

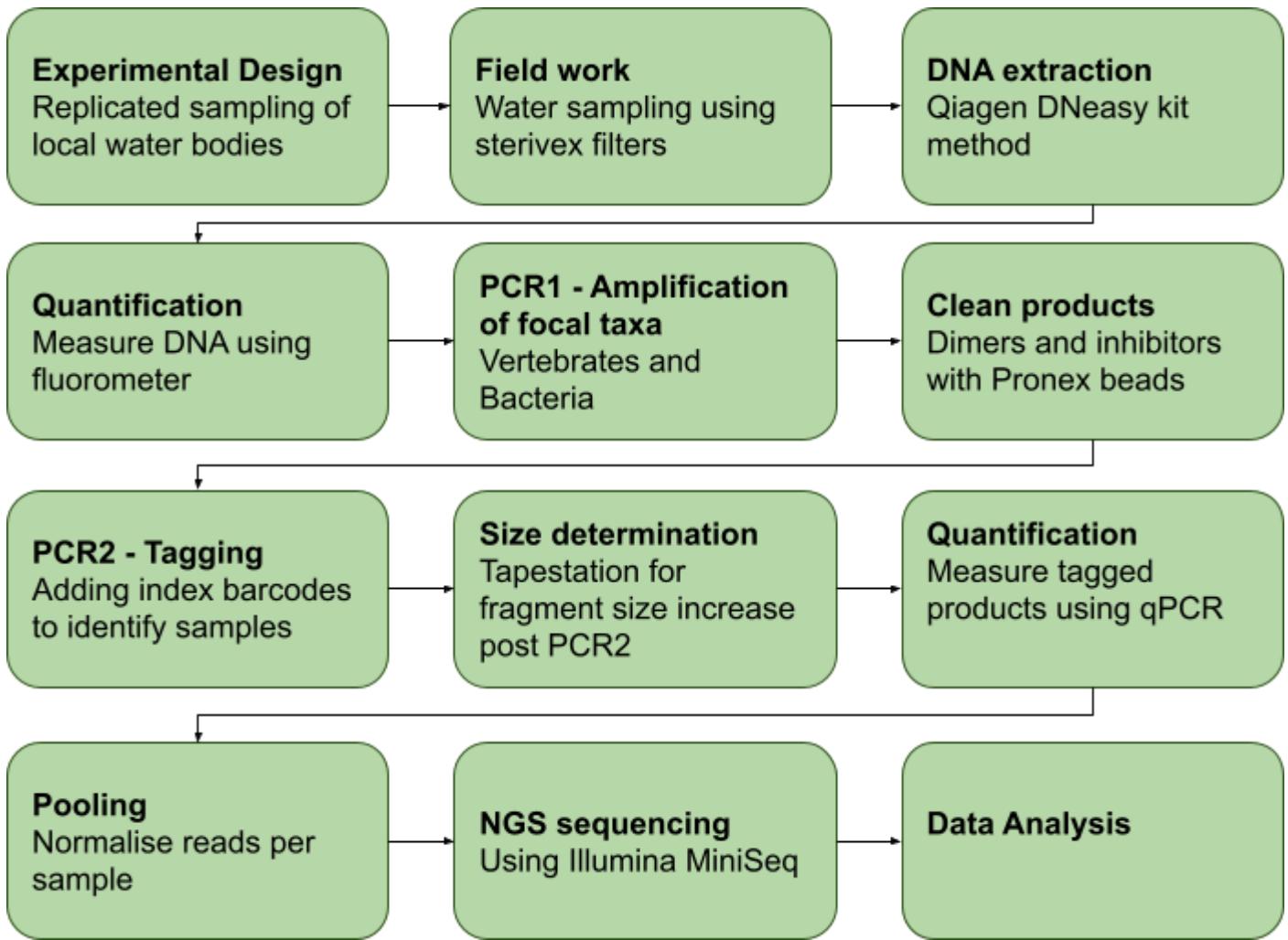
# Metabarcoding and Environmental DNA

## Workshop:

*Assessing biodiversity of a local pond using DNA metabarcoding*

Date	Time	Activity	Location
04/09	From 12:30	Arrival - Welcome and Introductions	Conference Room
04/09	1:30 - 15:00	1. Experimental Design and Field Sampling	Conference Room/Weston Park
04/09	15:00 - 15:30	Refreshments	Conference Room
04/09	15:30 - 17:00	2. DNA Extraction	Perak 2
05/09	9:30 - 11:00	3. DNA Extraction (continued)	Perak 2
05/09	11:00 - 11:30	Refreshments	Conference Room
05/09	11:30 -13:00	4. Quantification	Perak 2
05/09	13:00	Lunch	Conference Room
05/09	14:00 - 15:00	5. PCR1 and Gel electrophoresis	Perak 2
05/09	15:00 - 15:30	Refreshments	Conference Room
05/09	15:30 - 17:00	6. Visualise Gel and Lab Tour	Genomics Lab
06/09	9:30 - 10:30	7. Purification and PCR2 indexing	Perak 2
06/09	10:30 - 11:00	8. Pooling & Library Quantification	Perak 2
06/09	11:00 - 11:30	Refreshments	Common Room
06/09	11:30 - 12:15	9. Sequencing and Questions	Common Room
06/09	12:15	Walk to Pam Liversidge Building for Lunch	Atrium

# Workflow



# Introduction to the Metabarcoding Process

Metabarcoding allows us to:

- Identify a range of species in an environmental sample simultaneously, through genetic sequencing.
- This allows scientists to carry out a range of studies including diet analysis and population surveys.

How does it work:

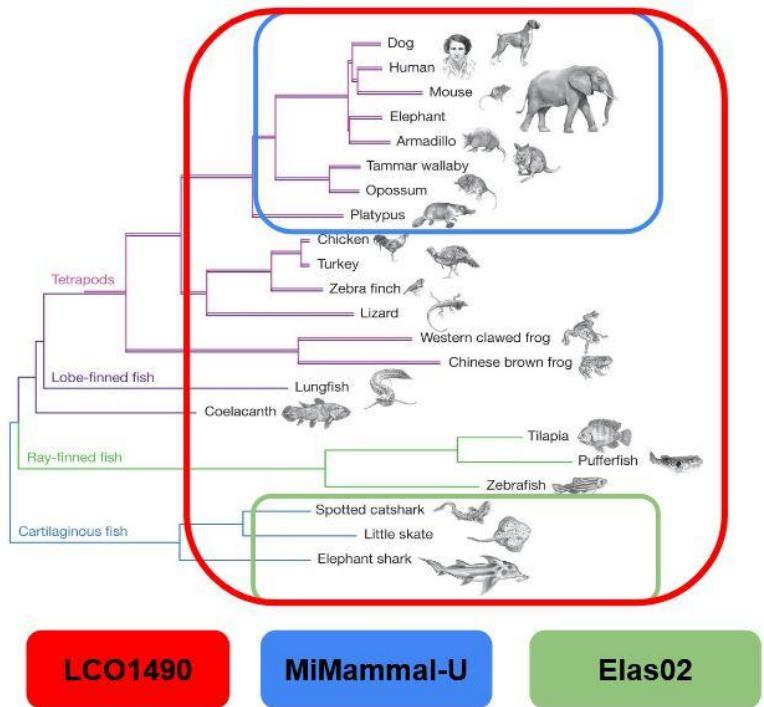
- Generic primers are used to target whole taxonomic groups, whilst bioinformatic analysis allows species identification.
- To be useful as a barcoding gene, the gene must be highly conserved within a species, but variable between species.
- The barcoding gene should be flanked by sequences that are conserved within the target phylogeny.
- Primers are designed to target the conserved sequences.
- Resulting sequence data can be compared to existing barcode libraries for species identification.

## Primer selection and the Diversity vs. Precision trade off

Evolutionary trees can show us how closely related different species are to each other. There are highly conserved regions of the genome that will be shared with all these species, and genetic regions that vary between species.

When designing your project, you will want to determine which taxonomic groups you wish to detect using genetic analysis. But bear in mind, the broader the taxonomic range you target, the more species you are likely to detect. And herein lies a tradeoff, between diversity and sequencing depth.

The flow cell on which your sequencing is run offers a limited number of reads, which can be allocated either to sequencing a few species multiple times - the sequencing depth, which increases the accuracy of the result - or allocated to sequencing many species fewer times, reducing the sequencing depth. If you want to sequence all species, at great sequencing depth, you will require multiple flow cells, which will increase your costs. We must therefore calculate the number of species we can sequence at a reasonable depth to generate reliable results.



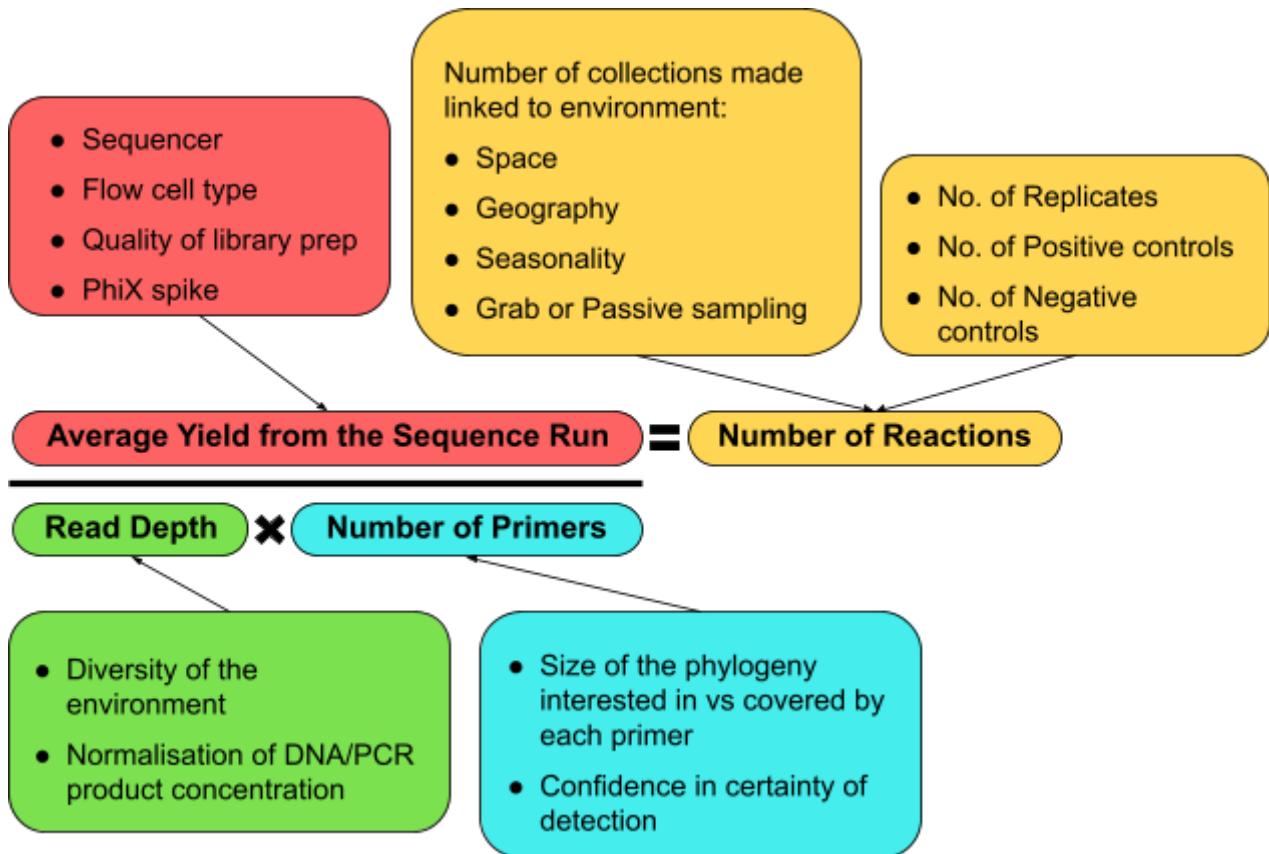
LCO1490

MiMammal-U

Elas02

# 1. Experimental Design

When planning your experiment it is important to think about your project holistically, considering your research aims, the power you will need to answer your research questions, and the cost both in terms of finance and time.



**Space** The number of collections you make should be appropriate for the space you are monitoring.  
i.e. Larger area = Larger number of samples.

**Geography** The number of collections you make should be appropriate for the niche diversity of an area.

**Seasonality** Collections should be made in a time series if diversity is likely to be impacted by Season/migration patterns.

**Grab or Passive sampling** Grab sampling is limited to that point in time. Passive sampling occurs over a greater period of time/ potentially over a larger area so may require less replication.

*Samples, replicates, positives, negatives.*

**Example study**

Miseqv2 2x150bp = 12million reads

2 primers of interest both of which require 50,000 reads

12 million x 0.8 (presume adding 20% PhiX ) = 96 samples

50,000 \* 2

**PhiX** - PhiX is spiked in at higher concentration in metabarcoding runs in order to increase diversity.

**Limited normalisation of DNA/PCR product concentration** - Normalisation helps reduce the chance that highly concentrated samples will swamp the run.

**Sequencer and flow cell size** - Different flow cell sizes yield different numbers of overall reads.

**Number of Primers** - Having a greater number of primers increases the power to detect a greater number of taxa.

Having more than 1 primer with overlapping species coverage - If the species captured by two primers overlap this leads to a) more certainty of detection, b) increases the chance of capturing rare species and c) a greater power to calculate abundance.

Diversity of the environment/size of the phylogeny covered by the primer - 50,000 reads per sample per primer is a good rule of thumb, however, this may need to be increased for primers that target a greater numbers of species, such as Riaz12s which captures almost all vertebrate groups. Conversely, a marker like EwA-F 16s might be limited to family or order and so need fewer reads to be confident.

**Unwanted species/blocking primers** - If species of no interest are abundant in the sample that can be amplified by the chosen primer set (e.g. host insects in a parasitoid wasp study). Reads will be wasted unless blocking primers are incorporated into the experimental design.

**Sample replicate** - Different sample collections from the same location.

**Extraction replicate** - Independent extractions from the same sample.

**PCR replicate** - Separate PCR reactions from the same extraction.

#### **Positive (Xeno) controls:**

If a positive fails to produce a signal then something has likely gone wrong at that stage in the process. These should be DNA of high enough quality that should produce clear and quantifiable amounts of PCR product every time. However, ideally they should be a) alien either synthetic DNA or species not present in your targeted environment. This is to minimise any risk of cross contamination from the positive leading to false assignments of species in the unknown samples b) ideally be a mixture (mesocosm) of a number of species c) ideally each species should be quantified and input at known concentrations. d) the amount of Positive in the final pool should be normalised so it does not “use up” an excess of available NGS reads (swamping).

#### **Negatives:**

You generally want to incorporate a new negative at every stage of the process. This enables one to establish if contamination occurs and at what stage it happened.

**Field Negative** - A negative that has been through field processes (e.g. Sterile water passed through a filter in the field at the same time as sample collection). Tests for clean technique within the field

**DNA Extraction Negative** - A blank (again normally sterile water) that is extracted at the same time as the samples. Tests for clean extraction technique

**PCR negative** - A blank (again normally sterile water) not extracted but simply loaded as a sample at the PCR stage.

Positives and negatives should ideally be distributed at random across the plate and should be of reasonable number. This helps sample tracking during analysis.

## 2. Field work

### Field Equipment:

- |  |  |
|--|--|
| <input type="checkbox"/> Ladle                 | <input type="checkbox"/> Caulking gun      |
| <input type="checkbox"/> Sterivex filter unit  | <input type="checkbox"/> Box of ice        |
| <input type="checkbox"/> Syringe               | <input type="checkbox"/> 10% Bleach        |
| <input type="checkbox"/> 250ml bottles         | <input type="checkbox"/> Tissue paper roll |
| <input type="checkbox"/> Field negative bottle | <input type="checkbox"/> Gloves            |

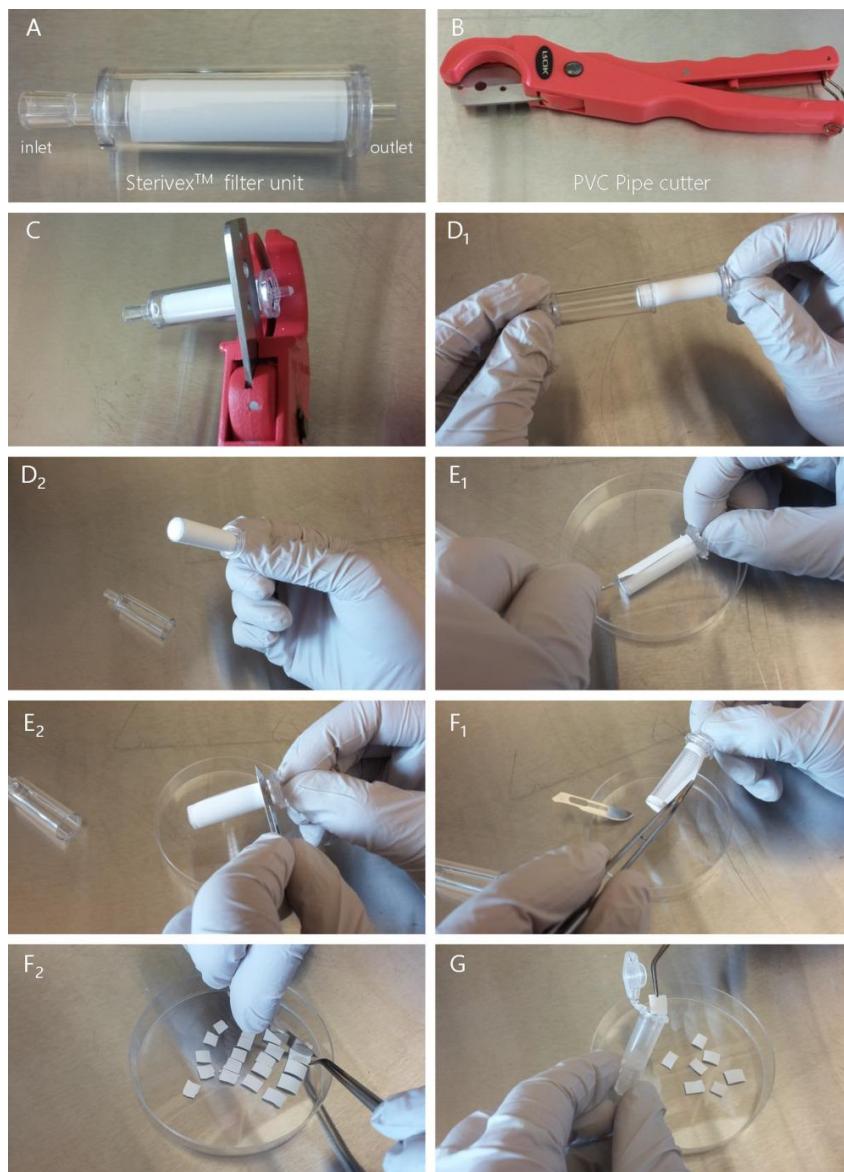
1. To be performed in groups of three. Complete and sign a risk assessment. Label bottles and filter units.
2. Gloves should be worn at all times during the sampling process and samples should be collected without entering the water, preventing disturbance of the substrate and limiting contamination from other sources.
3. Within the sampling site, identify locations where the water will be collected. The location of subsamples should be spaced as evenly as possible.
4. Use the ladle to collect water and pour into a labelled bottle.
5. Clean the outside of the bottle with bleach and wipe dry with clean tissue paper.
6. Before filtration, shake the bottle in order to mix the DNA across the whole volume of the water sample.
7. Attach a sterile filter capsule to a sterile syringe. Pull the top part off the syringe, pour 50 ml of water (measure with the marks on the syringe) and push the water through the filter capsule. A caulking gun may be used to help filter samples with a lot of sediment.
8. Using the same filter and syringe, repeat this until the desired amount of water has been filtered.
9. Store the filter inside the labelled package it came in, using masking/sellotape to close the packaging, and keep on ice
10. For each water sample, repeat this 3x using new bottles, syringes and filter units each time.
11. Store samples in a -20°C freezer until extraction (or store in the fridge if DNA extraction is performed on the same day)



### 3. DNA Extractions

#### 3.1 Removing the filter paper from the plastic unit

1. Clean the bench and equipment with 10% bleach.
2. Prepare labelled tubes with **400 µl Buffer ATL** and **10 µl proteinase K**. The lysis buffer ATL breaks open cell and nuclear membranes which exposes DNA to proteins which the enzyme proteinase K denatures. **This is where we create an extraction negative using the same reagents.**



(Cruaud et al. 2017)

**(A)** Sterivex filter unit, showing inlet and outlet. **(B)** PVC pipe cutter.

**(C)** Cutting of the bottom of the Sterivex unit (outlet end) with the PVC pipe cutter.

**(D1, D2)** Removal of the filter from the casing.

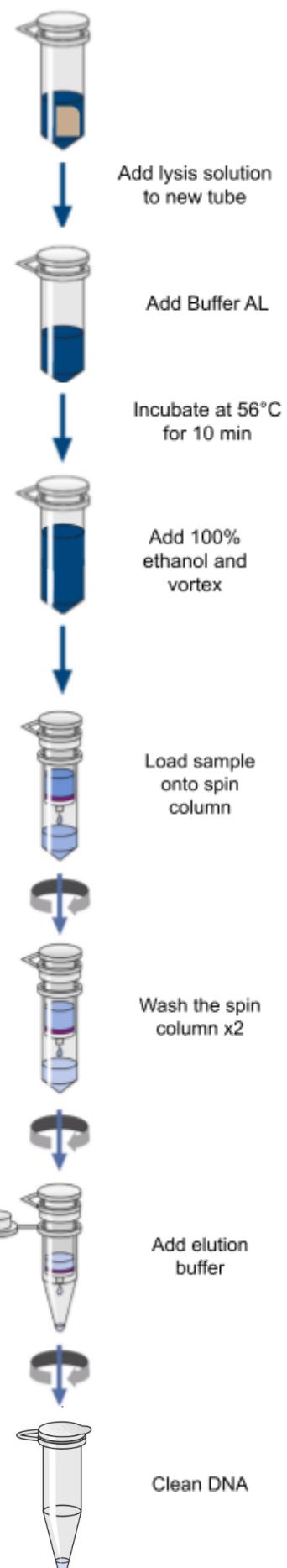
**(E1, E2)** Cutting of the filter attached to the inner part of the casing: first, longitudinal cut (E1) and second, transverse cut (E2).

**(F1, F2)** Detachment of the filter from the casing with sterilised forceps into a Petri dish and the dissection of the filter into small pieces with a sterile scalpel.

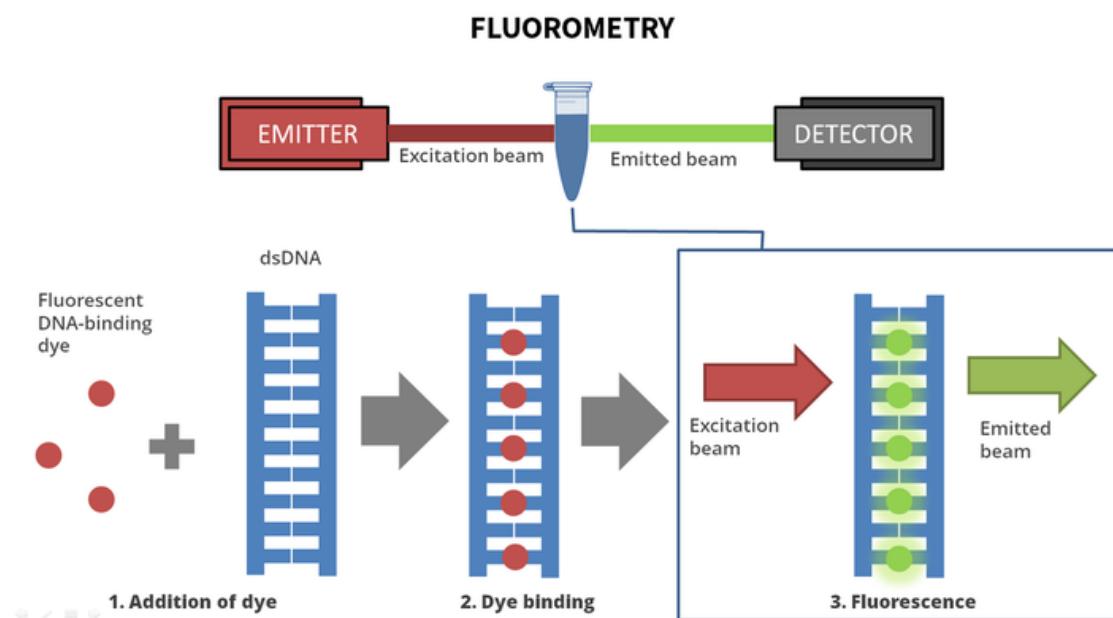
**(G)** Insert into the labelled microcentrifuge tube containing the lysis buffer. Incubate overnight at 56°C.

### 3.2 DNA extraction using a Qiagen DNeasy Kit

1. Clean the bench and equipment with 10% bleach.
2. Label a new 1.5 mL tube.
3. Vortex the microfuge tube containing the filter sample for **15 seconds**. Pipette as much solution as possible into the new tube.
4. Add **400 µl Buffer AL** to the 1.5 mL microfuge tubes with the lysis solution and vortex. Incubate at **56°C for 10 minutes**. This inactivates nucleases and promotes nucleic acid binding to the silica membrane.
5. Add **400 µl 100% ethanol** & vortex to precipitate the DNA.
6. Label a DNeasy spin column.
7. Add **700 µl of the sample solution** to a DNeasy spin column. Centrifuge at **8000 rpm for 1 minute**. Material will pass through the silica membrane, which attracts DNA and allows debris to pass through.
8. Place the spin column in a new collection tube and repeat step 7 so all the solution has passed through the spin column.
9. Place each spin column into a new collection tube and add **500 µl Buffer AW1**. Centrifuge at **8000 rpm for 1 minute**.
10. Place each spin column in a new collection tube and add **500 µl Buffer AW2**. Centrifuge at **11,000rpm for 3 minutes**. These washes help remove residual impurities.
11. Add **30 µl Buffer AE** to the spin column and incubate at **room temperature for 5 min**. This rehydrates the membrane and DNA allowing the DNA to become unbound and elute into solution. Centrifuge at **8000 rpm for 1 minute**.

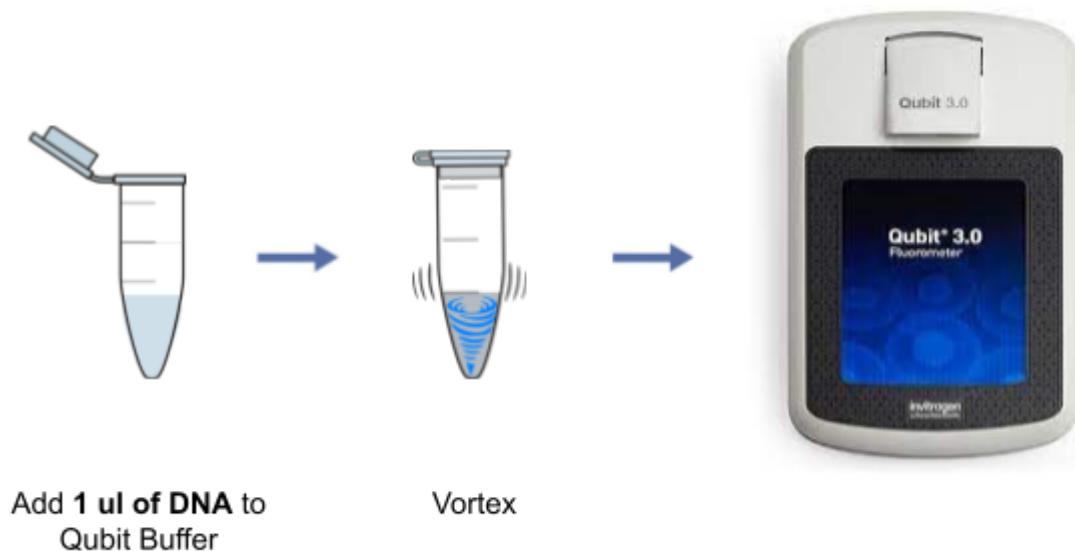


## 4. Quantification of DNA Extractions



Fluorometers utilise fluorescent dyes that bind to the DNA. Fluorescent dyes absorb a particular wavelength of light then emit light of another wavelength. The amount of fluorescence is directly proportional to the amount of DNA in the sample.

1. Add **1  $\mu$ l DNA sample** to the Qubit Buffer in 0.2 mL tube.
2. Close and vortex for **15 seconds**.
3. Place the tube on the Qubit Fluorometer and press “Read tube”.



## 5. PCR1 & Gel Electrophoresis

1. **Denaturation** - The double-stranded DNA is separated into single strands when heated.
2. **Annealing** - The primers bind to the DNA at your sequence of interest when the temperature is lowered. The exact temperature depends on the melting temperature of your primers.
3. **Extension** - The primers then direct the genetic machinery to the sequence in your DNA sample. When the temperature is raised a copy of the DNA strand is made by the polymerase enzyme. Extension time is influenced by the length of your target sequence

The PCR process then replicates this sequence repeatedly over 20 to 30 cycles. The number of replicates grows exponentially, resulting in millions of exact copies of your genetic sequence, known as amplicons.

The Polymerase Chain Reaction is achieved in a thermocycler. We programme the thermocycler to control the timings and temperature conditions of the sample. These settings are tailored to the sequence you are replicating.

### 5.1 Primer design

Vertebrates (mtDNA):

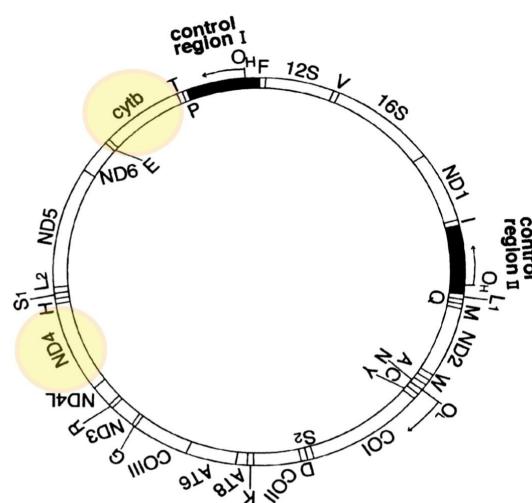
- COI
- 16S
- 12S
- ND4
- Cytb

Plants (chloroplast):

- ITS
- RBCL

Fungi - ITS

Bacteria - 16s or 18s



- Gene choice

It is vital to have reference sequences to compare against.

- Primer length

We design primers to be as specific as possible to the target region, however there is always a chance that the primer will bind to similar sequences, replicating non-desired regions of the DNA. The specificity is generally controlled by the length of the primer and the annealing temperature of the PCR reaction. Shorter primers will be less specific and therefore more likely to target a wider

range of genetic regions, or species. Longer primers will be more specific, but avoid making them so long that they fail to capture the range of individuals being sampled, which vary by their unique DNA mutations.

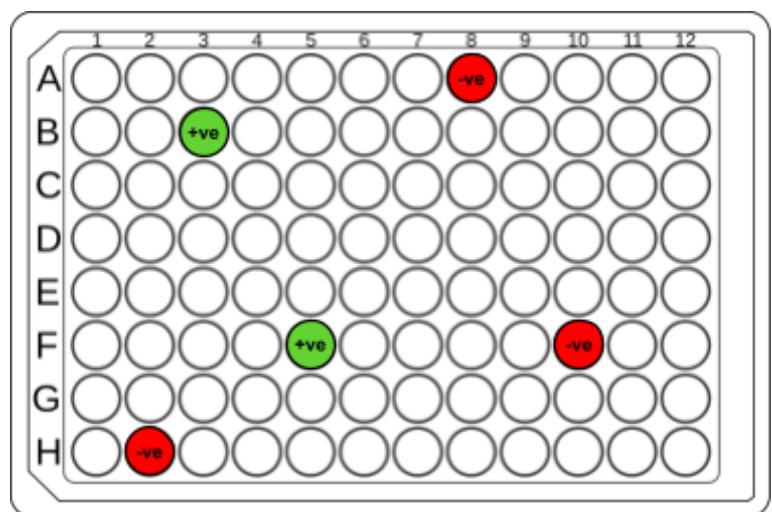
- **Amplicon length**

Our primers come in pairs, forward and reverse. They bind to either end of the DNA sequence of interest. This is the length of the DNA sequence being targeted for amplification, known as the amplicon. Shorter amplicons are more sensitive to the amplification of degraded DNA. Clearly though the shorter the amplicon the less sequence information present. Conversely, the longer the amplicon the more power you have to discriminate between species. This assumes an equal abundance of variants / mutations in a gene.

*In silico* software can help us in all stages of primer design, validating our primers against libraries of genetic sequence information.

## 5.2 Quality Control

- Including a known **positive** will help determine if the programme ran as expected
  - Positives should be synthetic DNA or species not present in your targeted environment that you have confirmed will be amplified by your PCR1 Primers.
- Including a **negative control** will highlight any contamination, which might influence all the samples in your run. Types of Negative
  - Field negative - A negative that has been through field processes.
  - DNA Extraction negative - A blank that is extracted at the same time as the samples.
  - PCR negative - A blank not extracted but simply loaded as a sample at the PCR stage.
- Positives and negatives should ideally be distributed at random across the plate and should be of reasonable number. This helps sample tracking during analysis.

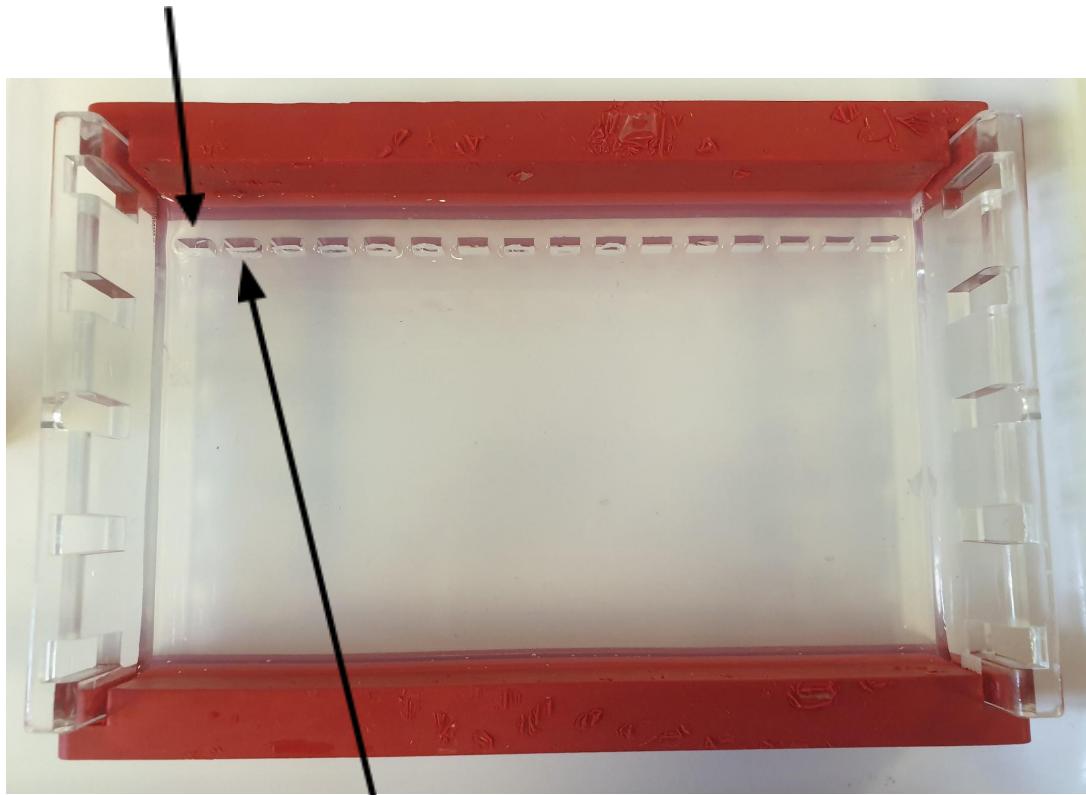


### 5.3 Gel Electrophoresis

Split into 3 groups. You are provided with a pre-made 1% agarose gel, 100bp ladder and a tube of PCR product mixed with loading dye.

**As a group:**

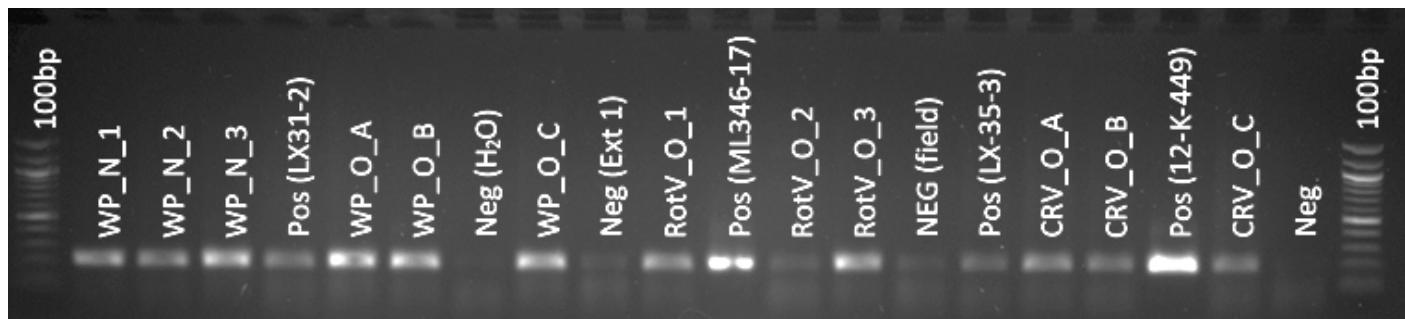
1. Remove red gel bumpers and carefully lower the tray and gel into the tank.
2. Add **5µl of the 100bp ladder** to the first well, being careful not to pierce the gel.



**Per individual:**

- Pipette mix and add **10µl of the PCR product mix** to a well.
- Repeat step 3 adding to the next well.
- Pass to the next group member.

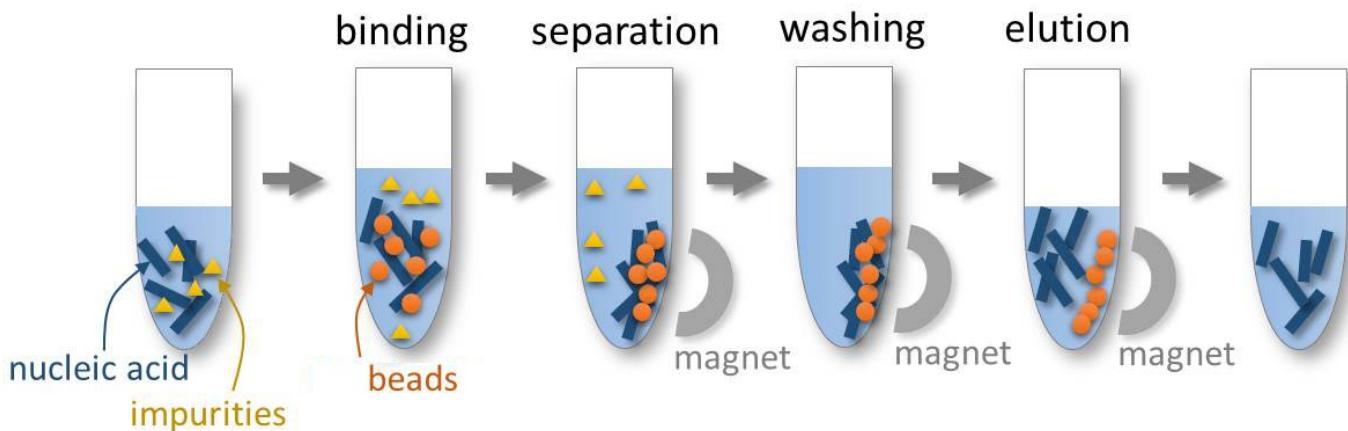
- PCR products can be visualised on an agarose gel.
- As seen in the gel image below, DNA samples that successfully amplified during the PCR process show up as white bands.
- By comparing the bands to a ‘DNA ladder’ which itself consists of known DNA fragment lengths, we can roughly determine the length of our PCR amplicons.
- The intensity of the band is proportional to the quantity of DNA loaded into the gel. A lane without a band tells us no DNA was amplified, as in the negative control.



*Gel image post-PCR 1. DNA ladders positioned at either end, samples and negative are in between.*

## 6. Bead Cleaning

- After the PCR reaction, samples will consist not only of our desired amplicons, but also excess reagents and extraneous DNA fragments.
- Bead cleaning allows us to remove waste products and size select our desired amplicons using a specific ratio of magnetic beads to PCR product.
- The beads more readily bind to longer fragments of DNA, therefore we select a ratio that will capture our amplicons whilst removing shorter extraneous fragments.

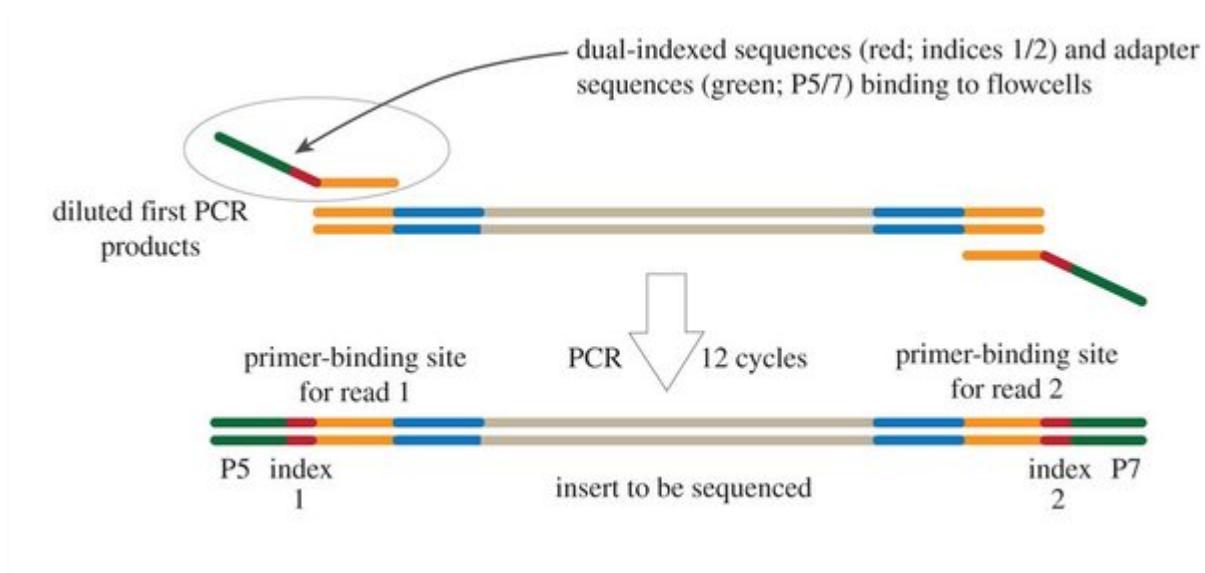


1. Clean the bench and equipment with 10% bleach. **Vortex and spin down** the Pronex Beads.
2. Add **50 µl Pronex Beads** to the tube containing the PCR product.
3. Mix well by pipetting up and down 10 times and incubate at **RT for at least 5 minutes**.
4. Place on a magnetic rack to separate beads from the solution.
5. When the liquid is completely clear, **aspirate the supernatant and discard**.
6. With the samples still on the magnetic rack, add **500 µl of Wash Buffer** and incubate at **RT for 30-60 seconds**.
7. Repeat step 6.
8. Allow the beads to dry at **RT for at least 5 minutes**, while on the magnetic rack.
9. Remove samples from the magnetic rack and elute with **25 µl of Elution Buffer**.
10. Mix well by pipetting up and down 10 times and incubate at **RT for at least 5 minutes**.
11. Place on a magnetic rack to separate beads from the solution.
12. When the liquid is completely clear, aspirate the solution containing your DNA into a fresh tube.

## 7. PCR2

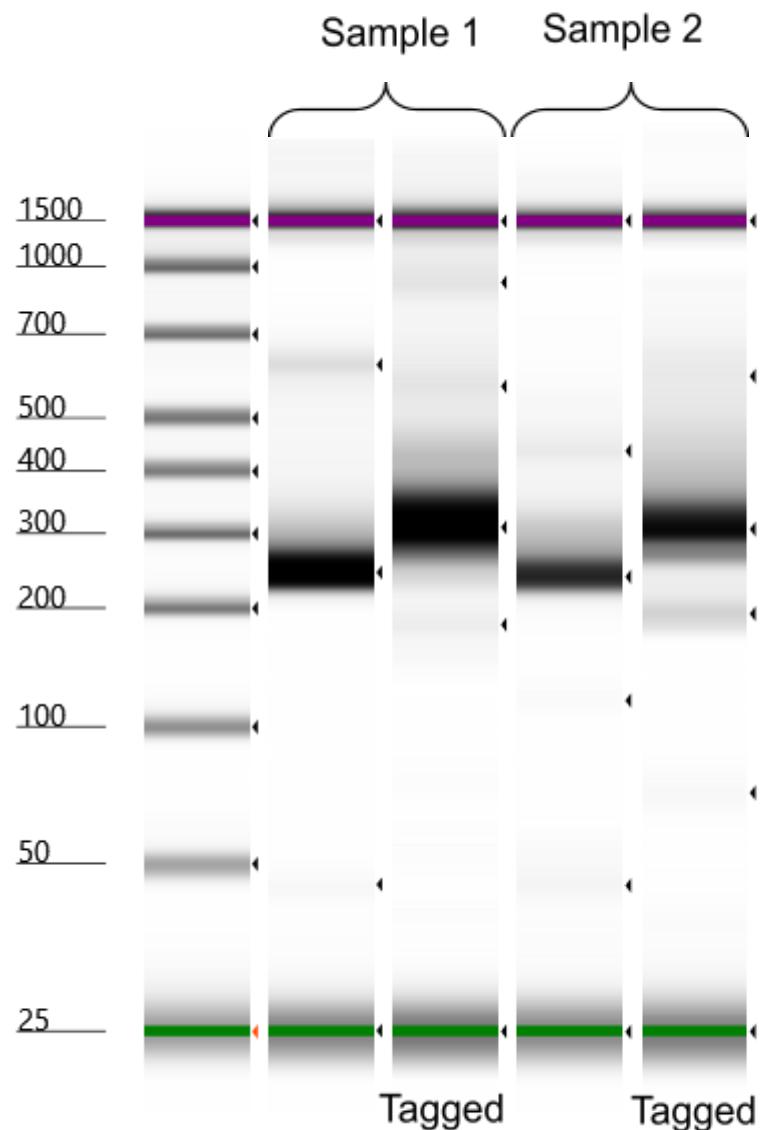
### 7.1 Unique Identifier Sequences

- In this second PCR reaction, identifier sequences are attached to our cleaned PCR1 products.
- These identifier sequences are unique to each sample.
- Once bound, all the tagged PCR products can be combined into one pool for sequencing, and identified in the bioinformatic data produced post-sequencing.



## 7.2 TapeStation QC

- The Tapestation is a quick, automated electrophoresis platform for nucleic acid sample quality control.
- In this case we use it to compare pre- and post- PCR2 products to determine if the unique identifier sequences have been successfully added to the amplicon fragments, and therefore increased in size.



## 8. Pooling and qPCR

### 8.1 Pooling

Pooling helps normalise the coverage of reads per sample and reduces the number of samples needing to be quantified using qPCR. Using the formula below we can calculate the correct volumes of each sample so that each sample is evenly represented when pooling together.

$$\frac{(\text{Final Volume } (\mu\text{l}) \times \text{Final Concentration } (\text{ng}/\mu\text{l}))}{(\text{Number of Samples} \times \text{Initial Concentration } (\text{ng}/\mu\text{l}))} = \text{Volume to Pool } (\mu\text{l})$$

Sample ID	Final Volume ( $\mu\text{l}$ )	Initial Concentration ( $\text{ng}/\mu\text{l}$ )	Final Concentration ( $\text{ng}/\mu\text{l}$ )	Number of Samples	Volume to Pool ( $\mu\text{l}$ )
X01	50	6.90	4	6	4.83
X02	50	2.49	4	6	
X03	50	4.85	4	6	
X04	50	4.01	4	6	
X05	50	9.12	4	6	
X06	50	5.74	4	6	

Total Volume to Pool ( $\mu\text{l}$ ) = \_\_\_\_\_

We then make up the pool with water to reach the final volume:

Volume of  $\text{H}_2\text{O}$  ( $\mu\text{l}$ ) = \_\_\_\_\_

### 8.2 Quantification using qPCR

Due to the presence of unlabelled DNA from PCR2 we must quantify products by qPCR. Using a commercial kit we can quantify products for Illumina sequencing on the qPCR machine that only target DNA with i5 and i7 indexes (identifier sequences) attached.

Samples and standards are run in triplicate to obtain averages that should cancel out any minor deviations of pipetting error. From these results we can pool the PCR2 products together as each sample now has unique identifiers.



## 9. Next Generation Sequencing

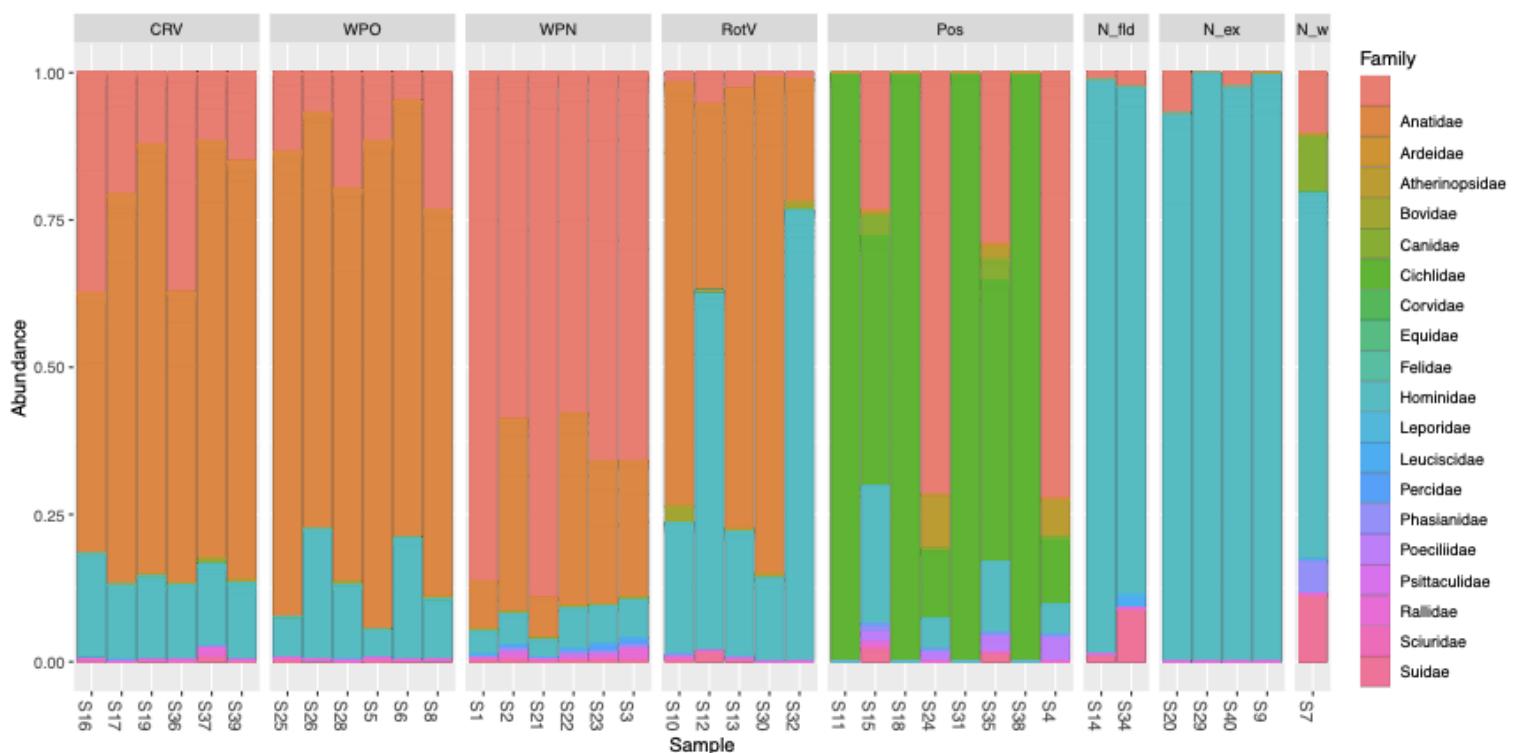
Different sequencing platforms can be used depending on the number of reads you require but also the length of the products you want to sequence.

Platform	Read length	Output
iSeq 100	2 x 150bp	1.2 Gb
MiniSeq	2 X 150bp	2.1 -2.4 Gb, 6.6-7.5 Gb
MiSeq	2 x 150bp, 2 x 250bp, 2 x 300bp	4.5-5.1 Gb, 7.5-8.5 Gb, 13.2-15 Gb*
NovaSeq	2 x 150bp	200 - 3000 Gb



## Previous eDNA Study

- In Autumn 2022 we carried out an eDNA metabarcoding experiment to test what species can be found in three local ponds.
- Samples
  - Weston Park October (**WP\_O**)
  - Rother Valley (**RotV\_O**)
  - Crookes Valley (**CRV\_O**)
  - Positive controls - Mexican river samples, African Cichlid stomachs (**Pos**)
  - Negatives
    - Field (**N\_fld**)
    - Extraction (**N\_ex**)
    - PCR (**N\_w**)
- Riaz 12S-V5 primer set (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3241669/>)
- Results:



# **Appendix I: Amplicon PCR & library preparation for Illumina sequencing**

## **1. PCR1**

### **1.1. PCR the amplicon/s from your DNA.**

Using amplicon-specific primers (XX) tailed with Illumina sequencing primer sites (F – small RNA primer; R – read 2 primer), e.g.:

F 5'-3'; ACACCTTTCCCTACACGACGCTTCCGATCTNNNNNXXXXXXXXXXXXXX  
R 5'-3'; GTGACTGGAGTTCAGACGTGCTCTCCGATCTXXXXXXXXXXXXXX

\*PCR recipe, in a total volume of 20 µl:

- ◆ 1 µl of DNA (at 10 ng/µl)
- 10 µl of Qiagen Multiplex PCR Master Mix (2x)
- 2 µl of F primer (at 1 to 5 µM)\*
- 2 µl of R primer (at 1 to 5 µM)\*
- 5 µl of sterile double-distilled water

[\*You may need to optimise the amount of primer used to avoid excess/unincorporated/dimer being carried through to the next step, while still getting good/strong amplification.

◆ If your DNA concentration is particularly low (which can be the case in studies on diet) increase the amount of DNA you use and decrease the amount of ddH<sub>2</sub>O accordingly.]

PCR program:

1 cycle of 95 °C for 15 min (Qiagen Multiplex PCR master mix requires this step)

then 25-35 cycles of

94 °C - 30 sec

Tm°C\* - 30 secs to 1min 30sec \*this depends on the Tm of your specific primers

72 °C - 30 secs to 2 min (depending on length).

72°C - 10 min

### **1.2. Positive and negative controls**

When running any PCR amplification reaction, positive and negative controls should be added to determine if the PCR is working as expected (+ve control) and to make sure no contamination is being introduced (-ve control). Negative controls from the DNA extraction may also be included.

### **1.3. Run an agarose gel**

Run 4µl of PCR product on a 1% agarose gel along with appropriate DNA size standard. We need to make sure samples have amplified and that there is a minimal amount of primer dimer.

### **1.4. Bead cleaning**

Prior to PCR2 we need to bead clean the samples to remove reagents left over from PCR1. If this isn't done PCR2 will result in a smear rather than a definite band.

### 1.5. Purification with Promega Pronex beads

1. Allow an aliquot of beads to reach room temperature, and vortex to resuspend them.
2. Make your sample up to 25  $\mu$ l with Low TE and add 37.5  $\mu$ l of resuspended Pronex beads – mix well by pipetting up and down 10 times and incubate at room temperature for 5 minutes. *Using 1.5x bead concentration will remove any reagents left over from the first PCR which could affect the following PCR.*
3. Place on a magnetic rack to separate beads from the solution. When the liquid is completely clear, aspirate **the supernatant and discard**. Do not disturb the pellet of separated magnetic beads and do not remove the samples from the magnetic plate.
4. With the samples still on the magnetic place, add 100  $\mu$ l 80% ethanol (make up fresh by separately measuring 80 ml of ethanol and 20 ml of water before combining them). Incubate at room temperature for 30 seconds, then carefully aspirate out and discard.
5. Repeat step 4.
6. Allow the beads to dry at room temperature for a few minutes, while on the magnetic stand. *Drying will allow traces of ethanol to evaporate, but over-drying the beads (if the pellet cracks) can significantly decrease elution efficiency.*
7. Remove samples from the magnetic plate and elute with 15  $\mu$ l of low TE (get a fresh sterile aliquot to ensure no contamination)- mix well by pipetting up and down 10 times (ensure that the beads are fully immersed & mixed with the low TE).
8. Place on a magnetic rack to separate beads from the solution ( $\sim$  1 minute).
9. Remove solution containing your DNA to a fresh 1.5ml tube without carrying over any beads.

## 2. PCR2

### 2.1. PCR to add unique identifying sequences to each sample.

Using tailed PCR to add unique identifier sequences (dual-plexed: Fi5 and Ri7 primers in unique combination for each sample) and Illumina sequencing sites to the amplicon products.

The general sequences of the forward and reverse primers with included 10bp barcode (red) are illustrated below.

Fi5\_01

5'CAAGCAGAAGACGGCATACGAGAT**TATCTTCTCG**GTGACTGGAGTTAGACGTGTGCTTCCGATC\*T3'

Ri7\_01

5'AATGATA~~CGCGACCACGAGATCTACACCGTCGCCTAT~~ACACTCTTCCCTACACGACGCTTCCGATC\*T3'

Set up the PCR on ice. Each well on the plate will use a different combination of Fi5 and Ri7 primers. Pipette these into the plate first – Fi5 at the bottom of each well and Ri7 on the side – so you can check both are in each well.

PCR recipe, in a total volume of 20 µl:

8 µl of template from PCR1

10 µl of Qiagen Multiplex PCR Master Mix

1 µl of Fi5/Fi7 Primer (at 10 µM)\*

1ul ddH<sub>2</sub>O

The PCR program to use is:

95 °C for 15 min

Followed by P8 to 12 cycles of:

98 °C for 10 sec

65 °C for 30 sec

72 °C for 30 sec

Then:

72 °C for 5 min

[\* Fi5/Fi7 primers are aliquoted together in plates which are ready for use. Ask Gav, Paul or Rachel where these are kept and how to use them]

[P Determine how many cycles you need from the concentration]

## **2.1. Tapestation**

Perform a run on the Tapestation using pre and post PCR2 samples so that you can check that the samples have increased in size from the addition of the PCR2 primers.

## **2.2. Fluorometer each sample & combine samples in groups of 8**

- Quantify 2 µl of each sample using the fluorimeter (to check the DNA concentration).
- Pool the same amount (150 ng\*) of each of the 8 samples from each of 12 columns for each 96-well plate, resulting in 12 samples to take forward to the purification step. [\*Aim for this amount – but you may have to lower how much you use, depending on how much DNA you have as revealed by the quantification earlier step.]
- The volume needed for the next step is 50 µl. If the volume of any of the pools exceeds this, use the speed-vac to concentrate up the DNA by reducing the volume. If speed-vac'd to lower than 50 µl, re-suspend to this volume using sterile water (NOT low TE) thus preventing too much EDTA in the sample.

## **3. Purification with Promega Pronex beads**

1. Allow an aliquot of beads to reach room temperature, and vortex to resuspend them.
2. Mix 50µl of each pool of PCR2 products with 75 µl resuspended Pronex beads – mix well by pipetting up and down 10 times and incubate at room temperature for 5 minutes.

3. Place on a magnetic rack to separate beads from the solution. When the liquid is completely clear, aspirate **the supernatant and discard**. Do not disturb the pellet of separated magnetic beads and do not remove the samples from the magnetic plate.
4. With the samples still on the magnetic place, add 100 µl 80% ethanol (make up fresh by separately measuring 80 ml of ethanol and 20 ml of water before combining them). Incubate at room temperature for 30 seconds, then carefully aspirate out and discard.
5. Repeat step 4.
6. Allow the beads to dry at room temperature for a few minutes, while on the magnetic stand. *Drying will allow traces of ethanol to evaporate, but over-drying the beads (if the pellet cracks) can significantly decrease elution efficiency.*
7. Remove samples from the magnetic plate and elute with 15 µl of low TE (get a fresh sterile aliquot to ensure no contamination)- mix well by pipetting up and down 10 times (ensure that the beads are fully immersed & mixed with the low TE).
8. Place on a magnetic rack to separate beads from the solution (~ 5 minutes).
9. Remove solution containing your DNA to a fresh 1.5ml tube without carrying over any beads.

#### **4. Quantification with qPCR**

##### **Materials:**

- 96-well PCR plate, Semi-Skirted, ABI Fast systems
- Clear optical adhesive plate cover (different from general sticky lids)
- Library quantification kit (KK4873, KAPA Biosystems)
- Sample dilution buffer: 10 mM Tris, pH 8.0 + 0.05% Tween 20

##### **Procedure:**

1. Clean work area before and after procedure. Use filter tips when pipetting. Also use appropriate plastics for the qPCR machine, they are not the same as the plates and adhesive seals we use for general PCR as they have optical properties.
2. Make a serial dilution of each library: 100, 1000 and 10000 -fold.
  - a. For 100-fold dilution, Combine 99 µl of dilution buffer with 1 µl of the library. Vortex.
  - b. For 1000-fold dilution, take out 1 µl from 1<sup>st</sup> dilution (100-fold) and add 9 µl of dilution buffer. Vortex.
  - c. For 10000-fold, take out 1 µl from 2nd dilution (100-fold) and add 9 µl of dilution buffer. Vortex.
3. Repeat step 2 two times to produce three independent dilutions of the library.
4. Prepare SYBR master mix as follows:

Reagent	µL/reaction
SYBR FAST mix + primers	6
Water	2

*Make sure that the primer mix has been added to the SYBR solution. Note that the six standards, a no template control, and your samples are run in triplicate. Also, it is usually necessary to add an extra 10% to the number of reactions/wells to account for pipette loss.*

5. Add 2 µl of the kit standards, diluted sample libraries, or dilution buffer (no template control) to appropriate wells in the 96-well plate.
6. Dispense 8 µl of the master mix to the appropriate wells in the 96-well plate.
7. Spin plate and ensure that there are no bubbles at the bottom of wells or debris on the bottom of the plate. Cover plate with a clear adhesive film.
8. Set up reaction on ABI's Quantstudio software. Define the wells used for the standards (task = standard, set appropriate values), no template control (task = NTC), and samples (task = unknown).
9. Set the reaction volume to 10 µl and the following qPCR profile:

5 min at 95°C  
35 cycles of the following:  
    30 sec at 95°C  
    45 sec at 60°C

10. Save file once the run is finished. Analyze data using the KAPA spreadsheet. Check triplicates for precision and remove obvious outliers. Concentrations for unknown samples will be output in pM (matching unit of standards), but will need to be multiplied by 1000 to account for the initial 1000-fold dilution. Therefore, the units can just be converted from pM to nM.
11. Pool samples in equimolar amounts – aim for 4 nM.
12. The final pool is now ready to submit for Illumina sequencing.

## Final product size

If your PCR product with normal primers is say 300bp the adapters and indexes add on 145bp. If using primers which include adapters then you add on 73 bp which is the length added on due to the size of the index files not including the overlap with the adapters.

## Regents and suppliers

QIAGEN Multiplex PCR Kit (100), cat# 206143, Qiagen, £174

AMPure XP, 60 mL, cat# A63881, Beckman Coulter, £835.80

Kapa qPCR quantification kit (for Quant Studio 12), KK4873, Cat# 07960336001, £495.00

Ethanol absolute 2.5 litres, cat# 10437341, Fisher Scientific

Proteinase K, Recomb., PCR grade, Roche, cat# 3115801001, SLS

EDTA 1kg, cat# BP120-1, Fisher Scientific

NaCl, cat# S/3120/60, Fisher Scientific

Tris-HCl solution 1M pH 8.0, cat# 15855478, Fisher Scientific

20% SDS, cat# BP1311-200, Fisher Scientific

## **Equipment required**

PCR machine  
Gel electrophoresis set up  
Magnetic rack  
Tapestation  
Fluorometer  
qPCR machine

## **Recipes**

### **Low TE (500 mL):**

5ml of Tris- HCL 1M  
100ul of 0.5M EDTA  
495ml of ddH2O  
Autoclave

### **Sample Dilution Buffer (500 mL):**

0.6 g Tris  
~490 mL water  
250 µl Tween 20  
Adjust pH and bring solution to 500 mL