Using “DNA Barcoding” to Help with the Taxonomical Gap

Laboratory Handbook

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1. Experimental Context

1.1 TEACHING OBJECTIVES

The purpose of this laboratory practical is to introduce you to the basic techniques of DNA analysis. The techniques that you will use include:

1) DNA extraction/isolation from Eukaryotic tissues using a standard protocol
2) PCR techniques targeting common gene markers used in animal barcoding studies
3) DNA quantification and quality check using spectrophotometry
4) PCR product visualisation techniques (gel electrophoresis)
5) Data analysis of sequenced PCR products

Particular attention should be paid to the following points:

[1] The manual should be used as a guide to assist you with the experiments rather than as a complete recipe book.

[2] There are certain safety requirements (in addition to those appropriate to normal laboratory practice) that must be adhered to (see next page). **It is a requirement that you read the section on safety before commencing any work**.

2. Timetable

Practical sessions will be held in the DCV laboratory located at the ground floor at the Medicine Faculty Building. The bioinformatic analysis can be conducted online and guidance can be provided after sequencing is done. Sessions will start promptly at the defined schedule. If you cannot attend any part of the practical, please inform the lecturer as soon as possible. The timetable is given below in figure 1. The following activities will be developed:

Activity 1: **DNA extraction and isolation (Eukaryotic tissues) and DNA quality check;**
Activity 2: **PCR preparation and run;**
Activity 3: **PCR product visualisation using Gel Electrophoresis;**
Activity 4 (Facultative): **Bioinformatic analysis –** Sequence curation, blast, alignment and species determination.

Figure 1. Timetable for laboratory practical on the molecular identification of collected specimens (highlighted in yellow).

	Day 6 (9/10/23)	Day 7 (10/10/23)	Day 8 (11/10/23)	Day 9 (12/10/23)	Day 10 (13/10/23)
Morning	***9h30 – Invertebrate identification*** Laboratory practical: - *Identification of the collected material using taxonomic (morphological) methods*	***9h30 – Flower Biology and Pollination Ecology*** Laboratory practical: - *Flower biology and breeding systems* - *Pollinator processing and identification*	***9h30 – Soil functions and plant-soil interactions*** Short lecture & Field practical: - *Measuring soil respiration and carbon balance* - *Assessing water infiltration and soil compaction* - *Assessing soil fauna feeding activity in situ*	***9h30 – Molecular techniques in ecology*** Laboratory practical: - *Identification of the collected material using molecular methods (barcoding) – part 2*	***9h30 - Methods and techniques on Vertebrates – Data processing and Data analysis*** Data analysis practical: - *Data processing of audio recordings* - *Data processing of camera trap photos and videos*
Lunch	12h30 – 14h00	12h30 – 14h00	12h30 – 14h00	12h30 – 14h00	12h30 – 14h00
Afternoon	***14h00 – Invertebrate identification*** Laboratory practical (cont) -	***14h00 – Flower Biology and Pollination Ecology*** Field practical: - *Pollinator sampling methods* ***15h00 – Flower Biology and Pollination Ecology*** Data analysis practical: - *Assessing breeding systems using genetic data*	***14h00 – Molecular techniques in ecology*** Laboratory practical: - *Identification of the collected material using molecular methods (barcoding) – part 1*	***14h00 – Molecular techniques in ecology*** Laboratory practical: - *Identification of the collected material using molecular methods (barcoding) – part 3*	***14h00 - Methods and techniques on Vertebrates – Data processing and Data analysis*** Data analysis practical (cont.)

3. Experimental Overview, "*DNA barcoding*"

You have collected a set of sample/s of edaphic invertebrates using a variety of sampling techniques at Monte Barata Field Station. Edaphic fauna are ideal organisms for terrestrial ecosystem monitoring. On the one hand, they are significant contributors to the biomass and fertility of many temperate soils. On the other hand, their lifestyles and body structure render them susceptible to human disturbance and poisoning. You should have recorded the metadata about your specimen (label, date, position) and kept your specimen in ethanol for further processing. Your challenge is determining the genetic identity (when possible) of your collected animals using standard gene barcoding techniques. You will be expected to determine your sample's potential species name (or at least the closest relative).

A reliable recovery of the mitochondrial marker, the **cytochrome oxidase subunit 1 (COI)** gene is critical for the identification of a given sample. In this study, you will be able to use a set of universal primers for increased efficiency of barcode recovery in a variety of different invertebrate species. You will then use the barcode sequence to recover an identification using sequence information available in an open data repository (e.g. NCBI). The laboratory practical you will undertake is based on an extension of these principles.

3.1 Laboratory Practice and Molecular Biology

(i) PURPOSE

To provide procedures that will optimise the results generated by molecular biological methods.

(ii) SCOPE

Procedures are designed to protect both your sample, the enzymes/reagents being used and minimise the cost of the experiments.

(iii) RESPONSIBILITIES

All glassware and solutions should be autoclaved before use.

When work is finished, waste should be autoclaved and disposed of through the waste management system.

Gloves and lab coat should be worn at all times.

(IV) METHOD

Essentially, the basic techniques of DNA extraction are straightforward providing that certain protocols are followed. These practical points are important if you are to obtain successful results.

[1] Nucleases are ubiquitous, and some are extremely stable enzymes. It is vital that these enzymes do not come in contact with your samples. Therefore, apparatus (eppendorfs and tips etc) and solutions that come in contact with your samples must be sterilised. Furthermore, always wear disposable gloves - the skin is an excellent source of nucleases, and there is enough enzyme in a fingerprint to ruin your experiments.

[2] Experiments must be carried out using good laboratory practice. This means that the normal safety precautions applicable to the handling of animals' tissues apply to the experiments described in this manual. Furthermore, all media and apparatus used for handling tissues should be sterile.

[3] Assume all your nucleic acid samples and the modification enzyme that are heat labile and keep them on ice unless indicated otherwise by protocols.

[4] The key to many of the techniques described in this manual is the ability to pipette small volumes carefully and accurately. It is essential when pipetting small volumes to ensure that the tip of the pipette is touching the surface of the container before expelling the solution. Always use clean pipette tips to avoid cross-contamination. Pulse spin Eppendorfs to ensure that all of the added components of reaction mixtures are mixed at the bottom of the tube.

[5] The transilluminator should be used for the minimum time possible as prolonged exposure of DNA samples to UV radiation causes random strand breakage - also the lamp has a finite life. Please avoid scratching the surface of the transilluminator as this will destroy the effectiveness of the filter - a new transilluminator costs more than €1500.

A general point about DNA work is that reagents are extremely expensive. Please use reagents/enzymes as sparingly as possible.

3.2 Notes About Preparing Materials for Use with DNA

(i) PURPOSE

To ensure that the environment, equipment and solutions do not contain contaminating nucleases that may attack your samples.

(ii) SCOPE

Nucleases retain the majority of their activity when heated at 95°C for 10 minutes. Therefore, treatment of materials for use with DNA/RNA must be more stringent. See below for preparation of materials for work with DNA and RNA.

(iii) RESPONSIBILITIES

- Good laboratory practice needed (e.g., **NO FOOD is allowed in the lab at any point**).
- The working area must be clean during work and maintained accordingly. Try and leave the laboratory space a little better than you have found it.
- **No mobile phones on benches and completely avoid using it during the practical work.**
- In case you need to record some data with your phone, ask first.
- Wear a lab coat and gloves to protect DNA from nucleases
- Waste disposables and solutions in autoclave bags

(iv) EQUIPMENT REQUIRED

Autoclave (operated by a specialized technician)

(vi) METHOD

Sterilisation (this will be done for you)

Preparation for DNA

All solutions, glassware and water used with DNA should be autoclaved before use. However, solutions and equipment used to prepare an agarose gel (see agarose gel electrophoresis) for analysis does not require sterilisation. All surfaces should be wiped down with 70% Ethanol. Lab coat and gloves must be worn at all times.

3.3 Genomic DNA isolation (animal tissues) – Activity 1

(i) PURPOSE

The purification of genomic DNA (nuclear and mitochondrial) from eukaryotic tissue.

(ii) SCOPE

DNA purified by this method is appropriate as a target for amplification of DNA barcodes but can also be used to a variety of other applications.

(iii) RESPONSIBILITIES

- Ensure all plasticware (tubes and tips) used are free of nucleases, and solution have been sterilised.
- Perform all centrifugation steps at room temperature (15–25°C).
- Re-dissolve any precipitates in **Buffer NL and Buffer NT1**.
- Add ethanol to Buffer NW1, and Buffer NW2 concentrates (**this has been performed**).
- Equilibrate frozen tissue or cell pellets to room temperature.

(i) EQUIPMENT REQUIRED

Microfuge (Eppendorf centrifuge)

Preheat an incubator to 56°C.

Vortex

(v) MATERIALS/SOLUTIONS REQUIRED

NZY Tissue gDNA Isolation kit Contains:

Component	50 columns	200 columns
Buffer NT1	20 mL	80 mL
Buffer NL	15 mL	60 mL
Buffer NW1	30 mL	120 mL
Buffer NW2 (concentrate)	2 x 7 mL	2 x 25 mL
Buffer NE	15 mL	60 mL
Proteinase K (lyophilized)	30 mg	4 x 30 mg
Proteinase buffer	1.8 mL	7 mL
NZYSpin Tissue columns (light green ring)	50	200
Collection tubes (2 mL)	100	400

(vi) METHOD

You will use a small piece of tissue 1-3mm^3 (stored in 96-100% ethanol) or full body depending on specimen size. Remove ALL excess ethanol by wiping the desired section over clean blue roll paper. The animal tissue you have to cut consists in 3mm^3.

[1] Add 180 µl of Buffer **NT1** to a clean Eppendorf tube, and only then add the tissue material. Add 25 µl proteinase K, mix by vortexing, and incubate at 56°C until completely lysed (usually takes 1-3 hours, **we will wait 1.5 hour**). Vortex occasionally during incubation. Vortex 15s directly before proceeding to step 2. If tissue is still visible, spin it down using a centrifuge (1 min at 1000 g) and carefully move the supernatant to a clean tube before proceeding with next step (without touching the debris pellet).

[2] Add 200 µl **Buffer NL**. Mix thoroughly by vortexing.

[3] Add 210 µl **ethanol (96–100%)**. Mix thoroughly by vortexing and incubate for a further 10 mins.

[4] Pipet the mixture into a spin column placed in a 2 ml collection tube (the empty tubes provided with the extraction kit). Centrifuge at ≥ 11 000 g x for 1 min. Discard the flow-through and collection tube.

[5] Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer NW1. Centrifuge for 1 min at ≥11 000 x g. Discard the flow-through and collection tube.

[6] Place the spin column in a new 2 ml collection tube, add 600 µl Buffer NW2, and centrifuge for 3 min at 11 000 x g. Discard the flow-through and collection tube.

[7] Transfer the spin column to a new 1.5 ml or 2 ml sterile Eppendorf tube.

[8] Elute the DNA by adding 40 µl Buffer NE to the centre of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥11 000 x g. The DNA is now eluted from the column. Discard the spin column and label the Eppendorf. You successfully purified your DNA sample.

For each sample of DNA extraction, Nanodrop procedure can be performed to observe the success of the respective DNA extraction and any evidence of contamination. Use your DNA elution buffer as blank (necessary to calibrate the machine). Use 1.2µl of each sample to do the measurement. Ideally, both ratios (260/280 and 260/230) should fall within the range of 1.7 and 2.0.

3.4 PCR for DNA Barcoding – Activity 2

(i) PURPOSE

Amplification of specific DNA sequences can be used to phylogenetically characterise an organism. Although many different loci may be considered, the most common are the ribosomal 16S for bacteria and mitochondrial COI and COII for eukaryotes. The general methodology is compatible with amplification of other targets given appropriate primers flanking the fragment of interest and optimising the annealing temperature to the Tm of the primers and allowing an extension time of ~1 min for every kb of the target amplicon.

(ii) SCOPE

The thermostable enzyme Taq DNA Polymerase is a protein of approximately 94kDa isolated from *Thermus aquaticus*. This enzyme replicates DNA at 74°C, and exhibits a half-life of 40 minutes at 95°C. The enzyme catalyses the polymerisation of nucleotides into duplex DNA in the 5´ -> 3´ direction in the presence of magnesium and also possesses a 3´ -> 5´ exonuclease activity. In this practical, you will be preparing your PCR using a ready to go mastermix. However, keep in mind that in your research (e.g. your final year research project) you might need individual PCR components (*Taq* DNA polymerase, nucleotides mix, Magnesium chloride), so you can tweak the reaction conditions. In this case, the PCR conditions have been optimised and a NZYTaq II 2× Green Master Mix can be used for your benefit.

(iii) EQUIPMENT REQUIRED

PCR Machine (heated lid)

(IV) MATERIALS/SOLUTIONS REQUIRED

You will need:

Template DNA (your DNA extracts)

Supreme NZYTaq II 2× Green Master Mix

COI Universal primers for invertebrates at 10 µM stock (check Table 1).

Sterile Materials:

- 1ml, 200 µl and 10 µl tips
- 1.5ml eppendorfs
- Sterile H_2O
- 0.2 ml thin wall PCR tubes

Table 1. Primers used in the cocktail for fish identification (using COI as gene marker).

Primer Name	Direction	Primer sequence 5′–3′	Position in the mtDNA	Reference
LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	1490	*See Folmer et al., 1994 **
HC02198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	2198	

* Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994 Oct;3(5):294-9. PMID: 7881515.

(v) METHOD

[1] This is the PCR master mix preparation step. We are going to prepare a PCR solution enough for **2x** PCR reactions (1 per student + negative control with no DNA template). Keep tubes on the ice at all times. Add the following components to a new and labelled 1.5 ml Eppendorf tube (keep tubes in ice when not using):

a) 15.8 µl Sterile H_2O

b) 20.8 µl 2x NZYTaq II 2× Green Master Mix

c) 3.4 µl 10 µM mix of Primers

42 µl Total

Be very precise when pipetting small volumes. Pipet slowly and release the liquid to the side of the tube because the volumes are very small.

[2] Aliquot 18 µl of final mix into each of 2 thin-walled 0.2ml PCR tubes. Add 2 µl of your DNA extract to the first PCR tube and label it. To the remaining tube, add 2ul of sterile water, and this makes your negative control. Place your reaction on ice and give your tubes to the lecturer/demonstrator.

[3] The tubes will be placed by the lecturer/demonstrator in a thermal cycler. Make notes of the program settings of the thermocycler. The reaction will take about 2hrs and tubes will be taken out by the lecturer/demonstrator. Briefly, the PCR solutions inside the tubes will pass cyclically through different temperatures at different times. The first step is called **denaturation**, and it is needed to separate the double strand of template DNA. It is set at 95°C for 30 sec (with an initial denaturation of 5 min), the

second step is called **annealing**; during this phase, the primers attach to their complementary regions on the DNA strands. This is set at 50°C for 40 sec. The third step is called **elongation**; The polymerase extends the attached primers. This is set at 72°C for 1 minute (primer extension). These temperature stages are set to occur cyclically **35 times**. A **final extensio**n time at 72°C for 10 minutes, is included.

[4] At the end of the cycling period, remove samples and put them in the fridge for later analysis (electrophoresis on agarose gels).

3.5 Agarose Gel Electrophoresis – Activity 3

(i) PURPOSE

To determine the size of a linearised fragment of DNA. To be sure that the PCR actually worked, we need to check the amplified DNA on a gel.

(ii) SCOPE.

DNA fractionation is performed according to molecular size by electrophoresis through an agarose gel (1% agarose is optimal for separation of DNA fragments between ~200-1000 bp). This method will be used to analyse DNA fragments produced by PCR. DNA is visualised by incorporating a stain into the molten gel. NZYTech® GreenSafe Premium DNA Stain is a staining agent with high affinity for DNA and fluoresces when irradiated with a UV source (it is a new nucleic acid stain which can be used as a safer alternative to the traditional ethidium bromide for detecting nucleic acids in agarose gels. It is as sensitive as ethidium bromide and can be used precisely in the same way in agarose gel electrophoresis); it is therefore used to visualise the migration of DNA molecules in the agarose gel.

(iii) EQUIPMENT REQUIRED

Transilluminator with gel doc system

55°C water bath

Electrophoresis apparatus

Electrophoresis Power Source

(iv) MATERIALS/SOLUTIONS REQUIRED

50 ml molten agarose (1%) in 1x TAE at 55ºC

Electrophoresis buffer – 1x TAE

Sterile Materials:

- 1 ml, 200 µl and 10 µl tips
- 1.5 ml eppendorfs
- 5x Loading dye
- NZYTech® GreenSafe Premium DNA Stain

(v) METHOD

[1] Seal the ends of the gel holder with rubber ends (masking tape can be used if the apparatus does not contain rubber sealing ends). Also, **make sure that the wells do not touch the bottom**, if gel is perforated you will lose your loaded sample.

[2] Place comb in the correct orientation across the end of the gel holder.

[3] Agarose will be melted (50 ml) in 1xTAE in an autoclave or using a microwave. Once agarose is molten then place in a 55°C water bath. **(this will be performed for you in the teaching laboratory).**

[4] Check that agarose is no hotter than 55°C, add 2.5 µl of NZYTech® GreenSafe Premium DNA Stain mix by swirling and pour into the holder taking care to avoid air bubbles. Allow to set (approx. 15 min). Do this with the gel holder on the bench - not in the electrophoresis apparatus.

[5] Carefully remove the comb and seal ends and place the gel holder into the electrophoresis apparatus. Ensure that the wells are placed over the red band. **Take care that the agarose gel does not slip off the plastic tray!**

[6] Pour 1x TAE into the apparatus until the gel is just covered. Ensure that no air remains in the wells by washing with 1x TAE.

[7] Prepare your standard by the addition of an appropriate volume of 5x loading dye (i.e. 5 µl sample and 1 µl of loading dye) into a piece of parafilm. If using the Supreme NZYTaq II 2× Green Master Mix buffer, the sample can be directly loaded into the gel (5ul). The Standard used is a 100 bp DNA ladder. Place the lid and connect the power supply.

[8] Run the gels at 90-120 V until the dye has migrated approx. half the length of the gel (~30 min).

[9] **Switch off and disconnect the power supply**.

[12] Remove holder gel on holder and transport to transilluminator. Place gel on the transilluminator, ensure the door is closed and visualise fluorescence using CCD camera. If recording the picture digitally use your student number and the date as the file name.

[14] Switch off the transilluminator, remove your gel and leave the equipment clean and dry.

Appendix 1 – PCR

PCR involves two single-stranded oligonucleotide primers, approximately 20-25 nucleotides in length, which flank the DNA sequence to be amplified (Figure 1). The primers hybridise to opposite strands of denatured DNA and DNA synthesis by a DNA polymerase proceeds between the two primers. PCR is a sensitive technique for the amplification of DNA and the increase in DNA fragments is exponential. From a single target DNA molecule, 268,435,456 fragments will be generated after 30 cycles of the PCR reaction.

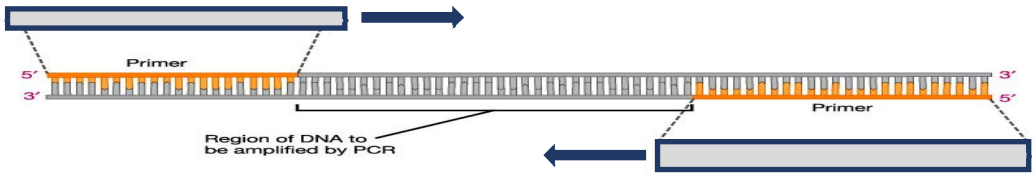

Figure 1: PCR primers for the amplification of *T.aquaticus* DNA polymerase.

PCR reactions involve some cycles, with each cycle composed of three steps. Figure 2 shows one cycle, with each cycle having 3 steps – denaturation, annealing and extension. Firstly, **DENATURATION**. The primers can only bind to their complementary sequence within the target if the DNA is single-stranded. The DNA is heated to ~95°C and the hydrogen bonds holding the two strands together are broken. At this stage, the primers are not bound to the DNA. For the primers to bind the PCR must be cooled to about 60°C; **ANNEALING.** Different primers have different nucleotides sequences and different lengths, so the annealing temperature needs to be calculated for the specific primer you are using. For the DNA polymerase in the PCR to begin DNA synthesis (**EXTENSION**), the mix needs to be at 72°C. This is the optimal temperature for the DNA polymerase used in the reaction to work.

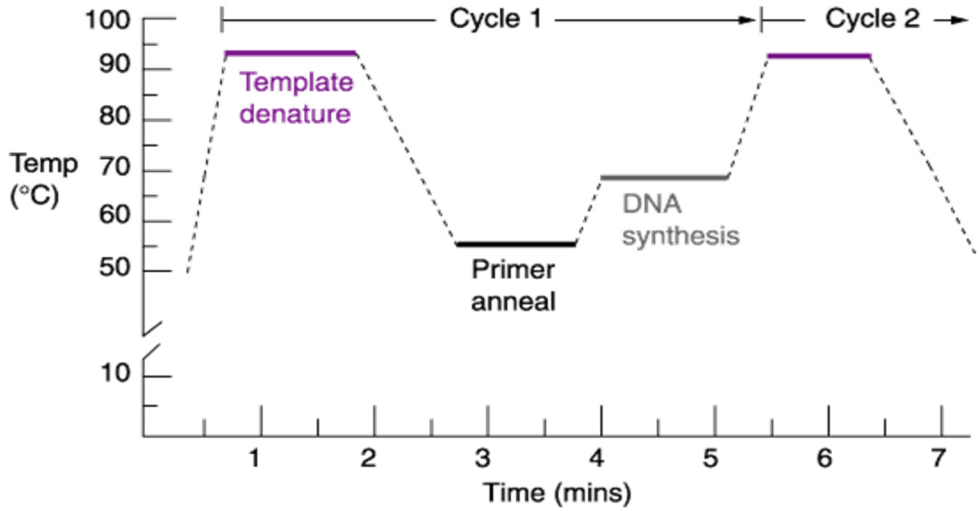

Figure 2: PCR profile for one cycle. The denaturation, annealing and extension (DNA synthesis) steps within the cycle are shown.

Each cycle of the PCR generates double-stranded DNA product, each of which can be used as template in the next cycle PCR cycle (figure 3). So, by repeated cycles of denaturation, annealing and extension, there is a rapid exponential accumulation of the specific target fragment of DNA. The DNA polymerase used must survive the repeated cycles of the PCR from 99°C to 60°C. Mammalian DNA polymerase works most efficiently at 37°C, but denatures (loses its protein structure) above ~50°C. So, the DNA polymerase must survive at 99°C (thermostable). *Taq* polymerase isolated from the thermophilic bacteria, *Thermus aquaticus,* is used in PCR. *Thermus aquaticus* is found in hot springs in Yellowstone National parks, as such its DNA polymerase does not denature at high temperatures.

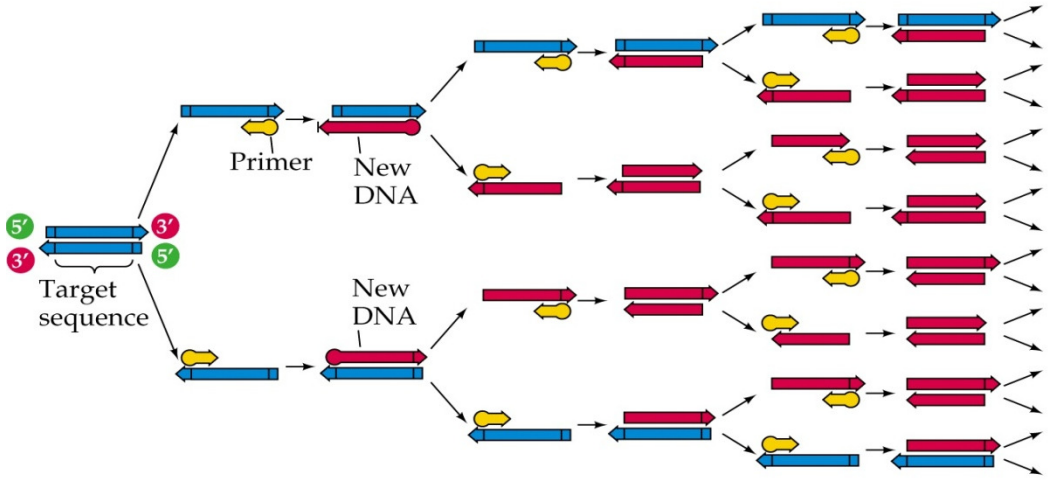

Figure 3: Exponential amplification of a single target DNA molecule during PCR

Appendix 2 – Agarose Gel Electrophoresis

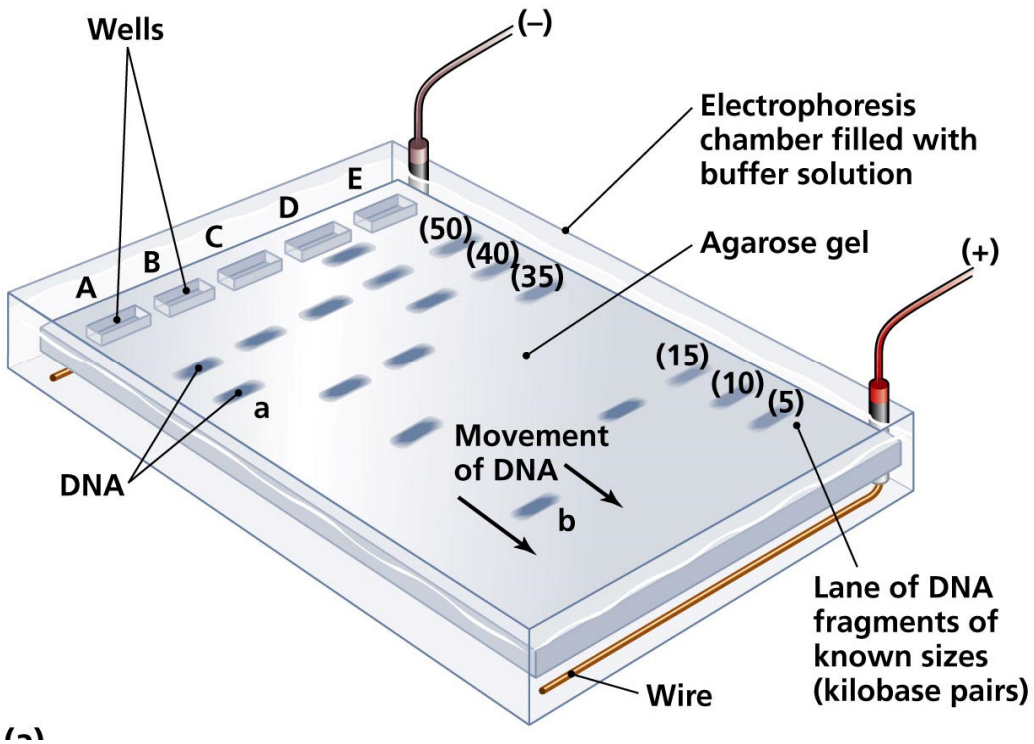

Figure 4: Agarose gel electrophoresis

An agarose gel is made up of a complex network of polymeric molecules with pores in between. The size of the pores depends on the type of buffer used and the concentration of the agarose used. The DNA molecules move through the gel matrix in the direction of the positive electrode, as DNA has a net negative charge from the phosphate groups in the sugar-phosphate backbone. The DNA fragments are then seperated on the gel by their size. Smaller DNA fragments migrate through the network of pores in the gel quickly, whilst larger fragments take much longer to move through the network. From figure 4 you can see lane E has DNA fragments of known size (DNA marker), with large fragments (50Kb) at the top and smaller fragments (5Kb) migrating further.

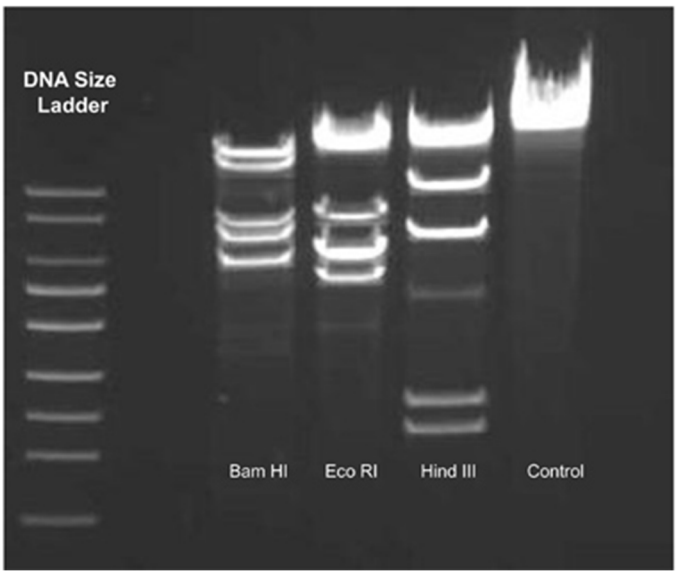

Figure 5: Example of DNA gel with DNA samples digested by restriction enzymes. The sizes of the DNA can be compared to DNA fragments of known size (DNA ladder).

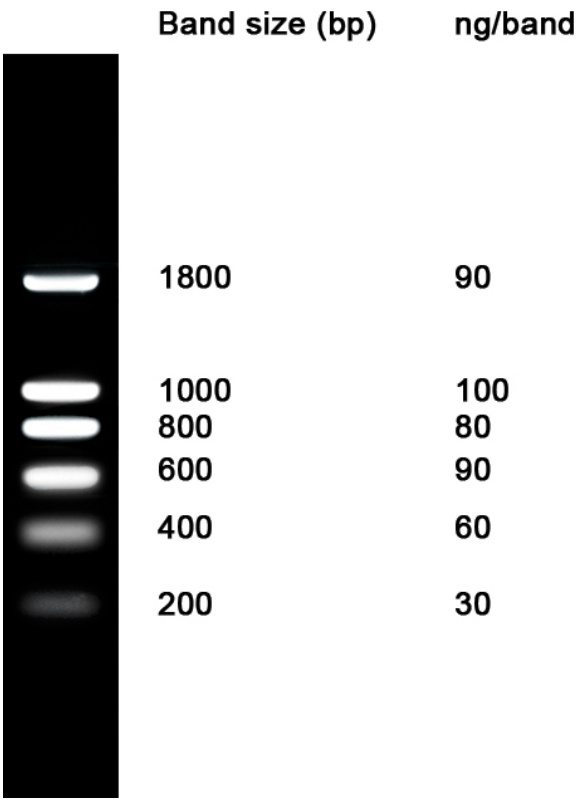

Figure 6: "NZYDNA Ladder I" DNA ladder.