanol in a fresh 1.5ml tube	
7	After lysis, vortex your samples for 15 seconds, then add your AL/ethanol mix to the sample and vortex again	
8	Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube	
9	Centrifuge at 8000rpm for 1 minute.	

10	Discard the flow-through and the collection tube into your waste pot, but keep the top bit that you put your sample in	
11	Place the DNeasy Mini spin column in a new 2 ml collection tube	
12	Add 500µl Buffer **AW1** to wash the membrane	
13	Centrifuge for 1 minute at 8000 rpm. Discard flow-through and collection tube.	
14	Place the DNeasy Mini spin column in a new 2 ml collection tube	
15	Add 500µl Buffer **AW2** to wash the membrane	
16	Centrifuge for 3 minutes at 13,000 rpm to dry the DNeasy membrane. Discard flow-through and collection tube.*	
17	Place the DNeasy Mini spin column into a clean, **labelled** 1.5 ml microcentrifuge tube	
18	Pipet 100µl Buffer AE onto the DNeasy membrane (*without touching the membrane*).	
19	Incubate at room temperature for 1 minute	
20	Centrifuge for 1 minute at 8000 rpm to elute. If the centrifuge is full, you don't have to worry too much about the lid, but if not, make sure you find the arrow that shows which way the rotor spins, and align your tube so that the lid is going with the spin rather than against it. Ask a demonstrator for help if you are not sure.	
21	Discard the spin column, and keep your labelled tube that now contains your DNA.	

*It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol will interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column **carefully** so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol

Kary Mullis developed PCR in 1983, whilst working at the Cetus Corporation. In doing so, he revolutionised molecular biology and won a Nobel Prize. PCR produces exponentially large amounts of a specific piece of DNA from trace amounts of starting material (template). The template can be any form of double-stranded DNA, such as genomic DNA and, in theory, only a single template strand is needed to generate millions of new DNA molecules.

PCR makes use of two basic processes:

1. Complementary DNA strand hybridization
2. DNA strand synthesis via DNA polymerase

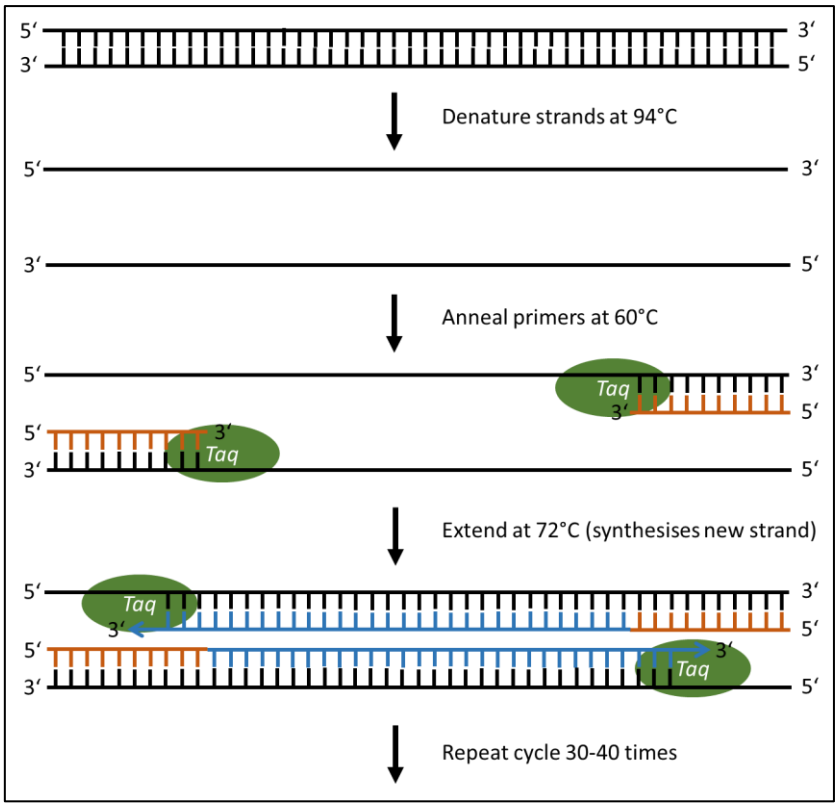
In the case of PCR, complementary strand hybridization takes place when two different oligonucleotide primers anneal to each of their respective complementary base pair sequences on the template. The two primers are designed with a specific sequence of nucleotides such that they can anneal at the opposite ends and on the opposite strands of the stretch of double-stranded DNA (template strand) to be amplified. Primers are complimentary to the up- and downstream regions of the sequence to be amplified, so they stick, or anneal, to those regions. Primers are needed because DNA polymerases can only add nucleotides to the end of a pre-existing chain.
The DNA polymerase must be a thermally stable polymerase because the polymerase chain reaction cycles between temperatures of 60°C and 94°C. The thermostable DNA polymerase (*Taq*) used in PCR was isolated from a thermophilic bacterium, *Thermus aquaticus*, which lives in high-temperature steam vents such as those found in Yellowstone National Park.

Following sample preparation, the template DNA, oligonucleotide primers, thermostable DNA polymerase (*Taq*), the four deoxynucleotides (A, T, G, C), and reaction buffer are mixed in a single microcentrifuge tube. The tube is placed into a thermal cycler – a machine containing an aluminum block that holds the samples and can be rapidly heated and cooled across extreme temperature differences.

PCR involves a repetitive series of cycles, each of which traditionally consists of three steps:

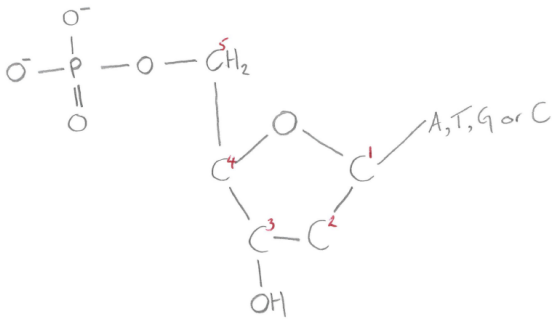
1. Template denaturation
2. Primer annealing
3. Extension

In the first step, the sample is heated to 94°C. At this temperature, the template strands separate (denature) and this is therefore called the **denaturation** step. The thermal cycler then rapidly cools to typically somewhere between 50-60°C to allow the primers to anneal to the separated template strands. This is called the **annealing** step. The two original template strands may reanneal to each other or compete with the primers for the primers complementary binding sites. However, the primers are added in excess such that the primers actually out-compete the original DNA strands for the primers' complementary binding sites. Lastly, the thermal cycler heats the sample to 72°C for *Taq* DNA polymerase to extend the primers and make complete copies of each template DNA strand (*Taq* polymerase works most efficiently at this temperature). This is called the **extension** step. Two new copies of each complementary strand are created and there are now two sets of double-stranded DNA (dsDNA). These two sets of dsDNA can be used for another cycle and subsequent strand synthesis.



Two new template strands are created from the original double-stranded template on each complete cycle of the strand synthesis reaction. This causes exponential growth of the number of template molecules, i.e., the number of DNA strands doubles at each cycle. Therefore, after 30 cycles there will be 2^{30} (over 1 billion) times more copies than at the beginning. PCR generates DNA of a precise length and sequence. On the first cycle, the two primers anneal to the original genomic template DNA strands at opposite ends and on opposite strands. After the first complete temperature cycle, two new strands are generated that are shorter than the original template strands but still longer than the length of the DNA that we want to amplify. It isn't until the third thermal cycle that fragments of the precise length are generated.

DNA is only synthesised in the 5' to 3' direction. For those of you unfamiliar with these terms, they refer to the numbering of the carbon atoms in the individual sugar molecules that make up the backbone of DNA. These sugars bond to each other via the third and fifth carbon atoms, so there is always a “free” carbon #5 at one end of a DNA strand, and a “free” carbon #3 on the other.



You’ll be using standard insect/spider DNA barcoding primers to amplify a 710bp fragment of the Cytochrome C Oxidase Subunit I (COX1, COI) gene: LepF1 (5’-ATTCAACCAATCATAAAGATATTGG-3’) and LepR1 (5’-TAAACTTCTGGATGTCCAAAAAATCA-3’). These primers were designed for Lepidoptera, as described in the below publication, but work for a much wider variety of taxa.

Hebert, P.D., Penton, E.H., Burns, J.M., Janzen, D.H. and Hallwachs, W., 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences*, *101*(41), pp.14812-14817.

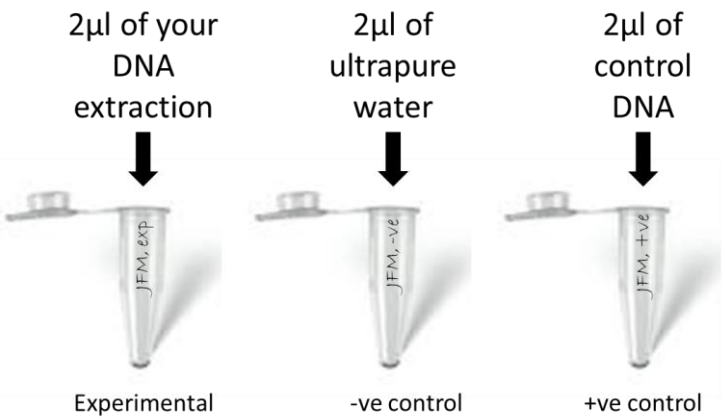
PCR is very sensitive, so it is important to consider suitable controls. A negative control would be a no template control, where you add water instead of your extracted DNA. If this works, you have some sort of contamination in your reagents. A positive control would be the use of some DNA that you know works with your primers and reaction conditions. If this doesn’t work, you’ve set something up wrong.

PCR

You have some 0.2ml PCR tubes on your bench, and you will need <u>three</u> of them. Label each one carefully on the side as *either* your experiment, your +ve control, or your -ve control. Add your initials or some other identifier, as they will get pooled into the PCR thermocycler with lots of other tubes.

Using the appropriate pipette, transfer 2µl of either your extracted DNA sample, ultrapure water, or positive control DNA to the appropriate tube. Try to pipette this into the bottom of the tube, and watch what you are doing very carefully, as static charges from your hands and gloves can make these small volumes of liquid move around. Cap the tube and carefully flick it to ensure the liquid is in the bottom of the tube.

Leave the tube on your bench for your demonstrator to collect and add the DNA polymerase, primers, dNTPs etc.



Part 4. Agarose gel electrophoresis

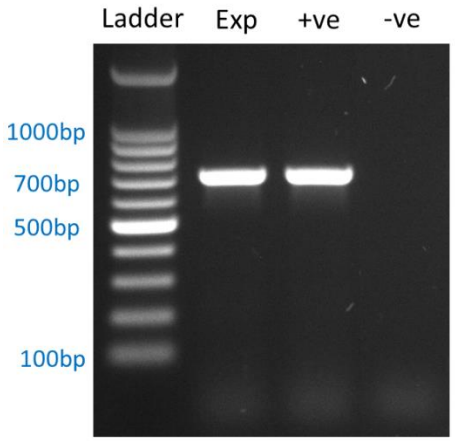
Rationale:
Electrophoresis will enable you to see if your DNA extractions and PCR worked and will (hopefully) give you a nice image to include in your report.

Purpose:
This practical will give you hands on experience of standard molecular biology techniques, and an opportunity to put your pipetting skills to good use.

Electrophoresis separates DNA fragments according to their relative size, based on their migration through an agarose gel matrix submerged in a conductive buffer solution. When an electrical current is passed through the buffer and gel, the DNA moves towards the positive pole as it has an overall negative charge. The gel matrix acts as a molecular sieve, with small fragments moving through it more easily than large ones, and fragments of the same size migrating together to produce distinct bands.

You'll run your experimental PCRs alongside your positive and negative controls on a 1.5% TAE agarose gel. The percentage of the gel determines how good the resolution is, with lower percentages of agarose better for large fragments, and high percentages for large fragments. The TAE (Tris-acetate-EDTA) buffer maintains pH, and the EDTA chelates divalent cations, which helps to prevent DNA degradation.

You'll also load a DNA ladder, which is made up of lots of DNA fragments of know size. By comparing your PCR product to the ladder you can roughly determine how big your product is, and whether it matches your expected size (710bp in this case). Your results should look like the example below.



You'll need to work as a group to make a gel for your bench. Make sure you share the work, and that everyone gets to see what is going on.

Preparing the gel

Collect your samples from your demonstrator. The PCR master mix we used has a red loading dye in it. As well as letting you see how far your sample has travelled, this helps it to sink into the gel so that it doesn't end up floating around the tank. This is why your samples are red.

To prepare the gel:

1. Weigh out sufficient agarose powder to make 150ml of 1.5% agarose in TAE buffer. You have 50x TAE stock, so you will need to dilute it to a 1x solution to make your gel.

2. *Carefully* heat the solution in the microwave until the power has dissolved. Try not to let it boil too much as you will lose volume. Also watch for superboiling. Take care when handling the flasks as they will be very hot.

3. Prepare your gel tray – ask your demonstrator if you are unsure.

4. When your solution has cooled enough to be held comfortably in your hand, add 10µl of SafeView Nucleic Acid stain and mix by *gently* swirling the flask.

5. Gently pour your agarose solution into the gel tray. Start in one corner and pour in a slow continuous movement to avoid bubbles. If there are any bubbles, use a pipette tip to move them to the side of the tray. Don't forget to put the comb in.

6. Leave the gel to set.

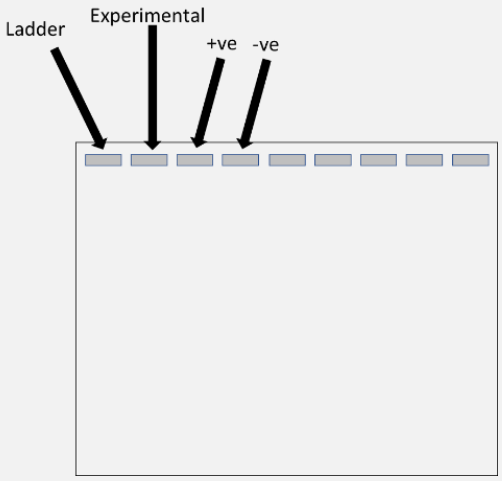
7. Place your gel in the gel tank, and cover it with 1x TAE, making sure not to go above the max fill line.

8. Remove the comb, taking care not to tear the gel.

Once the gel is ready, you'll need to load your samples. Try to be reasonably quick with this bit, as we need to get them running as soon as possible.

Loading your samples

1. Pipette 10µl of ladder into the first well. Then pipette 10µl of your experimental sample, your +ve control, and your -ve control into the next three wells.



Once everyone on your bench has loaded their samples, ask a demonstrator to help you get the gel running. At the end of the session we'll take some pictures of the gels for you to include in your report. Bring a USB stick with you to save your images.