Introduction to Molecular Biology and Bioinformatics Workshop
Biosciences eastern and central Africa - International Livestock Research Institute
Hub (BecA-ILRI Hub), Nairobi, Kenya
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Laboratory Manual

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http://hpc.ilri.cgiar.org/beca/training/IMBB/welcome.html
http://hub.africabiosciences.org/
Many of the solutions have been pre-prepared for use in the Workshop. Detailed recipes are included in the manual (Chapter 11).

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1. **Cautions**

**Chloroform**
Chloroform (CHCl$_3$) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses, and always use in a chemical fume hood.

**SDS**
SDS (sodium dodecyl sulphate) is toxic, an irritant, and poses a risk of severe damage to the eyes. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles. Do not breathe the dust.

**PureLink Genomic Lysis/Binding Buffer and Wash Buffer 1** contain guanidine hydrochloride. Guanidine hydrochloride is extremely hazardous in case of ingestion. Harmful in case of skin contact (irritant), of eye contact (irritant). Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering. Contact of Guanidine hydrochloride with acids or bleach liberates toxic gases. **DO NOT ADD acids or bleach to any liquid wastes containing this product.** Wear gloves and eye protection when handling these products.

**Binding Buffer in the GeneJET PCR Purification Kit** contains guanidinium thiocyanate. Wear gloves and eye protection when handling the Binding Buffer. It is harmful by inhalation, in contact with skin, eyes or if swallowed. Contact of guanidinium thiocyanate with acids or bleach liberates toxic gases. **DO NOT ADD acids or bleach to any liquid wastes containing this product.**

**UV transilluminator**
Radiation Hazard. UV Transilluminators are powerful sources of UV radiation that will cause serious damage to unprotected eyes and skin. Wear a UV face mask and gloves for protection

**Electrical equipment**
Never use electrical equipment with wet gloves on. Keep water and buffer away from all electrical connections. Do not use an electrical connection if it is wet.
2. Using a Micropipette

This chapter was taken from the University of Queensland website:


Introduction

When scientists need to accurately and precisely deliver smaller volumes of a liquid, they use a pipette – a calibrated glass tube into which the liquid is drawn and then released. Glass and plastic pipettes have been mainstays of chemistry and biology laboratories for decades, and they can be relied upon to dispense volumes down to 0.1 mL (100 µL).

Molecular biologists frequently use much smaller volumes of liquids in their work, even getting down to 0.1 µL (that’s one ten thousandth of a millilitre, or one ten millionth of a litre!). For such small volumes, they need to use a micropipette.

Micropipettes are called a lot of different names, most of which are based on the companies which manufacture. For example, you might hear them called “Gilsons”, as a large number of these devices used in laboratories are made by this company. *(In the Workshop, you will use Gilson Pipetman micropipettes).* Regardless of the manufacturer, micropipettes operate on the same principle: a plunger is depressed by the thumb and as it is released, liquid is drawn into a disposable plastic tip. When the plunger is pressed again, the liquid is dispensed.
**Tips**

The tips are an important part of the micropipette and allow the same device to be used for different samples (so long as you change your tip between samples) without washing. They come in a number of different sizes and colours, depending on the micropipette they are used with, and the volume to be dispensed.

The most commonly used tips are:

- D10 for use with P2 and P10 pipettes
- D20 for use with P20 pipette
- D100 for use with P100 pipette
- D200 for use with P200 pipette
- D1000 for use with P1000 pipette

They are loaded into tip boxes which are sterilized to prevent contamination. For this reason tip boxes should be kept closed if they are not in use. Tips are loaded onto the end of the micropipette by pushing the end of the device into the tip and giving two sharp taps. Once used, tips are ejected into a waste beaker or flask using the tip eject button. *(In the Workshop you must eject the tips into the flasks provided)*. Never touch the tip with your fingers, as this poses a contamination risk.

Also available in the Workshop are pipette tips with aerosol resistant filters, which prevent contamination of the pipette barrel with sample, and prevents cross-contamination between samples, which is especially important with PCR.

**Plunger positions** For each pipette, the plunger can sit at any one of three positions:

- **Position 1** is where the pipette is at rest
- **Position 2** is reached by pushing down on the plunger until resistance is met
- **Position 3** is reached by pushing down from position 2
Each of these positions play an important part in the proper use of the pipette.

**To Draw Up Liquid**

To remove the last drop of liquid from the tip, push down to Position 3. If delivering into a liquid, remove the tip from the liquid before releasing the plunger. Hold the micropipette with the thumb resting on the plunger and the fingers curled around the upper body.

- **Push down with the thumb until Position 2 is reached.**
- **Keeping the plunger at the second position, place the tip attached to the end of the micropipette beneath the surface of the liquid to be drawn up.** Try not to push right to the bottom (especially if you are removing supernatant from a centrifuged pellet), but ensure that the tip is far enough below the surface of the liquid that no air is drawn up.
- **Steadily release pressure on the plunger and allow it to return to Position 1.** Do this carefully, particularly with large volumes, as the liquid may shoot up into the tip and the body of the micropipette. If bubbles appear in the tip, return the liquid to the container by pushing down to Position 3 and start again (you may need to change to a dry tip).
**To Dispense Liquid**

Hold the micropipette so that the end of the tip containing tip is inside the vessel you want to deliver it to. When delivering smaller volumes into another liquid, you may need to put the end of the tip beneath the surface of the liquid (remember to change the tip afterwards if you do this to save contaminating stock). For smaller volumes you may also need to hold the tip against the side of the container.

Push the plunger down to Position 2. If you wish to mix two liquids together or resuspend a centrifuged pellet, release to Position 1 and push to Position 2 a few times to draw up and expel the mixed liquids

To remove the last drop of liquid from the tip, push down to Position 3. If delivering into a liquid, remove the tip from the liquid before releasing the plunger

Release the plunger and allow it to return to Position 1

---

**Changing the Volume**

Some micropipettes deliver fixed volumes, however the majority are adjustable. Each brand uses a slightly different method to do this – Gilsons have an adjustable wheel, others have a locking mechanism and turning the plunger adjusts the volume. All have a readout which tells you how much is being delivered and a range of volumes which can be dispensed. Trying to dispense less than the lower value of the range will result in inaccurate measurements. Trying to dispense over the upper range will completely fill the tip and allow liquid to enter the body of
the pipette. Do not overwind the volume adjustment, as this affects the calibration of the micropipette. The way to interpret the readout depends on the micropipette used:

In a 200-1000 µL micropipette (e.g. a Gilson P1000) the first red digit is thousands of µL (it should never go past 1), the middle digit is hundreds, while the third is tens. Therefore 1000 µL would read as 100, while 350 µL would read as 035.

In a 20-200 µL micropipette (e.g. a Gilson P200) the first digit is hundreds of µL (it should never go past 2), the second is tens and the third is units. Therefore 200 µL would read as 200, while 95 µL would read as 095.

In a 2-20 µL micropipette (e.g. a Gilson P20) the first digit is tens of µL (it should never go past 2), the second is units and the third red digit is tenths. Therefore 20µL would read as 200, while 2.5µL would read a 025

In a 0.2-2 µL micropipette (e.g. a Gilson P2) the first digit is units of µL (it should never go past 2), the second red digit is tenths and the third red digit is hundredths. Therefore, 2µL would read as 200, while 0.5 µL would read as 050

Internet resources and further information

1. Using a Micropipette - University of Leicester training video
   http://www.youtube.com/watch?v=2UQioYhOowM
2. Using a Micropipette
   http://www.di.uq.edu.au/sparqmicropipette
3. Pipetman maintenance
   http://oomyceteworld.net/protocols/RaininPipetmanGuide.pdf
4. Gilson Pipetman spare parts
   http://www.pipetman.com/ReplacementParts.aspx
3. Lab Math

Work in the biosciences lab requires a good knowledge of basic mathematical methods and formulae. In this chapter we have compiled the most common mathematical concepts and methods for use in the lab. Subjects include basics such as scientific notation and making solutions and dilutions.

Power Prefixes

It is essential that you learn the meaning of power prefixes. The most common ones used in the biology lab are kilo, milli, micro, nano and pico. In the laboratory you will encounter these when dealing with volumes (e.g. milliliters, ml or mL; microliters, µl or µl or µL), molarity (e.g. micomolar or µM), weights (e.g. kilograms, kg; nanograms, ng), molecular weights (e.g. kiloDaltons or kDa). You will also encounter kilo to describe 1000 nucleotide bases or base pairs (e.g. kb [kilo bases]; kbp [kilo base pairs]).

In bioinformatics you will encounter the prefix ‘mega’ which is used to describe a million bases (e.g. megabases or Mb).

<table>
<thead>
<tr>
<th>Prefix</th>
<th>Symbol</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>mega</td>
<td>M</td>
<td>$10^6$</td>
</tr>
<tr>
<td>kilo</td>
<td>k</td>
<td>$10^3$</td>
</tr>
<tr>
<td>hecto*</td>
<td>h</td>
<td>$10^2$</td>
</tr>
<tr>
<td>deka*</td>
<td>da</td>
<td>$10^1$</td>
</tr>
<tr>
<td>deci*</td>
<td>d</td>
<td>$10^{-1}$</td>
</tr>
<tr>
<td>centi</td>
<td>c</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>milli</td>
<td>m</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>micro</td>
<td>µ (or µ)</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>nano</td>
<td>n</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>pico</td>
<td>p</td>
<td>$10^{-12}$</td>
</tr>
</tbody>
</table>

*not used in the biosciences laboratory
Conversion Factors (grams)

<table>
<thead>
<tr>
<th>Conversion Factor</th>
<th>Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1\times10^3$ g</td>
<td>= 1 kg</td>
</tr>
<tr>
<td>$1\times10^{-3}$ g</td>
<td>= 1 mg</td>
</tr>
<tr>
<td>$1\times10^{-6}$ g</td>
<td>= $1,\mu$g</td>
</tr>
<tr>
<td>$1\times10^{-9}$ g</td>
<td>= 1 ng</td>
</tr>
<tr>
<td>$1\times10^{-12}$ g</td>
<td>= 1 pg</td>
</tr>
</tbody>
</table>

*Note: ‘$\mu$’ is often written as ‘u’

Conversion Factors (Molarity)

<table>
<thead>
<tr>
<th>Conversion Factor</th>
<th>Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1\times10^{-3}$ M</td>
<td>= 1 mM</td>
</tr>
<tr>
<td>$1\times10^{-6}$ M</td>
<td>= 1 $\mu$M</td>
</tr>
<tr>
<td>$1\times10^{-9}$ M</td>
<td>= 1 nM</td>
</tr>
<tr>
<td>$1\times10^{-12}$ M</td>
<td>= 1 pM</td>
</tr>
</tbody>
</table>

Concentration

- Concentration is the amount of a substance in a specific volume (or sometimes mass) of a solution or mixture.
- The substance that is dissolved is called the solute and the liquid is called the solvent. Remember ‘concentration’ and ‘amounts’ are NOT synonymous.
- ‘Amount’ is how much of a substance is present (e.g. 4 grams, 1 mole).
- ‘Concentration’ is a ratio with a numerator (amount) and a denominator (usually volume). e.g. 25 g NaCl per litre water, where the NaCl is the solute and water is the solvent.

The M words (Mole, Molar, Molarity)

- **Mole**: (symbol: mol) A name for $6.022 \times 10^{23}$ particles of something, e.g. a chemical. A mole of a chemical has a mass equal to the molecular weight (or formula weight) in grams.
- **Molar**: (symbol: M) The concentration, in moles per litre, of a solution. A one molar solution contains 1 mole of a substance in 1 litre (1 L). It is abbreviated M. For example one molar is written 1 M.
- **Molarity**: Molarity is the number of moles of a chemical in 1 L of solution and is thus a unit of concentration.
  A 1 Molar (1 M) solution is equivalent to one molecular weight (or formula weight) (MW or FW = g/mole) of a chemical dissolved in 1 L of solvent (usually water).
Molar solutions
A one molar solution of a chemical is one in which 1 litre of solution contains the number of grams of chemical equal to its molecular weight.

- **Example 1**
To make **1 litre** of a **1 M NaCl** solution, dissolve the molecular weight in grams of NaCl in a total volume of 1 litre of water:

**Formula:**
\[
\text{Grams required} = (\text{MW in g}) \times (\text{desired molarity in moles}) \times (\text{final volume in litres})
\]
58.46 (MW of NaCl) g x 1 moles x 1 litre = **58.46 g**
Dissolve **58.46 g** NaCl in water to a final volume of **1 litre**

- **Example 2**
To make **100 mL** of a **0.3 M NaCl** solution:

**Formula:**
\[
\text{Grams required} = (\text{MW in g}) \times (\text{desired molarity in moles}) \times (\text{final volume in litres})
\]
58.46 (MW of NaCl) g x 0.3 moles x 0.1 litre = **1.75 g**
Dissolve **1.75 g** of NaCl in water to a final volume of **100 mL**

Percent solutions
There are 2 types of percentage solutions: weight per volume (w/v) and volume per volume (v/v):

- **Percentage (w/v) = weight (g) in 100 mL of solution**
- **Percentage (v/v) = volume (mL) in 100 mL of solution**

- **Example 1 (w/v)**
To make a 3% solution of NaCl in water, weigh 3 g of NaCl and then make up the volume to 100 mL with water.

- **Example 2 (v/v)**
To make a 10% solution of glycerol in water, take 10 mL of glycerol and mix with 90 mL of water.

Concentrated stock solutions - using ‘X’ units
Many enzyme buffers and electrophoresis buffers are prepared and stored as concentrated solutions. For example, a solution 20 times more concentrated would be denoted as **20X** and would require a **1:20** dilution to attain the working concentration.
• **Example 1**  
  Using 50X electrophoresis buffer  
  To prepare 1 litre of 1X electrophoresis buffer from a 50X stock, take 20 mL of stock (i.e. 1/50 of the final volume) and mix with 980 mL of water.

• **Example 2**  
  Restriction enzyme digest using a 10X buffer  
  To set up a 25 µL restriction enzyme digestion, add 2.5 µL of a 10X buffer (i.e. 1/10 of the final volume), plus the other reaction components, and then water to a final volume of 25 µL.

**Preparation of working solutions from concentrated stock solutions**  
Many buffers in molecular biology require the same components but often in varying concentrations. To avoid having to make every buffer from scratch, it can be useful to prepare several concentrated stock solutions and dilute as needed.

The following formula is useful for calculating amounts of stock solution needed:  
\[ Ci \times Vi = Cf \times Vf \]  
Where:  
\( Ci \) = initial concentration, or concentration of stock solution  
\( Vi \) = initial volume, or amount of stock solution needed  
\( Cf \) = final concentration, or concentration of desired solution  
\( Vf \) = final volume, or volume of desired solution

You must first normalize the units, i.e. choose the same units for volumes and for concentrations: e.g. convert all volumes to mL and all molarities to mM

• **Example 1**  
  How do you prepare 100 mL of TE buffer containing 10 mM Tris and 1 mM EDTA using stocks of 1 M Tris and 0.5 M EDTA?

  **Tris**  
  \[ Ci \times Vi = Cf \times Vf \]  
  \[ 1000 \times Vi = 10 \times 100 \]  
  so \( Vi = (10 \times 100) / 1000 = 1 \) mL

  **EDTA**  
  \[ Ci \times Vi = Cf \times Vf \]  
  \[ 500 \times Vi = 1 \times 100 \]
so \( Vi = (1 \times 100) / 500 = 0.2 \text{ mL} \)

Take 1 mL of 1 M Tris and 0.2 mL 0.5 M EDTA and mix with 98.8 mL water. 
*Check your result. Does it make sense?*

- **Example 2**
  How do you prepare 250 mL of buffer containing 100 mM Tris and 10 mM NaCl using stocks of 0.5 M Tris and 0.1 M NaCl?
  **Tris**
  \[
  Ci \times Vi = Cf \times Vf \\
  500 \times Vi = 100 \times 250 \\
  \text{so } Vi = (100 \times 250) / 500 = 50 \text{ mL} 
  \]
  **NaCl**
  \[
  Ci \times Vi = Cf \times Vf \\
  100 \times Vi = 10 \times 250 \\
  \text{so } Vi = (10 \times 250) / 100 = 25 \text{ mL} 
  \]
  Therefore, mix 50 mL of 0.5 M Tris, 25 mL 0.1 M NaCl and 175 mL water.
  *Check your result. Does it make sense?*

- **Example 3**
  I have a 10% solution of NaCl in a tube and would like to make 65 mL of a 7% NaCl solution. What do I do?
  \[
  Ci \times Vi = Cf \times Vf \\
  10 \times Vi = 7 \times 65 \\
  Vi = (7 \times 65) / 10 = 45.5 \text{ mL} 
  \]
  Take 45.5 mL of 10% NaCl stock and mix with 19.5 mL of water.
  *Check your result. Does it make sense?*

**Simple Dilution**

A simple dilution is one in which a unit volume of a liquid material of interest is combined with an appropriate volume of a solvent liquid to achieve the desired concentration.

The dilution factor is the total number of unit volumes in which your material will be dissolved. The diluted material must then be thoroughly mixed to achieve the true dilution.
For example, for a 1:5 dilution combine 1 unit volume of stock solution (the material to be diluted) + 4 unit volumes of the solvent (the diluent). This is known as a 1+4 dilution, and has a dilution factor of 5. The dilution factor is frequently expressed using exponents: 1:5 would be $5^{-1}$; 1:100 would be $10^{-2}$, and so on.

Dilutions can be written in different ways. For example, one part in ten dilution can be written in any of these ways: 1:10

- 1 in 10
- 1 part in 10
- 1+9
- $10^{-1}$
- dilution factor of 10

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### Lab math cheat sheet

A **mole** of a chemical has a mass equal to the molecular weight (or formula weight) in grams.

A **one molar** solution contains 1 mole of a chemical in a total volume of 1 litre.

When making molar solutions use the formula:

**Grams required = (MW in g) x (desired molarity in moles) x (volume in litres)**

Percentage \( (w/v) \) = weight \( (g) \) in 100 ml of solution

Percentage \( (v/v) \) = volume \( (ml) \) in 100 ml of solution

A 20X solution is 20 times more concentrated and would require a 1:20 dilution (i.e. a 1 + 19 dilution) to attain the typical working concentration.

One part in ten dilution can be written

1:10 ; 1 in 10 ; 1+9 ; $10^{-1}$ ; and the dilution factor is 10.

When making working solutions from concentrated stock solutions use the formula: \( C_i x V_i = C_f x V_f \) with normalised units.
Internet resources and further information

1. Molarity
   http://en.wikipedia.org/wiki/Concentration#Molarity

2. Scientific notation

3. Lecture: David R. Caprette, Ph.D. Solutions and Dilutions
   http://www.bioedonline.org/presentations/index.cfm#presentation35

4. How to Make Simple Solutions and Dilutions
   http://abacus.bates.edu/~ganderso/biology/resources/dilutions.html

5. BioMath Calculators
   http://www.promega.com/biomath/default.htm

6. Introduction to lab math

7. Laboratory Math for Biologists
   http://www.rickhershberger.com/bioactivesite/lab/math/labmath.ppt#292

8. Notes on Basic Laboratory Mathematics, Laboratory Solutions & Some Practice Problems
   http://www.tracy.k12.ca.us/thsadvbio/pdfs/biotechmath.pdf

9. Chemistry Tutorial 10.2a: Concentration - Molarity
   http://www.youtube.com/watch?v=JwV3F708Zrl

10. Molarity
    http://www.youtube.com/watch?v=MH0pNcvfsm8

11. Maths for Science: Open University online course
    http://openlearn.open.ac.uk/mod/oucontent/view.php?id=398516&direct=1

12. Using numbers and handling data: Open University online course
    http://openlearn.open.ac.uk/mod/oucontent/view.php?id=398704

13. Mole and Avogadro’s Number: Khan Academy lecture
    http://www.khanacademy.org/science/chemistry/v/the-mole-and-avogadro-s-number
4. Genomic DNA extraction from animal materials

Introduction
Adapted from
• http://www.qiagen.com/literature/benchguide/pdf/1017778_benchguide_chap_2.pdf

Collection and storage of samples
The quality of the starting material affects the quality and yield of the isolated DNA. Optimal results are obtained with fresh material, or with material that has been immediately frozen (e.g. frozen in liquid nitrogen or dry ice) and stored at –80°C or liquid nitrogen. Repeated freezing and thawing of stored samples should be avoided, as this leads to reduced fragment size and precipitation of the DNA, and in diagnostic samples, to reduced yields of pathogen DNA (e.g. viral DNA). In general, genomic DNA yields will decrease if samples are stored at either 4°C (fridge temperature) or –20°C (general freezer temperature) without previous treatment. Animal tissues can also be stored in 70-90% ethanol at 4°C, often for many months without loss of DNA quality.

DNA purification
The purpose of DNA purification from the cell is to ensure it performs well in subsequent downstream applications such as PCR, AFLP, microsatellite analysis, SNP analysis etc. Ideally, the DNA should be free of contamination with protein, carbohydrate, lipids, or other nucleic acid (e.g. DNA free of RNA).

The initial release of the cellular material is achieved by breaking the cell and nuclear membranes (cell lysis). Lysis must take place in conditions that will not damage the nucleic acid. Following lysis, the DNA is purified. There are many methods that have been developed for the purification of DNA, which reflects the diversity of the sample sources (animal, plant, fungi, protozoa, bacteria, viruses).

After release of DNA from the cell, further purification requires removal of contaminating proteins, lipids, carbohydrates, RNA and any cell debris. Traditionally, this was accomplished by ‘organic extraction’ using a combination of high salt, low pH, and an organic mixture of phenol
and chloroform. The combination readily dissolves hydrophobic contaminants such as lipids and lipoproteins, collects cell debris, and strips away most DNA-associated proteins. After extraction with phenol/chloroform the DNA is then precipitated with alcohol (ethanol or isopropanol), and then washed with 70% ethanol to remove salts.

Safety concerns in the laboratory make the use of phenol undesirable. Methods of DNA isolation that do not require phenol extraction have been developed and are used in many laboratories. An example is given below.

**Silica-based column purification of DNA: Using commercial kits**

These days many labs use commercial spin column kits for the isolation of nucleic acids. The spin columns contain a silica resin that selectively binds DNA. Briefly, a cell lysate in high concentrations of chaotropic salts is applied to a silica column, and the DNA in solution adsorbs to the solid phase. After the immobilized DNA is washed with optimized wash buffer to remove cellular proteins and metabolites, the purified DNA is eluted in a specific volume of low salt buffer. No alcohol precipitation is required. This method is safer than methods where organic extraction is used.

Silica-based column kits are available for purification of genomic DNA from animal, plant and microbial sources. Kits are also available for the purification of RNA, plasmids and PCR products.
Chelex method of DNA extraction

Chelex offers a fast, cheap, and effective method of DNA extraction. Chelex 100 resin is a chelating material from Bio-Rad. It is noteworthy for its ability to bind transition metal ions. Chelex resin is often used for DNA extraction in preparation for PCR. The exact role of Chelex in DNA preparation is uncertain. The Chelex appears to protect the DNA from the effects of the heating used to release the DNA from the cells, perhaps through sequestering divalent heavy metals that would otherwise damage the DNA.

There are several reasons to prefer this method over others. In as little as 12 min, one can have DNA suitable for PCR. The risk of contamination resulting from opening and closing of tubes is greatly reduced because the tube is opened just one time to add the sample. It is inexpensive which is important when processing large numbers of samples. It requires little tissue; all that is needed for a successful reaction, is a piece of tissue approximately the size of the period at the end of this sentence. The Chelex method works with a variety of tissues including blood, muscle and whole insects.
DNA extraction from muscle tissue using a spin column kit

In the workshop you will purify genomic DNA from animal muscle tissues using an Invitrogen PureLink Genomic DNA Kit. The DNA obtained will be very pure with high molecular weight, suitable for PCR. DNA will then be used to PCR amplify the CO1 gene, the target DNA Barcoding gene for animals.

PureLink Genomic DNA Kit Overview

Equipment and materials

1. Microfuge (Eppendorf tube centrifuge, microcentrifuge)

2. Vortexer
3. Gilson Pipetman pipettes and aerosol resistant filter pipette tips (D10, D20, D100, D200, D1000)

4. Water bath set at 55°C. Eppendorf Snap-Cap Microcentrifuge Safe-Lock Tubes, 0.5 mL and 1.5 mL (Fisher Scientific # 05-402-18; 05-402-25).

5. PureLink Genomic DNA Kit (Invitrogen # K1820-01). Store at room temperature.
6. Absolute (100%) ethanol. Store at room temperature.
7. Sterile scalpel blades and clean microscope slides.
8. Nitrile gloves
9. Muscle tissue sample (stored in 80% ethanol at 4°C)

**Method**

- All centrifugations (spins) are done in a microfuge at room temperature. Ensure the centrifuge rotor is balanced before spinning.
- Make sure there is no precipitate visible in PureLink Genomic Digestion Buffer or PureLink Genomic Lysis/Binding Buffer. If any precipitate is visible in the buffers, warm the buffers at 37°C for 3-5 min and mix well to dissolve the precipitate before use.
- Add absolute ethanol to PureLink Genomic Wash Buffer 1 and PureLink Genomic Wash Buffer 2 according to instructions on each label. Mix well. Mark on the labels that ethanol is added. Store both wash buffers with ethanol at room temperature.
- Use filter tips throughout.
- PureLink Genomic Lysis/Binding Buffer and Wash Buffer 1 contain guanidine hydrochloride. Guanidine hydrochloride is extremely hazardous in case of ingestion. Harmful in case of skin contact (irritant), of eye contact (irritant). Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering. Contact of
Guanidine hydrochloride with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid wastes containing this product. Wear gloves and eye protection when handling these products.

1. You will each receive a small piece of muscle tissue (of unknown origin) in 75% ethanol in a 1.5 mL Eppendorf tube. Each muscle sample is about 25 mg. These samples are stored at 4°C.
2. Remove as much of the ethanol as possible using a 200 µL pipette tip. Dispose of the ethanol.
3. Using a sterile scalpel blade, chop the muscle sample as finely as possible on a clean glass microscope slide. Transfer the chopped tissue to a clean 1.5 mL Eppendorf tube.
4. Add 180 µL PureLink Genomic Digestion Buffer and 20 µL Proteinase K (supplied with the kit) to the tube. Ensure the tissue is completely immersed in the buffer mix.
5. Incubate at 55°C with occasional vortexing until lysis is complete (1-4 hours, although the lysis incubation can be left overnight if this is convenient).
6. To remove any particulate materials, centrifuge the lysate at 13,000 rpm in a microfuge for 3 min at room temperature.
7. Transfer supernatant to a new 1.5 mL Eppendorf tube.
8. Add 20 µL RNase A (supplied in the kit) to the lysate, mix well by brief vortexing, and incubate at room temperature for 2 min. Microfuge briefly.
9. Add 200 µL PureLink Genomic Lysis/Binding Buffer and mix well by vortexing for 5 sec.
10. Add 200 µL absolute ethanol to the lysate. Mix well by vortexing for 5 sec. Microfuge briefly. When processing multiple samples, you may prepare a master mix of the buffer and ethanol by mixing equal volumes of each relative to the number of samples.
11. Remove a PureLink Spin Column in a Collection Tube from the package.
12. Add the lysate (~640 µL) to the PureLink Spin Column.
13. Centrifuge the column at 13,000 rpm in a microfuge for 1 min at room temperature.
14. Discard the collection tube and place the spin column into a clean PureLink Collection Tube supplied with the kit.
15. Add 500 µL Wash Buffer 1 (already prepared with ethanol; see above) to the column.
16. Centrifuge column at room temperature at 13,000 rpm for 1 min at room temperature.
17. Discard the collection tube and place the spin column into a clean PureLink collection tube supplied with the kit.
18. Add 500 µL Wash Buffer 2 (already prepared with ethanol; see above) to the column.
19. Centrifuge the column at 13,000 rpm for 1 min at room temperature.
20. Discard collection tube. Place the spin column in a clean 1.5 mL Eppendorf tube (without lid).
21. Centrifuge the column at 13,000 rpm for 2 min at room temperature. This step is necessary to remove excess ethanol from the column. Any remnant ethanol will inhibit downstream DNA manipulations.
22. Discard collection tube. Place the spin column in a clean 1.5 mL Eppendorf tube (without a lid).
23. Add 25 µL of PureLink Genomic Elution Buffer to the column. Incubate at room temperature for 2 min.
24. Centrifuge the column at 13,000 rpm for 1 min at room temperature.
25. Leave the column in the tube. Add another 25 µL of PureLink Genomic Elution Buffer to the column. Incubate at room temperature for 2 min.
26. Centrifuge the column at 13,000 rpm for 1 min at room temperature. The eluate in the tube contains purified genomic DNA.
27. Transfer the DNA to a clean, labelled 0.5 mL Eppendorf tube.
28. Store the purified DNA at -20°C.

Agarose electrophoresis of genomic DNA (see Chapter 6 for more information on agarose gel electrophoresis)
1. Transfer 3 µL of the purified genomic DNA to a clean 0.5 mL Eppendorf tube and mix with 3 µL 2X DNA Gel Loading Buffer.
2. Load sample onto a 0.8% agarose gel with GelRed.
3. In three wells on the gel load 50 ng, 100 ng and 200 ng of Lambda DNA standards. These are provided at 10, 20 and 40 ng/µL in 1X DNA Gel Loading Buffer. Load 5 µL of each.
4. Also include a DNA Ladder in an outside well.
5. Run the gel at 50-80 V until the Bromophenol Blue dye has reached 2/3 the length of the gel or the Orange G dye has reached 3/4 the length of the gel.
6. Record an image of the gel using a gel documentation system.
7. Estimate the concentration of the genomic DNA by comparing with the Lambda Standards on the agarose gel.
**Nanodrop spectrophotometry of genomic DNA** (See Chapter 10 for instructions on using the Nanodrop). The NanoDrop is a cuvette-free spectrophotometer that uses just 1-2 µL to measure nucleic acid concentration and purity.

1. Blank the Nanodrop with 1.5 µL of diluent used for your genomic DNA sample.
2. Perform spectral measurement 1.5 µL of your samples.
3. Dilute an aliquot of the DNA in Nuclease Free Water in a labelled 0.5 mL Eppendorf tube to give 50 µL of 10 ng DNA/µL.
4. Store both the undiluted and diluted genomic DNA at -20°C.

**Example:** 0.8% agarose gel of high quality genomic DNA extracted from Cape Buffalo muscle using a PureLink kit.

![M 1](image)

M: GeneRuler 1 kb Plus DNA Ladder; 1: Cape Buffalo genomic DNA (gDNA)
Chelex method of DNA extraction from muscle tissue

This is a simple, rapid and cheap method that produces gDNA that is pure enough for PCR.

Equipment and materials

1. Chelex 100 Molecular Grade Resin (Bio-Rad # 142-1253)
2. Nuclease Free Water
3. Eppendorf Snap-Cap Microcentrifuge Safe-Lock Tubes, 0.5 mL (Eppendorf # 05-402-18)
4. Gilson Pipetman pipettes and aerosol-resistant filter pipette tips
5. PCR thermocycler with 0.5 mL tube block
6. Microcentrifuge
7. Vortexer
8. Nitrile gloves
9. Sterile scalpel blades and glass microscope slides
10. Muscle tissue sample (stored in 80% ethanol at 4°C)

Method

- Use filter tips throughout
- All centrifugations (spins) are done in a microfuge at room temperature. Ensure the centrifuge rotor is balanced before spinning.

1. You will each receive a very small piece of muscle tissue of unknown origin in a 0.5 mL Eppendorf tube. Each muscle sample is about 5 mg.
2. Add 100 µL of freshly prepared 20% (w/v) Chelex in Nuclease Free Water to the sample.
   *Chelex settles quickly so if the mixture if not well mixed your concentrations and results will be variable.*
3. Mix by briefly vortexing.
4. Heat the sample at 95°C for 5 min on a thermocycler with a heated lid.
5. Centrifuge the tubes at 14,000 rpm at room temperature for 5 min.
6. Discard the pellet and transfer supernatant to a new 0.5 mL Eppendorf tube. Label and store at -20°C.

**Agarose electrophoresis of genomic DNA** (see Chapter 6 for more information on agarose gel electrophoresis)
1. Transfer 3 µL of the purified genomic DNA to a clean 0.5 mL Eppendorf tube and mix with 3 µL 2X DNA Gel Loading Buffer.
2. Load sample onto a 0.8% mini agarose gel with GelRed.
3. In three wells on the gel load 50 ng, 100 ng and 200 ng of Lambda DNA standards. *These are provided at 10, 20 and 40 ng/µL in 1X DNA Gel Loading Buffer. Load 5 µL of each.*
4. Also include a DNA Ladder in an outside well.
5. Run the gel at 60-80 V until the Bromophenol Blue dye has reached 2/3 the length of the gel or the Orange G dye has reached 3/4 the length of the gel.
6. Record an image of the gel using a gel documentation system.
7. Estimate the concentration of the genomic DNA by comparing with the Lambda Standards on the agarose gel.

**Nanodrop spectrophotometry of genomic DNA** (See Chapter 10 for instructions on using the Nanodrop). The NanoDrop is a cuvette-free spectrophotometer that uses just 1-2 µL to measure nucleic acid concentration and purity.
1. Blank the Nanodrop with 1.5 µL of diluent used for your genomic DNA sample.
2. Perform spectral measurement 1.5 µL of your samples.
3. Dilute an aliquot of the DNA in Nuclease Free Water in a labelled 0.5 mL Eppendorf tube to give 50 µL of 10 ng DNA/µL.
4. Store both the undiluted and diluted genomic DNA at -20°C.

**Internet resources and further information**
1. Information on other methods of DNA purification
2. Animal sample FTA protocols
3. FTA general information
   http://www.whatman.com/References/51613%20FTA%20Cards%20Data%20Sheet%281%29.pdf
4. How silica spin columns work
5. Qiagen: Genomic DNA Purification Technical hints, applications, and protocols: Link
6. Promega: DNA Purification
   http://www.promega.com/~media/Files/Resources/PAGuide/Letter/chap9.ashx
7. DNA Extraction procedures
8. Walsh et al. (1991) Chelex 100 as a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material.
5. Genomic DNA extraction from plant materials

Introduction
Adapted from:

  http://cshprotocols.cshlp.org/content/2009/3/pdb.prot5177.abstract

Plant sample collection and storage before isolation of genomic DNA
Leaves are harvested from tissue cultured plants, the greenhouse or field-grown plants. It is preferable to use young leaves without necrotic areas or lesions, although older leaves which are not senescent may be used. If the midrib is thick and tough, remove it. Cut or fold leaves into 10-15 cm sections and place in a plastic screen mesh bag along with the tag identifying the sample. (Aluminium foil or paper bags may be substituted if holes are punched to allow good air flow.) Place bags in an ice chest or other container with ice to keep samples cool but do not allow them to freeze. Make sure samples do not get wet. Leaf samples can be dried using silica gel, and dried leaf samples may be stored in sealed plastic bags at room temperature for a few days or, preferably, at -20°C where the leaf materials will be stable for several years. Fresh leaf samples may be frozen and stored at -80°C for up to one year.

Fresh leaf samples yield the highest quality DNA, and in the Workshop you will use sample fresh young leaves from plants collected from the ILRI campus.

DNA purification from plant materials
Plant materials are among the most difficult for high quality DNA extractions. DNA extraction from plant tissue can vary depending on the material used. The key is to properly prepare the tissues for DNA extraction. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen in a mortar and pestle is employed to break
down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated.

Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, containing reagents such as cetyltrimethyl ammonium bromide (CTAB) or SDS (sodium Dodecyl Sulphate). The presence of polysaccharides in a plant DNA preparation can inhibit techniques such as polymerase chain reaction (PCR). Cetyltrimethyl ammonium bromide (CTAB) is a surfactant useful for isolation of DNA from tissues containing high amounts of polysaccharides. Under the high-salt conditions used in this protocol, the CTAB binds the polysaccharides, removing them from the solution. SDS is a strong anionic detergent useful for isolation of DNA from tissues containing high amounts of polysaccharides.

The solubilised DNA is then extracted with chloroform to remove lipids and proteins. Finally, DNA must then be precipitated from the aqueous phase and washed to remove contaminating salts.

**Genomic DNA extraction from plants: modified Dellaporta method**

The method used in the Workshop is a simplification of the Dellaporta (2003) method.

**Equipment and materials**

1. Nitrile gloves
2. Refrigerated and non-refrigerated microcentrifuges
3. 0.5 mL and 1.5 mL Eppendorf tubes
4. Eppendorf tube micro pestles
5. Scissors and forceps
6. Household bleach (10%) diluted in tap water
7. Shaking water bath at 65°C
8. Water bath at 37°C
9. SDS/DTT Extraction Buffer. Store at room temperature. Prewarm to 65°C before use.

11. Isopropanol. Store at -20°C in a 100 mL Duran bottle.

12. 70% ethanol and 100% (absolute) ethanol. Store both in 100 mL Duran bottles at -20°C.

13. Low salt TE buffer. Store at room temperature.

14. RNAse A (Merck Millipore # 70856). Supplied at 10 mg/ml in 10 mM Tris-HCl, 1 mM EDTA, 50% glycerol, pH 7.5. Store at -20°C.

15. 3 M sodium acetate (pH 8.0). Store at room temperature.

16. 5M potassium acetate (pH 8.0). Store at room temperature.

17. Lambda DNA standards. These are provided at 10, 20 and 40 ng/µL in 1X DNA Gel Loading Buffer. Load 5 µL of each. Store at -20°C.

18. Nuclease Free Water (PCR Grade Water; Roche # 3315932001). Store at room temperature.

**Method**

**Tissue extraction**

1. Collect a young fresh leaf from two different plants of your choice from the ILRI grounds. Take a photograph of the plants for your records.

2. Wrap the leaf in foil and take to the lab.

3. In the lab, cut ~200 mg of young leaf using clean scissors. Using clean forceps transfer the sample to a labelled 1.5 mL Eppendorf tube. Keep on ice until you are ready for the next step.

   *Rinse scissors and forceps in 10% bleach solution, tap water, then 70% ethanol (each in a beaker) and air dry after each sample. This will prevent cross-contamination between samples.*

4. When ready, remove tubes from ice and add 50 µL SDS/DTT Extraction Buffer (pre-warmed to 65°C) to each sample tube.

5. Grind the tissue using a microtube micro-pestle until a uniform fine pulp is produced. Leave pestle in tube after grinding.

6. Add 550 µL SDS/DTT Extraction Buffer (pre warmed to 65°C) while rinsing off the micro pestle. Remove the pestle, and then cap the tube.

7. Put the tube into a shaking water bath at 65°C for 30-60 min. Flick regularly to mix.

8. Centrifuge the tube at 14,000 rpm in a microfuge for 5 min at room temperature to pellet plant debris.
9. Transfer upper, greenish phase to a clean 1.5 mL Eppendorf tube.
   **Note:** Be sure NOT to pipette up any of the pellet or floating debris.

**Precipitation of Proteins and Polysaccharides**
10. Add 250 µL of ice-cold 5M potassium acetate. Mix by gently inverting 5-6 times.
11. Incubate on ice for 5 min.
12. Centrifuge in a microfuge at 14,000 rpm for 10 min at 4°C in a refrigerated microfuge.
13. Transfer 450 µL of supernatant to a new 1.5 mL Eppendorf tube.

**Solvent Extraction 1**
14. In a fume hood, add an equal volume (i.e. 450 µL) of chloroform:isoamyl alcohol (CIA-24:1) to each tube.
15. Cap the tube well. Then gently invert the tube gently for 3-5 min to extract thoroughly.
   **Note:** Do not shake the tubes otherwise the DNA will shear. During the extraction the solution will turn cloudy yellow/green. If you have many samples, then this step can be done with the tubes in a rack.
   **Caution:** Chloroform is a hazardous chemical, and must be handled in a fume hood. Please dispose of all used chloroform in the designated waste bottle in the fume hood.
   
16. Centrifuge the tube at 14,000 rpm for 10 min at 4°C in a refrigerated microfuge.
   *This produces two phases, an upper aqueous phase which contains the DNA, and a lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. The interface between these two phases contains most of the "junk" cell debris, degraded proteins, etc.*

17. Transfer 450 µL of the upper phase (which will be greenish in colour) taking care not to pipette up any CIA or interface material, to a new labelled 1.5 mL Eppendorf tube. Keep the tubes on ice.

**RNAse A treatment**
18. Remove RNAse A (10 mg/mL) from the freezer and keep on ice.
19. Add 3 µL RNAse A to each sample and mix by flicking the tube several times. Incubate in a water bath at 37°C for 30 min.
Solvent Extraction 2

20. In a fume hood, add an equal volume of chloroform:isoamyl alcohol (CIA-24:1) to each tube, i.e. if your sample is 450 µL then add 450 µL CIA.

21. Cap the tube well. Then gently invert the tube gently for 5 min to extract thoroughly. If you have many samples, then this step can be done with the tubes in a rack.
   **Note:** Do not shake the tubes otherwise the DNA will shear.

22. Centrifuge the tube at 14,000 rpm for 10 min at 4°C in a refrigerated microfuge. This produces two phases, a clear upper aqueous phase which contains the DNA, and a clear lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. The interface between these two phases contains most of the "junk" - cell debris, degraded proteins, etc.
   **Note:** If the aqueous layer appears cloudy, repeat the step until the solution is clear.

23. Transfer 400 µL to a new labelled 1.5 mL Eppendorf tube.
   **Note:** Make sure you do not disturb the interface layer as this is where proteins, polyphenols and polysaccharides are lurking!

Crude DNA pellet precipitation

24. Add an equal volume (400 µL) of isopropanol and mix by inverting gently 8-10 times
25. Incubate at -20°C for 1 hr.
   **Note:** The longer the chilled incubation, the more the precipitation. An overnight incubation at -20°C is preferred.

26. Centrifuge at 14,000 rpm for 15 min at 4°C.

27. Pipette off as much of the supernatant as possible without disturbing the pellet.

28. Dry the pellet by placing the open tube in a water bath at 80°C for 5 min.

70% ethanol wash

29. Add 200 µL 70% ethanol and invert the tube several times to wash the pellet.

30. Centrifuge tubes at 14,000 rpm for 10 min at 4°C.
   **If the pellet is dirty (e.g. a brown colour) then repeat the wash-step with 70% ethanol.**

31. Decant supernatant from each sample. Be very careful not to pour off the pellet.

32. Air-dry the DNA pellet for 30-60 min by leaving the open tube on its side on the bench or in a fume hood with the fan running. **Put the open tube on a piece of clean aluminium foil or Parafilm to prevent the tube from getting dirty.** Once dried, the pellet should be clear.
**Note:** Do not over-dry the pellet otherwise it may be difficult to re dissolve in the next step.

33. Add 50 µL of sterile low salt TE buffer to the pellet. Dissolve the pellet by incubating in a waterbath at 55°C with frequent mixing by flicking the tube. Or you can leave the tube at room temperature overnight to dissolve, and afterwards flick the tube to ensure complete dissolution.

**Note:** Do not vortex to mix as this will shear the DNA.

34. Store DNA at -20°C.

**Agarose gel electrophoresis of genomic DNA**

1. Remove 3 µL of the purified genomic DNA and add to a clean 0.5 mL Eppendorf tube. Mix with 3 µL 2X DNA Gel Loading Buffer.
2. Load on to a 0.8% mini agarose gel with GelRed.
3. In three wells on the gel load 50 ng, 100 ng and 200 ng of Lambda DNA standards. *These are provided at 10, 20 and 40 ng/µL in 1X DNA Gel Loading Buffer. Load 5 µL of each.*
4. Also include a DNA Ladder in an outside well.
5. Run the gel at 60-80 V for 1 hr.
6. Record an image of the gel using a gel documentation system.
7. Estimate the concentration of the genomic DNA by comparing with the Lambda Standards on the agarose gel.

**Nanodrop spectrophotometry of genomic DNA.** The NanoDrop is a cuvette-free spectrophotometer that uses just 1-2 µL to measure nucleic acid concentration and purity. See Chapter 10 for detailed instructions on using the Nanodrop.

1. Blank the Nanodrop with 1.5 µL of diluent used for your genomic DNA sample.
2. Perform spectral measurement 1.5 µL of your samples.
3. Dilute an aliquot of the DNA in Nuclease Free Water in a labelled 0.5 mL Eppendorf tube to give 50 µL of 20 ng DNA/µL.
4. Store both the undiluted and diluted genomic DNA at -20°C.
Example: 0.8% agarose gel of high quality genomic DNA extracted from various plants using the modified Dellaporta method.

M: GeneRuler 1kb Plus DNA Ladder;
1. Lambda DNA standard (200 ng); 2. Bougainvillea; 3. Hibiscus;
4. Loquat; 5. Cowpea

Internet resources and further information
3. Plant DNA extraction method [link]
4. Plant genomic DNA extraction using CTAB [link]
5. FTA protocols for plant samples [link]
6. DNA Extraction procedures [link]
6. **Agarose gel electrophoresis**

**Introduction**
Adapted from:
- [http://www.docstoc.com/docs/23538647/Agarose-Gel-Electrophoresis](http://www.docstoc.com/docs/23538647/Agarose-Gel-Electrophoresis)
- [http://cibt.bio.cornell.edu/workshops_and_summer_programs/0708alum/4-Gel.pdf](http://cibt.bio.cornell.edu/workshops_and_summer_programs/0708alum/4-Gel.pdf)

Gel electrophoresis is a widely used technique in molecular biology, used routinely for the analysis and preparation of DNA.

Electrophoresis is the separation of charged molecules in an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatine-like slab. DNA samples are loaded onto the gel in wells created by a sample comb during gel casting. For electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. DNA will move towards the positive electrode and away from the negative electrode.

Several factors influence how fast the DNA moves, including (i) the strength of the electrical field, (ii) the concentration of agarose in the gel and (iii) most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules thereby giving separation of the DNA molecules in the sample by virtue of DNA size. DNA in the gel is visualized with a DNA stain, which can either be added to the gel before casting, or can be used to stain the DNA on the gel after electrophoresis.

Ethidium bromide has been the predominant dye used for nucleic acid gel staining for decades because of its low price and generally sufficient sensitivity. However, ethidium bromide is a toxic and highly mutagenic material. The safety hazard and costs associated with decontamination and waste disposal can ultimately make the dye expensive to use. Alternative, safer gel stains, such as GelRed, have become commercially available in recent years. GelRed binds to DNA and illuminates when exposed to ultraviolet light, causing the DNA bands to fluoresce. GelRed will be used in the Workshop.
Equipment and materials

1. Nitrile gloves
2. Safety goggles
3. Heat resistant insulated gloves or Insulated Grippers.
4. P10, P20, P100, P200, P1000 Gilson Pipetman pipettes and appropriate pipette tips (D20, D20, D100, D200, D1000).
5. 0.5 mL Eppendorf tubes
6. Glassware: 500 mL flasks, 1 L & 100 mL measuring cylinders, 1 litre Duran bottle and 200 mL Duran bottles.
7. Agarose, Molecular Biology Grade (Merck Millipore # 121853). Store at room temperature.
8. GelRed DNA stain (10,000X in water) (Biotium # 41003). Store at room temperature, or at 4°C for long term storage.
9. Water bath set at 55°C
10. DNA Gel Loading Buffers. Two 6X buffers are supplied. Select either for use in the workshop.
   i. 6X DNA Gel Loading Buffer (Merck Millipore # 69180-3). 6X Loading Buffer is for gel electrophoresis of DNA fragments or PCR products, and contains Orange G, Xylene Xylanol FF, and glycerol. In 1% agarose gels Orange G co-migrates with a ~50 bp DNA fragment and Xylene Cyanol with ~4000 bp DNA fragment. Store at 4°C.
   ii. 6x Orange Loading Dye Solution (Fermentas # R0631) contains 10mM Tris-HCl (pH 7.6) 0.15% Orange G, 0.03% Xylene Cyanol FF, 60% glycerol and 60mM EDTA. Store at room temperature, or at 4°C for up to 12 months.
   Prepare a 2X DNA Gel Loading buffer by mixing 0.5 mL 6X buffer and 1 mL sterile deionised water. Store at 4°C.
11. DNA ladders. Four DNA ladders are supplied. Select any for use in the workshop.
   i. Quick-Load 100 bp DNA Ladder (50 μg/mL) (NEB # N0467S). This DNA Ladder is a pre-mixed, ready-to-load molecular weight marker containing Bromophenol Blue as a tracking dye, which migrates with DNA of 500 bp in a 1% agarose gel. It contains 12 bands suitable for use as molecular weight standards for agarose gel electrophoresis. Use 10 μL per well (lane). The ladder includes fragments ranging from 100-1,517 base pairs. The 500 and 1,000 bp bands have increased intensity to serve as reference bands. Quick-Load 100 bp DNA Ladder is stable for at least
6 months at 25°C. For long term storage, store at 4°C or -20°C. If stored at -20°C, mix well after thawing.

ii. **Low Molecular Weight DNA Ladder, 500 μg/mL (NEB # N3233S).** This ladder includes fragments ranging from 25-766 bp. The 200 bp band has increased intensity to serve as a reference point. For long term storage, store at -20°C; mix well after thawing. Gel Loading Dye, Blue (6X) is supplied with the ladder, and contains Bromophenol Blue which migrates with DNA of 500 bp in a 1% agarose gel. To prepare a ready-to-use solution of the ladder, mix 100 μL Low Molecular Weight DNA Ladder, 167 μL Gel Loading Dye, Blue (6X), and 733 μL TE buffer in a 1.5 mL Eppendorf tube. Mix well, microfuge briefly and prepare 200 μL aliquots in 0.2 mL Eppendorf tubes. For long term storage, store at -20°C; mix well after thawing. Keep an ‘in-use’ aliquot at 4°C. Use 10 μL per well (lane) on a gel.

iii. **Perfect DNA 100 bp Ladder (Merck Millipore # 70539).** The Perfect 100 bp DNA Ladder is supplied ready-to-use at 125 μg/ml in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. It contains 13 bands with fragments of the following sizes: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000 and 2500 bp. Bands at 500 and 1000 bp are brighter for easy reference on agarose gels. Use 5 μL per well (lane) on a gel. Storage: short term, store at 4°C; long term, store at -20°C.

iv. **GeneRuler™ 1 kb Plus DNA Ladder (Fermentas #SM1331)** is designed for sizing and quantification of DNA fragments in agarose gels. The ladder is composed of fifteen individual DNA fragments (in base pairs): 20000, 10000, 7000, 5000, 4000, 3000, 2000, 1500, 1000, 700, 500, 400, 300, 200, 75. It contains three reference bands (5000, 1500 and 500 bp) for easy orientation. The ladder is dissolved in TE buffer. The ladder is supplied with 6X DNA Loading Dye (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA). Before use, dilute the ladder to a 1X solution as follows: 25 μL Ladder, 25 μL 6X DNA Loading Dye, 150 μL sterile deionised water. Use 5 μL per well (lane) on a gel. Storage: short term, store at 4°C; long term, store at -20°C.
12. Lambda DNA Standards (10, 20 and 40 ng/µL in 1X DNA Gel Loading Buffer). Store at -20°C. Use 5 µL/well on a gel.
13. 10X TBE buffer (BecA-ILRI Hub Central Core)
14. Deionised water (BecA-ILRI Hub Central Core)
15. Microwave oven
16. Water bath at 55-60°C
17. Electrophoresis gel tank, power supply, gel casting tray, combs (Mupid-ex gel kit; Eurogentec #MU-0040)

18. **Gel documentation system.** Also known as a **Gel Doc System.** It is widely used in molecular biology laboratories for the imaging and documentation of nucleic acids on agarose gels stained with a fluorophore such as GelRed or ethidium bromide. Typically a Gel Doc System
is composed of an ultraviolet (UV) light transilluminator, a hood to shield external light sources and a camera for image capturing.

In the workshop we will use a Syngene Ingenius 3 UV light gel doc system:

We will also use a Blue LED Transilluminator which uses pure visible blue light as the excitation source - and there is no damaging and dangerous UV radiation to worry about. You can use a digital camera (e.g. on your mobile phone) to capture gel images from the Blue LED Transilluminator.

**Method**

**Preparing agarose gels (0.8%, 1.8%, 2%)**

1. Measure out 50 mL 10X TBE buffer in a 100 mL cylinder. Transfer to a 1 litre cylinder. Add ~100 mL deionised water to the 100 mL cylinder and transfer the water to the 1 litre cylinder (*this is to remove residual TBE in the 100 mL cylinder*). Add deionised water to the 1 litre cylinder to 1 litre (this will give a 0.5X TBE solution). Transfer the 0.5X TBE to a 1 litre Duran bottle. Mix by inverting the bottle a few times. Label the bottle with contents, your name and date.

2. Make three different concentrations of agarose gels:
   - To make 250 mL of **0.8%** agarose gel: Weigh out 2.0 g of agarose powder
   - To make 250 mL of **1.8%** gel: Weigh out 4.5 g of agarose powder
   - To make 250 mL of **2%** gel: Weigh out 5 g of agarose powder

   *0.8% gel will be used to analyse genomic DNA (gDNA); 1.8% gel will be used to analyse PCR products; 2% gel will be used to analyse PCR product restriction digests.*
3. Transfer the required amount of agarose to a 500 mL flask. Add 250 mL 0.5X TBE Buffer to the flask, and swirl to get an even suspension of agarose. Put a loose fitting Duran cap or similar on the top of the flask to prevent excessive evaporation during boiling.

4. Dissolve the agarose by boiling in a microwave until the solution becomes clear: Put tissues under the flask when you microwave, which will make an accidental boil-over spill easier to clean! Heat the agarose/buffer for 10 sec, then remove from the microwave and swirl briefly to mix. Repeat until the solution starts to boil. Continue to boil, with very short intervals to prevent ‘boiling over’, until the solution becomes clear. Eye protection and heat resistant gloves must be worn when removing hot items from the microwave. Do not hold hot glassware with tissues! Beware of boiling over and spilling hot liquid!

5. Dispense the molten gel into two 125 mL aliquots in 200 mL Duran bottles. Use immediately (see below) or store at room temperature until required, but be sure to loosen the cap before microwaving again otherwise the bottle may explode!

Casting the agarose gel
1. Set up the casting tray and combs according to the manufacturers’ instructions. In the Workshop we will use the Mupid-ex gel kit (see the Kit Instructions folder on the Workshop website for more information).

2. Place the casting stand on a level surface.
   Place gel tray(s) into the casting stand. Two gels can be cast simultaneously with normal size trays.
   Put the 13-toothed comb into the casting stand.
3. Melt a 125 mL agarose aliquot in a microwave oven as described above.
4. Cool in a water bath at 55-60°C for 30 min, gently swirling every 10 min so it cools evenly. **IMPORTANT! Failure to cool the solution sufficiently could deform the stand and gel tray.** Cooling can be done more rapidly under a running cold water tap, with continuous, gentle swirling so it cools evenly. The gel is at the correct temperature for pouring when the bottle can just be held comfortably. Note: excessive cooling will lead to the gel solidifying in the bottle; if this happens then you must re-melt by reheating in a microwave, and then cool again to 55-60°C. Avoid vigorous swirling to prevent introducing bubbles into the molten gel!

5. Add 3.75 μL GelRed to 125 mL agarose solution. Swirl gently to mix to give an even solution, but avoid introducing air bubbles.
6. Pour an appropriate volume of melted agarose into the casting tray fitted with a sample comb.  
   *The correct volume to use in a gel cast will be indicated in the manufacturers’ instructions. For the Mupid-ex gel kit, about 30 mL of solution is required to yield a gel 4 mm thick in small size trays (half gel), and about 60 mL of solution is required in large size trays (whole gel).*
For this alternative casting system (below), pour approximately 12.5 mL gel into the small gels, and 25 mL gel in the large gels.

Bubbles on the surface of the gel can be removed by ‘pricking’ with a syringe needle or pipette tip. Alternatively they can be moved to the edge of the gel with a syringe needle or a pipette tip.

Any unused gel in the Duran bottle can be stored at room temperature for later use.

7. Allow the gel to solidify. It will turn from clear to translucent as it solidifies, and will take about 30-40 min.
8. Remove the comb(s) with both hands by gently pulling upwards.

Note: for some gel kits the comb can be removed after the gel has been placed into the electrophoresis gel tank and overlaid with buffer.
9. Hold the gel tray on the both sides at the notches of the casting stand. Pull the tray upwards. Gel preparation is now complete.

Gels can be stored in the gel tray at 4°C for up to a week: Wrap in cling film and store in a ‘moist box’ (a lunch box with tissues soaked in water or 0.5X TBE) to prevent the gels from drying out.

Alternatively, to store the gel, keep it in the gel tray and casting stand and saturate with a small quantity of 0.5X TBE buffer and cover the whole gel and tray with cling film to protect it from drying out and contamination, and store at 4°C.

10. Place the gel and gel tray on the gel bed of the Gel Tank Unit, without removing it from the tray. Be careful the gel does not slide from the tray.

Be sure the sample wells are nearest to the negative (black) electrode. DNA is negatively charged and will migrate towards the positive (red) electrode.

11. Add 0.5X TBE to the tank so the gel is covered by ~ 2 mm of buffer. The buffer volume required for the chamber will be indicated in the manufacturers’ instructions. For the Mupid-exU gel kit, the volume required is approximately 300 mL. Always use fresh buffer for each gel run.
Loading the DNA samples onto the gel

1. To a 0.5 mL labelled Eppendorf tube, mix the required µL of your DNA sample with an equal volume of 2X DNA Gel Loading Buffer. Gently mix by gently pipetting up and down 3-4X, or by flicking the tube.

2. Carefully pipette each sample into separate wells in the gel. Make sure to record the order of the samples on the gel. Do not damage the wells with the pipette tip. Use a fresh tip for each sample, or wash the tip after each sample by pipetting multiple times in a beaker of sterile deionised water or in the buffer in the gel tank. Leave outside wells for the DNA size Ladder (1 well) or Lambda DNA Standards (3 wells).

3. Load the DNA size ladder or Lambda DNA Standards:
   a. When analyzing PCR products, pipette appropriate amount of DNA Ladder into one of the outside wells.
   b. When analyzing genomic DNA, pipette 5 µL of each of the Lambda DNA Standards (10, 20, 40 ng/µL in 1x DNA Gel Loading Buffer), into three outside wells. The Lambda DNA Standards, diluted to the appropriate concentration, are supplied.

4. Do not move the tank after the samples have been loaded, otherwise the samples may be washed out of the wells.

Running the gel (electrophoresis)

Caution: Electrical hazard! Never use electrical equipment with wet gloves on. Keep water and buffer away from all electrical connections. Do not use an electrical connection if it is wet.

1. Place the lid on the gel box (and where necessary connect the electrodes to the gel tank) and connect the power supply. Make sure the positive (red) and negative (black) electrodes are correctly connected to the power supply. DNA is negatively charged so will migrate towards the positive (red) electrode.

2. Turn on the power supply.
3. Set the voltage (usually between 50 and 100 V)
4. Set the time (usually between 30 and 60 min).
5. Start the run.
6. Ensure the current is running through the buffer by looking for bubbles forming on each electrode. Also ensure that the current is running in the correct direction by observing the movement of the dye in the sample buffer (it will take a minute or before you can see a movement). The dye should move towards the positive (red) electrode. *The dye will run in the same direction as the DNA.*
7. Run the gel until the Bromophenol Blue dye has reached 2/3 the length of the gel or the Orange G dye has run 3/4 the length of the gel.
8. Turn off the power on the power pack and at the socket (*and where necessary disconnect the wires from the power supply and the tank*).
9. Remove the lid of the gel tank.
10. Carefully remove the gel and the gel tray and transfer to a plastic box.
11. Rinse the gel in deionised water or tap water.

**Viewing and photographing the gel with the Gel Doc System**

*UV transilluminator Gel Doc system*

**Caution: Radiation Hazard!** UV Transilluminators are powerful sources of UV radiation that will cause serious damage to unprotected eyes and skin. Wear a UV face mask and gloves for protection when using an open transilluminator.
1. Place the gel on the UV transilluminator and turn on the UV light.
2. Observe the DNA bands on the screen and record the gel image with the digital video camera.
3. Turn off the UV light.
4. Save the file.
5. Dispose the gel in the appropriate bin.
6. Clean the UV box with damp tissues.

*Blue LED Transilluminator Gel Doc System*
1. Place the gel on the transilluminator.
2. Close the orange amber filter lid.
3. Turn on the LED light to view the fluorescence.
4. Record the gel image with a digital camera.
5. Turn off the LED light.
6. Dispose the gel in the appropriate bin.
7. Clean the LED box with damp tissues.
**Cleaning the gel equipment**

1. Dispose the tank buffer in the sink.
2. Rinse the tank with deionised water.
3. Clean the casting tray, gel tray and combs with deionised water. Be sure the trays and combs are clean of residual agarose gel.

**Internet resources and further information**

1. Making an Agarose Gel - University of Leicester
   [http://www.youtube.com/watch?v=wXiITW3pflM](http://www.youtube.com/watch?v=wXiITW3pflM)
2. Running an Agarose Gel - University of Leicester
   [http://www.youtube.com/watch?v=U2-5ukpKg_Q&feature=relmfu](http://www.youtube.com/watch?v=U2-5ukpKg_Q&feature=relmfu)
3. Agarose Gel Electrophoresis
   [http://www.youtube.com/watch?v=9f2VSyVhsGl](http://www.youtube.com/watch?v=9f2VSyVhsGl)
4. How to Make and Run an Agarose Gel (DNA Electrophoresis)
   [http://www.youtube.com/watch?v=2UQIoYhOowM](http://www.youtube.com/watch?v=2UQIoYhOowM)
5. Ask a biologist: agarose gel electrophoresis
   [http://askabiologist.asu.edu/agarose-gel-electroporesis](http://askabiologist.asu.edu/agarose-gel-electroporesis)
6. Agarose gel electrophoresis (basic method)
   [http://www.methodbook.net/dna/agarogel.html](http://www.methodbook.net/dna/agarogel.html)
7. DNA ladders (Wikipedia)
8. Overview of DNA stains
9. GelRed information
10. Mupid-exU gel system
11. Mupid ex gel system Manual
12. How to Make and Run an Agarose Gel (DNA Electrophoresis)
    [http://www.youtube.com/watch?v=2UQIoYhOowM](http://www.youtube.com/watch?v=2UQIoYhOowM)
13. Qiagen Benchguide Chapter 1
14. Agarose Gel Electrophoresis
7. **Polymerase Chain Reaction (PCR)**

**Introduction: PCR Basics**
Adapted from the Promega website:


The polymerase chain reaction (PCR) is a simple technique that amplifies a DNA template to produce multiple copies of a specific DNA fragment *in vitro*. Traditional methods of cloning a DNA sequence into a vector and replicating it in a living cell often require days or weeks of work, but amplification of DNA sequences by PCR requires only hours.

A typical amplification reaction includes target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Once assembled, the reaction is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures for set amounts of time. This series of temperatures and times is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence in the reaction. Ten cycles theoretically multiply the target by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of 1-2 hours.

Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension. The initial step denatures the target DNA by heating it to 94-95°C for 15-120 seconds. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermostable DNA polymerase. In the next step of a cycle, the temperature is reduced to approximately 45-65°C for 15-60 seconds. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase to synthesise a new DNA strand in the final step. The synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is about 72°C. The extension step is usually 30-120 seconds. The next cycle begins with a return to 94-95°C for denaturation.
The thermostable DNA polymerase most commonly used in PCR is Taq DNA polymerase, named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated. It is often abbreviated to "Taq Pol" or simply "Taq".

PCR image from scienceblogs.com:
http://scienceblogs.com/insolence/2007/06/the_autism_omnibus_the_difference_betwee.php
DNA barcoding: PCR amplification of the CO1 gene from animal DNA

Introduction: DNA Barcoding
Adapted from:

A DNA barcode is a short DNA sequence taken from standardized portions of the genome and are used to identify species. They can be obtained reasonably quickly and cheaply, especially through PCR.

Since Linnaeus, biologists have used distinguishing features in taxonomic keys to apply binomial species names, such as Homo sapiens. From insects to birds, evidence now shows that short DNA sequences from a uniform locality (e.g. the CO1 gene) on genomes can also be a distinguishing feature. As a Linnaean binomial is an abbreviated label for the morphology of a species, the short sequence is an abbreviated label for the genome of the species. The barcode of life thus provides a master key, in addition to the binomial species name, to knowledge about a species. Compiling a public library of sequences linked to named specimens, plus faster and cheaper PCR and DNA sequencing, will make this new barcode key increasingly practical and useful.

A portion of the cytochrome c oxidase subunit 1 mitochondrial gene (CO1) is the standard barcode region for higher animals. The barcode region is 658 nucleotide base pairs (bp) from the 5’ end of the CO1 gene, a very short sequence relative to 3 billion base pairs in the human genome, for example.

In the Workshop we will use DNA barcoding to illustrate the power of PCR, DNA sequencing and bioinformatics to identify animal species. We will use PCR using CO1 gene primers with a broad target group including mammals, reptiles and fish.

For PCR you will have the opportunity to (a) use the Bioneer Accupower PCR Premix Kit tubes, and (b) to make PCR mixes from individual components.
Equipment and materials

1. Nitrile gloves
2. Vortexer
3. Plate centrifuge
4. Microfuge
5. Ice bucket and ice
6. Pipettes and filtered pipette tips
7. 0.5 mL and 1.5 mL Eppendorf tubes
8. 0.2 mL PCR tubes
9. Tube racks
10. Bioneer Accupower PCR PreMix, 20 µL kit (Bioneer # K-2016).

   The Bioneer Accupower PCR PreMix kit contains 0.2 mL tubes, each containing lyophilised components of a PCR master mix (enzyme, buffer, Tween 20, MgCl2, dNTPs) and a gel tracking dye (Xylene Cyanol), plus a stabilizer (sorbitol) that maintains full activity for over one month at room temperature, and at least two years in a -20°C freezer. You simply add your primers, template DNA and Nuclease Free Water to a final volume of 20 µL, and then run the PCR. **Note: the PreMix contains Top DNA polymerase, not Taq DNA polymerase.*** Top DNA polymerase is a novel thermostable DNA polymerase that is more processive than Taq DNA polymerase. The extension rate of Top DNA polymerase is > 3X that of Taq DNA Polymerase. Top DNA polymerase can be used for a variety of PCR applications including TA cloning and is a robust enzyme for standard PCR. It contains no proofreading or 5'-3’ exonuclease activity. Store at -20°C.

11. Individual PCR reagents

   a) Three Taq DNA polymerases are available in the workshop:

      (i) **Crimson Taq DNA Polymerase** (5u/µL) with 5X Mg-free PCR Reaction Buffer (NEB # M0325L). Crimson Taq (Mg-free) Reaction Buffer contains a density reagent, which allows direct loading of PCR products onto a gel. In addition, Crimson Taq (Mg-free) Reaction Buffer has a trace amount of a red dye, which serves as a colour aid in gel loading and a tracking dye which migrates at about 10 bp on a 1% TBE agarose gel. Store at -20°C.

      (ii) **FastStart Taq DNA Polymerase** (5u/µL) with 10X Mg-free PCR Reaction Buffer (Roche # 12032929001). This modified recombinant Taq DNA Polymerase is inactive at temperatures below 75°C, but is activated by a 2- to 4-minute heat activation step at 95°C. This is known as ‘hot-start’
PCR. In hot-start PCR the polymerase is modified to ensure that it remains inactive at lower temperatures and only becomes active at the high temperatures at which primers specifically bind. This prevents the amplification of nonspecific products and increases the yield, or sensitivity, of the desired PCR product. Store at -20°C.

(iii) **DreamTaq DNA Polymerase** (5u/ µL) (Thermo Scientific #EP0701). This enzyme is supplied with a 10X reaction buffer that includes MgCl₂ at a concentration of 20 mM. For the purposes of this workshop, which you will determine the effect of MgCl₂ concentration on the PCR, do not use the DreamTaq 10X buffer. Instead use one of the Mg-free buffers supplied with the other Taq polymerases supplied in the workshop.

b) 25 mM MgCl₂ Supplied with Taq DNA polymerases. Store at -20°C.

c) dNTP Mix (Fermentas # R0191). dNTP Mix contains an aqueous solution of dATP, dCTP, dGTP and dTTP, each at a final concentration of 10 mM. Store at -20°C.

12. CO1 PCR primers (2 µM each in Nuclease Free Water). Store at -20°C.
   a) VF1d_t1 TCTCAACCAACCACAARGAYATYGG
   b) VR1d_t1 TAGACTTCTGGGGTGGCCRAARAAYCA
   Degenerate bases R= A/G; Y= C/T
   These primers have a broad target group including mammals, reptiles and fishes. Primers were obtained from Bioneer.


14. Your purified genomic DNA (gDNA) samples from muscle tissues diluted to 10 ng/µL. Store at -20°C.

15. +ve control gDNA (10 ng/µL). Store at -20°C.

16. ABI GeneAmp 9700 PCR thermocycler or equivalent.

**CO1 PCR using Bioneer Accupower PCR PreMix Kit tubes**

- PCR is very sensitive and prone to cross-contamination.
- Work as cleanly as possible.
- Use filtered pipette tips throughout.
- Always wear disposable gloves. Change gloves frequently.
- Work individually
Method

1. Thaw CO1 primers VF1d_t1 and VR1d_t1 (2 μM each) and DNA samples on ice. Flick the tubes to mix, then microfuge briefly. Return to ice.

Prepare a water/primer mix: To a 0.5 mL Eppendorf tube on ice add the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>280 μL</td>
<td>-</td>
</tr>
<tr>
<td>Primer VF1d_t1 (2 μM)</td>
<td>40 μL</td>
<td>0.20 μM</td>
</tr>
<tr>
<td>Primes VR1d_t1 (2 μM)</td>
<td>40 μL</td>
<td>0.20 μM</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>360 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

*This is sufficient for 20 reactions.*

Cap the tube, mix by flicking the a few times, then microfuge briefly. Store on ice.

2. Take two strips of 8 Bioneer Premix tubes from the freezer.
3. Label the strips of tubes using a fine black marker pen with numbers 1 to 16.
4. Add 18 μL water/primer mix to each of the 16 tubes. Cap tubes and put in a rack at room temperature.

5. To tubes 1, 2, 9, 10 add 2 μL Nuclease Free Water. Cap the tubes. *These are your negative controls.*
6. To each of tubes 3, 4, 5, 6 add 2 μL (20 ng) purified gDNA from muscle tissue (purified using the PureLink Kit). Cap tubes and return to ice.
7. To each of tubes 11, 12, 13, 14 add 2 μL (20 ng) purified gDNA from muscle tissue (purified using the Chelex method). Cap tubes and return to ice.
8. To each of tubes 7, 8, 15, 16 add 2 μL (20 ng) +ve control gDNA. Cap tubes and return to ice. *These are your positive controls.*

9. Place your tubes in the rack provided (see below), along with all PCR tubes from your group.
10. Place a lid on the rack. Vortex to dissolve the dried PreMix blue pellet by holding the rack with the tubes on the vortexer for a few seconds (below).

11. Spin the rack/tubes in a plate centrifuge at 2500 rpm for 2 min at room temp. Ensure the centrifuge is balanced before use (below):
12. Perform PCR of samples in an ABI GeneAmp 9700 Thermocycler with heated lid option (or equivalent PCR machine) with the following amplification program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>45°C</td>
<td>45 sec</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>Soak</td>
<td>20°C</td>
<td>soak</td>
<td>1</td>
</tr>
</tbody>
</table>

13. At the end of the amplification program put the PCR tubes on ice.
14. Label 16 x 0.5 mL Eppendorf tubes with numbers 1 to 16, and add 3 µL 2X DNA Gel Loading Buffer to each tube.
15. Add 3 µL of PCR products to tubes containing 2X DNA Gel Loading Buffer. Use a clean pipette tip for each PCR product.
16. Electrophorese on a 1.8% agarose gel prepared with GelRed. Include a DNA ladder in an outside lane of each row of wells. Run the gel at 50-100 V in 0.5X TBE buffer until the Bromophenol Blue has migrated 2/3 the length of the gel or the Orange G has migrated 3/4 the length of the gel.
17. Document the gel with the gel doc system. The specific CO1 PCR product is 709 bp.
18. Store PCR products at -20°C.

**Example: Agarose gel of CO1 PCR products** (1.8% gel run in 0.5X TBE buffer at 100 V for 35 min)

CO1 PCR using individual PCR reagents: titrating MgCl₂

- PCR is very sensitive and prone to cross-contamination.
- Work as cleanly as possible.
- Use filtered pipette tips throughout.
- Always wear disposable gloves. Change gloves frequently.
- Work individually

**Method**

1. On ice, thaw the following reagents: primers **VF1d_t1** and **VR1d_t1** (2 µM each), **10X (or 5X) Taq Buffer**, 25 mM MgCl₂, 10 mM dNTP. Flick the tubes to mix contents, then microfuge briefly.

2. Remove the DNA polymerase from the freezer and microfuge briefly to settle the contents, and then keep on ice.

3. Label eight 0.5 mL Eppendorf tubes: M1-M8. Put in a rack at room temperature. Add the following to the tubes:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water (µL)</td>
<td>18</td>
<td>16</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>25 mM MgCl₂ (µL)</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td><strong>Final Mg conc. In PCR (mM)</strong></td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>2.5</td>
<td>3</td>
<td>3.5</td>
<td>4</td>
</tr>
</tbody>
</table>

Cap the tubes. Mix by flicking and then microfuge briefly. Then leave at room temperature.
To a labelled 0.5 mL Eppendorf tube on ice add the following to prepare a PCR Master Mix. This is sufficient for 10 reactions of 25 µL.

Note: some groups will use 10X buffer and others will use 5X buffer. Adjust volumes of buffer and water according to this table:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>92.5 µL (67.5 µL)</td>
<td>-</td>
</tr>
<tr>
<td>*10X *(5X) Taq Buffer</td>
<td>25 µL (50 µL)</td>
<td>1X</td>
</tr>
<tr>
<td>10 mM dNTP Mix</td>
<td>5 µL</td>
<td>200 µM</td>
</tr>
<tr>
<td>Primer VF1d_t1 (2 µM)</td>
<td>25 µL</td>
<td>0.20 µM</td>
</tr>
<tr>
<td>Primer VR1d_t1 (2 µM)</td>
<td>25 µL</td>
<td>0.20 µM</td>
</tr>
<tr>
<td>*Taq DNA Polymerase (5u/µL)</td>
<td>2.5 µL</td>
<td>1.25u/20µL</td>
</tr>
<tr>
<td>Genomic DNA (10 ng/µL)</td>
<td>25 µL</td>
<td>1 ng/µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>200 µL</td>
<td></td>
</tr>
</tbody>
</table>

* FastStart Taq DNA Polymerase is supplied with 10X buffer
* Crimson Taq DNA Polymerase is supplied with 5X buffer
**Use either buffer with DreamTaq
1 Use either of your DNA samples

5. Cap the tube and mix by flicking the tube. Microfuge briefly. Return the tube to ice.
6. Return the PCR reagent stock solutions to the -20°C freezer.

7. Label eight 0.2 mL PCR tubes: 1 to 8.
8. Add 20 µL Master Mix to each tube. Cap the tubes and leave on ice.

9. Transfer 5 µL of MgCl₂ from M1 to PCR tube 1
   Transfer 5 µL of MgCl₂ from M2 to PCR tube 2
   Transfer 5 µL of MgCl₂ from M3 to PCR tube 3 etc.

10. Cap the PCR tubes and mix each by gently flicking. Microfuge briefly.
11. Perform PCR of samples in an ABI GeneAmp 9700 Thermocycler with heated lid (or equivalent PCR machine) with the following amplification program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>45°C</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>Soak</td>
<td>20°C</td>
<td>soak</td>
<td>1</td>
</tr>
</tbody>
</table>

12. At the end of the amplification program put the PCR tubes on ice.
13. Label 5 x 0.5 mL Eppendorf tubes 1 to 5, and add 3 μL 2X DNA Gel Loading Buffer to each tube.
14. After the PCR has completed, add 3 μL of PCR products to tubes containing 2X DNA Gel Loading Buffer. Use a clean pipette tip for each PCR product.
15. Electrophorese on a 1.8% agarose gel prepared with GelRed. Include a DNA ladder in an outside lane.
16. Run the gel at 50-100 V in 0.5X TBE buffer until the Bromophenol Blue has migrated 2/3 the length of the gel or the Orange G has migrated 3/4 the length of the gel.
17. Document the gel with the gel doc system. A specific CO1 PCR product is 709 bp.
18. Store the remaining PCR products at -20°C.

Example: CO1 PCR products from PCR with a range of MgCl₂ concentrations (0.5 – 4.0 mM MgCl₂). Gel: 1.8% agarose. DNA ladder: 1Kb Plus.
Example 2: CO1 DNA sequence showing positions of PCR primers and the CO1 barcode region

> Himantopus mexicanus (Black-necked Stilt) voucher JGS1761 cytochrome oxidase subunit I (COI) gene, complete cds; mitochondrial. GenBank: DQ385166.1

GTGACTTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC
ATTGCCATTTCTATCAACCGATGTATATTCTCAACCAACCACAAAGGCTAGCTCCGTATATTGTACGGTACAGGTTATGGGCTGCTCAGGTACCACAGGAACCCAGCCAGTGAGGAGAC

Key:
Primer VF1d_t1  TCTCAACCAACCACAAARGAYATYGG
Primer VR1d_t1  TAGACTTCTGGGTGCCRAARAAYCA
R= A/G; Y= C/T

The size of a PCR product with primers VF1d_t1 and VR1d_t1 is 709 bp. The size of the barcoding region (in blue) is 658 bp.
PCR of the ribulose-1, 5-bisphosphate carboxylase oxygenase large subunit (rbcL) for DNA Barcoding of plants

Introduction: DNA Barcoding
DNA barcoding is the use of short DNA sequences of standard segment(s) of the genome for species identification. These DNA barcode sequences can be obtained reasonably quickly and cheaply, especially through PCR. The ribulose-1, 5-bisphosphate carboxylase oxygenase large subunit gene (rbcL) and the Maturase K gene (matK) from the plastid genome are recommended as the standard barcode regions for higher (land) plants based on assessments of sequence quality and levels of species discrimination (CBOL Plant Working Group, 2009; Burgess et al., 2011).

Since Linnaeus, biologists have used distinguishing features in taxonomic keys to apply binomial species names, such as *Zea mays* (maize). Then, as a master key opens all the rooms in a building, the binomial species name accesses all knowledge about a species. From ferns to trees, evidence now shows that short DNA sequences from uniform localities (e.g. rbcL, matK for higher plants) on genomes can also be a distinguishing feature. As a Linnaean binomial is an abbreviated label for the morphology of a species, the short sequence is an abbreviated label for the genome of the species. The barcode of life thus provides an additional master key to identifying a species. Compiling a public library of sequences linked to named specimens, plus faster and cheaper sequencing, will make barcoding to identify species increasingly practical and useful.

In the Workshop we will use DNA barcoding of the *rbcL* gene to illustrate the power of PCR, DNA sequencing and bioinformatics.

Equipment and materials

1. **rbcL primers** (2 µM each in Nuclease Free Water)
   a. rbcL-forM13 `TGTAAAACGACGGCCAGTATGTCACCACAAACAGAGACTAAAGC`
   b. rbcL-revM13 `CAGGAAACAGCTATGACGTAAAATCAAGTCCACRCGC`
   Primers were obtained from Bioneer.
   Underlined sequences are M13 tails that are included in the primers to facilitate DNA sequencing of the rbcL PCR products.

2. Your plant gDNA sample (20 ng/µL)
3. +ve control gDNA (20 ng/µL)
4. Vortexer
5. Nitrile gloves
6. Plate centrifuge
7. Microfuge
8. Ice bucket and ice
9. Pipettes and filtered tips
10. 0.5 mL and 1.5 mL Eppendorf tubes
11. 0.2 mL PCR tubes
12. Tube racks
13. Bioneer Accupower PCR PreMix, 20 µL kit (Bioneer # K-2016). See above for details. Store at -20°C.
14. Individual PCR reagents
   a) Three Taq DNA polymerases are available in the workshop:
      (i) **Crimson Taq DNA Polymerase** (5u/µL) with 5X Mg-free PCR Reaction Buffer (NEB # M0325L). Crimson *Taq* (Mg-free) Reaction Buffer contains a density reagent, which allows direct loading of PCR products onto a gel. In addition, Crimson *Taq* (Mg-free) Reaction Buffer has a trace amount of a red dye, which serves as a colour aid in gel loading and a tracking dye which migrates at about 10 bp on a 1% TBE agarose gel. Store at -20°C.
      (ii) **FastStart Taq DNA Polymerase** (5u/µL) with 10X Mg-free PCR Reaction Buffer (Roche # 12032929001). This modified recombinant *Taq* DNA Polymerase is inactive at temperatures below 75°C, but is activated by a 2- to 4-minute heat activation step at 95°C. This is known as ‘hot-start’ PCR. In hot-start PCR the polymerase is modified to ensure that it remains inactive at lower temperatures and only becomes active at the high temperatures at which primers specifically bind. This prevents the amplification of nonspecific products and increases the yield, or sensitivity, of the desired PCR product. Store at -20°C.
      (iii) **DreamTaq DNA Polymerase** (5u/ µL) (Thermo Scientific #EP0701). This enzyme is supplied with a 10X reaction buffer that includes MgCl₂ at a concentration of 20 mM. For the purposes of this workshop, which you will determine the effect of MgCl₂ concentration on the PCR, do not use the DreamTaq 10X buffer. Instead use one of the Mg-free buffers supplied with the other Taq polymerases supplied in the workshop.
   b) 25 mM MgCl₂ Supplied with *Taq* DNA polymerases. Store at -20°C.
c) dNTP Mix (Fermentas # R0191). dNTP Mix contains an aqueous solution of dATP, dCTP, dGTP and dTTP, each at a final concentration of 10 mM. Store at -20°C.

15. Nuclease Free Water. Store at room temperature.
16. ABI GeneAmp 9700 PCR thermocycler or similar, with 96 well block
17. 2X DNA Gel Loading Buffer
18. 1.8% agarose gel with GelRed

rbcL PCR using Bioneer Accupower PCR PreMix Kit tubes

- PCR is very sensitive and prone to cross-contamination.
- Work as cleanly as possible.
- Use filtered pipette tips throughout.
- Always wear disposable gloves. Change gloves frequently.
- Work individually

Method
1. Remove the primer stocks, Nuclease Free Water from the -20°C freezer.
2. Thaw the primer stocks on ice. Flick the tube a few times to mix. Microfuge briefly. Return the tubes to ice.

3. Prepare a water/primer mix:
   To a 0.5 mL Eppendorf tube on ice add the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>280 μL</td>
<td>-</td>
</tr>
<tr>
<td>rbcLa-fM13 (2 μM)</td>
<td>40 μL</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>rbcLa-revM13 (2 μM)</td>
<td>40 μL</td>
<td>0.2 μM</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>360 μL</td>
<td></td>
</tr>
</tbody>
</table>

   *This is sufficient for 20 reactions.*

4. Cap the tube, mix by flicking the tube a few times, then microfuge briefly. Keep on ice.

5. Take two strips of 8 Bioneer Premix tubes from the freezer.
6. Label the strips of tubes using a fine black marker pen with numbers 1 to 16.
7. Add 18 µL water/primer mix to each of the 16 tubes. Cap tubes and put in a rack at room temperature.

8. To tubes 1, 2, 9, 10 add 2 µL Nuclease Free Water. Cap the tubes. These are your negative controls.

9. To each of tubes 3, 4, 5, 6 add 2 µL (40 ng) purified gDNA from plant sample 1. Cap tubes and return to ice.

10. To each of tubes 11, 12, 13, 14 add 2 µL (40 ng) purified gDNA from plant sample 2. Cap tubes and return to ice.

11. To each of tubes 7, 8, 15, 16 add 2 µL (40 ng) +ve control gDNA. Cap tubes and return to ice. These are your positive controls.

12. Place in the tube rack (below).

13. Place a lid on the rack. Vortex to dissolve the dried PreMix blue pellet by holding the rack with the tubes on the vortexer for a few seconds (below).

14. Spin the rack/tubes in a plate centrifuge at 2500 rpm for 2 min at room temp. Ensure the centrifuge is balanced before use (below):
19. Perform PCR of samples in an ABI GeneAmp 9700 Thermocycler with heated lid (or equivalent PCR machine) with the following amplification program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>Soak</td>
<td>20°C</td>
<td>soak</td>
<td>1</td>
</tr>
</tbody>
</table>

20. At the end of the amplification program put the PCR tubes on ice.
21. Label 16 x 0.5 mL Eppendorf tubes with numbers 1 to 16, and add 3 µL 2X DNA Gel Loading Buffer to each tube.
22. After the PCR has completed, add 3 µL of PCR products to the tubes containing 2X DNA Gel Loading Buffer. Use a clean pipette tip for each PCR product.
23. Electrophorese on a 1.8% agarose gel with GelRed. Include a DNA ladder in an outside lane of each row.
24. Run the gel at 50-100 V in 0.5X TBE buffer until the Bromophenol Blue has migrated 2/3 the length of the gel or the Orange G has migrated 3/4 the length of the gel.
25. Document the gel with the gel doc system. A specific rbcL PCR product is 634 bp.
26. Store PCR products at -20°C.
Example: 1.8% agarose gel of maize rbcL PCR products generated with Bioneer PreMix tubes

M: GeneRuler 1 kb Plus DNA ruler
1 – 6: rbcL maize PCR product

rbcL PCR product
(634 bp)
rbcL PCR using individual PCR reagents: titrating MgCl₂

- PCR is very sensitive and prone to cross-contamination.
- Work as cleanly as possible.
- Use filtered pipette tips throughout.
- Always wear disposable gloves. Change gloves frequently.
- Work individually

**Method**

1. On ice, thaw primers rbcLa-fM13 and rbcLa-revM13 (2 µM each), **10X (or 5X) Taq Buffer**, 10 mM dNTP Mix and 25mM MgCl₂. Flick the tubes to mix contents, then microfuge briefly. Keep on ice.
2. Remove the DNA polymerase from the freezer and microfuge briefly to settle the contents, and then keep on ice.
3. Label eight 0.5 mL Eppendorf tubes: M1-M8. Put in a rack at room temperature. Add the following to the tubes:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water (µL)</td>
<td>18</td>
<td>16</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>25 mM MgCl₂ (µL)</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td><strong>Final Mg conc. In PCR (mM)</strong></td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>2.5</td>
<td>3</td>
<td>3.5</td>
<td>4</td>
</tr>
</tbody>
</table>

4. Cap the tubes. Mix by flicking and microfuge briefly. Then leave at room temperature.
5. To a labelled 0.5 mL Eppendorf tube on ice add the following to prepare a PCR Master Mix. *This is sufficient for 10 reactions, each with a final volume of 25 µL.*

**Note:** some groups will use 10X buffer and others will use 5X buffer. Adjust volumes of buffer and water according to this table.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>92.5 µL (67.5 µL)</td>
<td>-</td>
</tr>
<tr>
<td>*10X *(5X) Taq Buffer</td>
<td>25 µL (50 µL)</td>
<td>1X</td>
</tr>
<tr>
<td>10 mM dNTP Mix</td>
<td>5 µL</td>
<td>200 µM</td>
</tr>
<tr>
<td>Primer rbcLa-FM13 (2 µM)</td>
<td>25 µL</td>
<td>0.25 µM</td>
</tr>
<tr>
<td>Primer rbcLa-RevM13 (2 µM)</td>
<td>25 µL</td>
<td>0.25 µM</td>
</tr>
<tr>
<td><strong>Taq DNA Polymerase (5u/µL)</strong></td>
<td>2.5 µL</td>
<td>1.25u/20µL</td>
</tr>
<tr>
<td>Genomic DNA (20 ng/µL)</td>
<td>25 µL</td>
<td>2 ng/µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>200 µL</strong></td>
<td></td>
</tr>
</tbody>
</table>

* FastStart Taq DNA Polymerase is supplied with 10X buffer
* Crimson Taq DNA Polymerase is supplied with 5X buffer
**Use either buffer with DreamTaq
1 Use either of your DNA samples

6. Cap the Master Mix tube and mix by flicking the tube. Microfuge briefly. Return the tube to ice.

7. Return your PCR stock solutions to the -20°C freezer.

8. Label eight 0.2 mL PCR tubes: 1 to 8.

9. Add 20 µL Master Mix to each tube. Cap the tubes and leave on ice.

10. Transfer 5 µL of MgCl₂ from tube M1 to PCR tube 1
    Transfer 5 µL of MgCl₂ from tube M2 to PCR tube 2
    Transfer 5 µL of MgCl₂ from tube M3 to PCR tube 3
    etc.
11. Cap the PCR tubes and mix each by flicking. Microfuge briefly.

Perform PCR of samples in an ABI GeneAmp 9700 Thermocycler with heated lid (or equivalent PCR machine) using the following amplification program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>3 min</td>
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<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>1 min</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Soak</td>
<td>20°C</td>
<td>soak</td>
<td>1</td>
</tr>
</tbody>
</table>

12. At the end of the amplification programme put the PCR tubes on ice.

13. Label eight 0.5 mL Eppendorf tubes: 1 to 8, and add 3 µL 2X DNA Gel Loading Buffer to each tube.

14. After the PCR has completed, add 3 µL of PCR products to tubes containing 2X DNA Gel Loading Buffer. Use a clean pipette tip for each PCR product.

15. Run on a 1.8% agarose gel with GelRed. Run the gel at 50-100 V in 0.5X TBE buffer until the Bromophenol Blue dye has run 2/3 the length of the gel or the Orange G dye has run 3/4 the length of the gel.

16. Document the gel with the gel doc system.

17. A predicted rbcl PCR product for Zea mays is 634 bp (using primers with M13 tails).

Sizes of rbcl PCR products from other plant species may vary slightly.

18. Store the remaining PCR products at -20°C.

Example: rbcl PCR with titrated MgCl₂ 1.8% agarose gel.
Example 2: *Zea mays* rbcL gene sequence and positions of the PCR primers

>gi|18035|emb|Z11973.1| Zea mays chloroplast rbcL gene for ribulose bisphosphate carboxylase

```
ATGTCAACCACAAACAGAAACTAAAGC AAGTTGTTGGAATTAAAGCTGCTGTAATAGGATTATAAAATTGACCT
ACTACACCCGGAGATCAGAAACCAAGGATACGTAGATCTCGGCACATCGGATGCTACTCTCGACTCGG
GGTTCCGCTCTGATGACAGGACTGTCGGCCAATTCTTACTCTCAGATGATGAACTGGGATAAAGTTGACGA
ACTGATGGATTTCTTCTTAGCTGTTGCTACACATACATGCCACCGTTCCTGGGACC
CAGATCAATATTATCTGTTATGTTATCCATTGACACTATTATTGAGAGGTAGTTCTTCCTTTG
TACTCCATGTTGGTAACGTTATTTGCTTTCAAAGCCTTACAGGCTTACGTTAATT
CCCTCGTTAATTCAAAAACTTTTCAAGTCCGCCCTTCAGGTTATCAAGTTGAACAGTTGACAG
AGTACGGTCATTCCTTTATAGGTATCTACTATAAACAAATGGGATATATTACGCAAAAAATTACGGTAG
AGCGGTGTATTAGGTGTCTACGCCGTGGACTCTTTATTACCAAAGAAGGTGAAACGCTAAACTCAAAACCA
TTATGCCTGGAGAACCCGCTTTTCGCTTTTTGTGCCGAAGCAATTATAAAGGCAAAGGGGAAAGTTGGT
AAATCAAAGGGGAATTACTTGGATGCTGAGATGAGCTGACATCGAGGAAATGATAGGAGGCTGTATTTGGC
AAGGGAAATTAGGGTTCTCTTCTGAGATGTACAGACTACTTTAACAAGGAGATCCCGCACAATACTACCTT
GCTCATTATTGGGCCACGCCGCTTTACCCGCTACTTTTTCTACATCCACAGGACATGCTGATATTGAAGAC
AGAAAAATCATGTTATTGCATTTCCGCTGTATTAGCTAAAGCATTGTGGCTGTGAGGAGGATCATATCCA
CTCCGCTACAGTAGTAGTAGTAGTGAAGATGAAAGGGAACGGCAAATACTTTAGGTGAATTTATTGCGG
GATGATTTTTATGAAAGAGATGCTTCCGCTGTTATCTTTTTACCTCAGGGATGCTTATCCATTGCAAGGT
TTATACGGGTGCTCTGCGGATATCAGTGTGGCCATATCCAGACGCTGAGAAACTTTTGGGAGATGA
TTCCGCTATCAATTTGTTGGGAGACCTTTAGGACACATCTTGGGAAATAAGCAGTCTGGTGACAGCTATAT
CTGTTGGCCCTTAAGAGCCCTGTGATACGCTTGGATAGAGCGGAGGCTACCTTGCTGCTGATGATGATG
TTATCAAGCAGCTTGCAATGAGTGCTGAACTACGCAGCTTGTGAAATATGGAAGGAGATCATAATT
TGATGTTTCAAAAGCGATGATCCATATAAAA
```

**KEY**

**Primers**

- rbcLa-fM13: \textcolor{red}{\textbf{TGTAAAAACGACGCCAGT ATGTCAACCACAAACAGAGACTAAAGC}}
- rbcLa-revM13: \textcolor{red}{\textbf{CAGGAAAACAGCTATAGGCTAAAATCAAGTCCACCRCG}}

Degenerate position R = A or G

Sequence in **BROWN** is the M13 tail

For maize the size of a rbcL PCR product, generated using primers with M13 tails, is 634 bp. The size of the rbcL barcode region (yellow) is 553 bp.
Internet resources and further information

1. PCR (Wikipedia)  
   http://en.wikipedia.org/wiki/Polymerase_chain_reaction
2. PCR Primer: Widely used manual from Cold Spring Harbor Press:  
   http://books.google.co.ke/books?id=IbAcGxyD06MC&dq=PCR+manual&printsec=frontcover&source=ин&hl=en&ei=pzHATfKsLXrQf70Z32Aw&sa=X&oi=book_result&ct=result&resnum=11&ved=0CF4Q6AEwCg#v=onepage&q&f=false
3. PCR video  
   http://www.youtube.com/watch?v=eEcy9k_KsDo&feature=related
4. Bioneer Accupower PCR PreMix instructions  
5. Bioneer AccuPower 2010 Brochure  
6. Standard PCR protocols  
   http://www.protocol-online.org/prot/Molecular_Biology/PCR/Standard_PCR/index.html
7. PCR Amplification-Promega overview of different PCR applications  
9. CO1: The ideal barcoding gene  
10. DNA barcoding (Wikipedia)  
    http://en.wikipedia.org/wiki/DNA_barcoding
11. What is DNA barcoding?  
    http://www.barcodeoflife.org/content/about/what-dna-barcoding
12. Barcode of Life - Outreach Materials  
    http://www.barcodeoflife.org/content/resources/outreach-materials
13. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species  
    http://rspb.royalsocietypublishing.org/content/270/Suppl_1/S96.full.pdf
14. NEBcutter V2.0: DNA sequence analysis to find restriction enzymes sites  
    http://tools.neb.com/NEBcutter2/
    http://www.pnas.org/content/106/31/12794.full
16. Bafeel et al. (2012). Ribulose-1, 5-biphosphate carboxylase (rbcL) gene sequence and random amplification of polymorphic DNA (RAPD) profile of regionally endangered tree

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8. Purification of PCR products

Introduction

PCR products must be purified before they can be used in downstream applications such as DNA sequencing, ligation and cloning. There are many suitable methods available including PEG precipitation, filtration, organic extraction, alcohol precipitation, and Exonuclease I - Shrimp Alkaline Phosphatase (Exo-SAP) treatment.

In the Workshop we will use a commercial kit, and also an ethanol precipitation method.

The GeneJET PCR Purification Kit is designed for rapid and efficient purification of DNA from PCR and other enzymatic reaction mixtures. The kit utilizes silica-based membrane technology in the form of a convenient spin column, eliminating the need for toxic phenol-chloroform extractions. The GeneJET PCR Purification Kit effectively removes primers, dNTPs, enzymes and salts from PCR and other reaction mixtures. The kit can be used for purification of DNA fragments from 25 bp to 20 kbp. The recovery rates are 90-100% in a 100 bp – 10 kb DNA fragment size range. Each GeneJET purification column has a total binding capacity of up to 25 µg of DNA and the entire procedure takes just 5 min. The purified DNA can be used in common downstream applications such as sequencing, restriction digestion, ligation and cloning.

GeneJET PCR Product Purification

Equipment and materials
1. Nitrile gloves
2. Absolute ethanol. Store at room temperature.
3. Nuclease Free Water
4. Microfuge
5. 1.5 mL and 0.5 mL Eppendorf tubes
6. GeneJET PCR Purification Kit (Fermentas # K0701). Store at room temperature.
7. Pipettes and filter pipette tips
Method

• NOTE: Prior to the initial use of the GeneJET PCR Purification Kit, dilute the concentrated Wash Buffer with ethanol (96-100%): To 9 mL of concentrated Wash Buffer add 45 mL of ethanol. Mix thoroughly. Store at room temperature.

• Binding Buffer in the GeneJET PCR Purification Kit contains guanidinium thiocyanate. Wear gloves and eye protection when handling the Binding Buffer. It is harmful by inhalation, in contact with skin, eyes or if swallowed. Contact of guanidinium thiocyanate with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid wastes containing this product.

• Work individually.

1. Combine PCR products to be purified in a 1.5 mL Eppendorf tube.

2. Measure the volume of your combined PCR products using a P200 pipette and the appropriate tip.

3. Add an equal volume of Binding Buffer to the PCR products (e.g. for 100 µL of PCR product, add 100 µL of Binding Buffer). Mix thoroughly by flicking and inverting the tube several times. Microfuge briefly.

4. Transfer the mixture to the GeneJET purification column. Microfuge for 1 min at 13,000 rpm. Discard the flow-through.

5. Add 700 µL of Wash Buffer (diluted with the ethanol as described above) to the GeneJET purification column. Let it stand for 1 min. Microfuge for 1 min at 13,000 rpm at room temperature. Discard the flow-through and place the purification column back into the collection tube.

6. Centrifuge the empty GeneJET purification column for an additional 1 min at maximum speed to completely remove any residual wash buffer. 

   **Note. This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.**

7. Transfer the GeneJET purification column to a clean 1.5 mL Eppendorf tube (without a lid).

8. Add 30 µL of Nuclease Free Water to the centre of the GeneJET purification column membrane and microfuge for 1 min at 13,000 rpm at room temperature.

9. Discard the GeneJET purification column. Transfer the purified PCR product to a clean, labelled 0.5 mL Eppendorf tube.

10. Agarose gel analysis:
   
   a) Remove 3 µL of purified PCR product and mix with 3 µL 2X DNA Gel Loading Buffer in a 0.5 mL Eppendorf tube.
Load onto a 1.8% agarose gel prepared with 0.5X TBE and GelRed.

Also load a DNA ladder into one of the outside wells.

Run at 100 V for ~30-40 min.

Record the gel image.

Determine the size and integrity of your product.

For plants the size of a rbcL PCR product, generated using primers with M13 tails, is approx. 634 bp. For animals the size of a CO1 PCR product is approx. 709 bp.

11. If the yield and integrity of the purified PCR product looks acceptable from the gel, then proceed to Nanodrop Spectrophotometry.
   
a. A pure sample of DNA has a 260/280 ratio of 1.8.
   
b. An ideal concentration is at least 25 ng/µL.

12. DNA sequencing.
   
a. For sequencing, your purified PCR product should be at least 25 ng/µL with a 260/280 ratio of 1.8-1.9.
   
b. Submit an aliquot (10 µL in a labelled 0.5 mL Eppendorf tube) to SegoliP for direct DNA sequencing.
   
c. The PCR product will be directly sequenced using the forwards and reverse PCR primers. Aliquots of the PCR primers (5 µM) have already been given to the SegoliP unit for sequencing.

13. Store the remaining purified PCR product at -20°C.

Example: 1.8% agarose gel showing GeneJET purified rbcL PCR products (634 bp)

M: GeneRuler 1 kb Plus DNA ladder; 1 – 4: purified rubcL PCR products
PCR Product Purification by Precipitation

Equipment and materials
1. Nitrile gloves
2. Microfuge and refrigerated microfuge
3. Pipettes and tips
4. 0.5 mL Eppendorf tubes
5. 3M Sodium Acetate pH 5.2. Store at room temperature.
6. Absolute ethanol. Store at room temperature.
7. 70% ethanol. Store at room temperature.

Method
1. Combine the PCR products for purification in a 0.5 mL Eppendorf tube.
2. Add 1/10\(^{th}\) volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol to the PCR product. For example, if your PCR product volume is 100 µL then add 10 µL sodium acetate and 250 µL absolute ethanol. If you are purifying a large number of samples then you can prepare a “mastermix” of the two solutions according to the number of samples for purification.
3. Mix thoroughly by inverting the tube several times.
4. Incubate the tube at -20°C for 1 hr.
5. Centrifuge at 14,000 rpm for 20 min at 4°C.
6. Carefully discard the supernatant and add 300 µL of 70% ethanol to the pellet.
7. Mix thoroughly by inverting the tube several times.
8. Centrifuge at 14000 rpm for 15 min at 4°C.
9. Carefully discard the supernatant and air dry the pellet.
10. Dissolve the pellet with 20 µL of Nuclease Free Water.
11. Agarose gel analysis:
   a. Remove 3 µL of purified PCR product and mix with 3 µL 2X DNA Gel Loading Buffer in a 0.5 mL Eppendorf tube.
   b. Load onto a 1.8% agarose gel prepared with 0.5X TBE and GelRed.
   c. Also load 5 µL of 1kb Plus DNA ladder into one of the outside wells.
   d. Run gel at 50-100 V.
   e. Record the gel image.
   f. Determine the size and integrity of your product.
12. If the yield and integrity of the purified PCR product looks acceptable from the gel, then proceed to Nanodrop Spectrophotometry. A pure sample of DNA has a 260/280 ratio of 1.8 and is relatively free from protein contamination.

13. DNA sequencing.
   a. For sequencing, your purified PCR product should be at least 25 ng/µL with a 260/280 ratio of 1.8-1.9.
   b. Submit an aliquot (10 µL in a labelled 0.5 mL Eppendorf tube) to BecA’s SegoliP Unit for direct DNA sequencing.
   c. The PCR product will be directly sequenced using forwards and reverse sequencing primers (for sequencing of the CO1 PCR products, the PCR primers will be used; for sequencing the rbcL PCR products the M13 primers will be used). Aliquots of the PCR primers (5 µM) have already been given to the SegoliP unit for sequencing.

14. Store the remaining purified PCR product at -20°C.

Example: 1.8% agarose gel showing PCR products purified by precipitation.

![1.8% agarose gel showing PCR products purified by precipitation.](image)

M: GeneRuler™ 1 kb Plus DNA Ladder; 1-4: Purified PCR products
Example **NanoDrop readout** showing concentrations in ng/µL of the PCR primers and purified PCR products based on the A260 reading, and purity based on the A260/A280 ratio.

<table>
<thead>
<tr>
<th>Plate ID</th>
<th>Well</th>
<th>Sample ID</th>
<th>Date</th>
<th>Time</th>
<th>Conc.</th>
<th>Units</th>
<th>A260</th>
<th>A280</th>
<th>260/280</th>
<th>NA Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>A1</td>
<td>i7983</td>
<td>05/10/2011</td>
<td>4:03 PM</td>
<td>37.54 ng/ul</td>
<td>1.138</td>
<td>0.693</td>
<td>1.64</td>
<td>ssDNA-33</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B1</td>
<td>MWG 70</td>
<td>05/10/2011</td>
<td>4:03 PM</td>
<td>35.94 ng/ul</td>
<td>1.039</td>
<td>0.685</td>
<td>1.58</td>
<td>ssDNA-33</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C1</td>
<td>M5F</td>
<td>05/10/2011</td>
<td>4:23 PM</td>
<td>73.38 ng/ul</td>
<td>2.224</td>
<td>1.154</td>
<td>1.93</td>
<td>ssDNA-33</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>D1</td>
<td>M6R</td>
<td>05/10/2011</td>
<td>4:23 PM</td>
<td>68.67 ng/ul</td>
<td>2.061</td>
<td>1.256</td>
<td>1.66</td>
<td>ssDNA-33</td>
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<tr>
<td>6</td>
<td>A3</td>
<td>SRM68</td>
<td>05/10/2011</td>
<td>5:05 PM</td>
<td>142.4 ng/ul</td>
<td>2.847</td>
<td>1.517</td>
<td>1.88</td>
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<tr>
<td>7</td>
<td>B3</td>
<td>SRMFO</td>
<td>05/10/2011</td>
<td>5:05 PM</td>
<td>131.9 ng/ul</td>
<td>2.638</td>
<td>1.4</td>
<td>1.88</td>
<td>DNA-50</td>
<td></td>
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<tr>
<td>8</td>
<td>C3</td>
<td>SRM95</td>
<td>05/10/2011</td>
<td>5:05 PM</td>
<td>121.7 ng/ul</td>
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<td>9</td>
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<td>90.12 ng/ul</td>
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<td>1.83</td>
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<td>10</td>
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<td>114.8 ng/ul</td>
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<td>11</td>
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<td>SRMCKM</td>
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<td>5:05 PM</td>
<td>82.84 ng/ul</td>
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<td>0.882</td>
<td>1.88</td>
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<td>12</td>
<td>G3</td>
<td>FTA16</td>
<td>05/10/2011</td>
<td>5:05 PM</td>
<td>94.06 ng/ul</td>
<td>1.881</td>
<td>0.996</td>
<td>1.89</td>
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<td></td>
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<tr>
<td>13</td>
<td>H3</td>
<td>FTA17</td>
<td>05/10/2011</td>
<td>5:05 PM</td>
<td>89.21 ng/ul</td>
<td>1.784</td>
<td>0.952</td>
<td>1.87</td>
<td>DNA-50</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>A4</td>
<td>FTA19</td>
<td>05/10/2011</td>
<td>5:08 PM</td>
<td>134.5 ng/ul</td>
<td>2.69</td>
<td>1.43</td>
<td>1.88</td>
<td>DNA-50</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>B4</td>
<td>FTA24</td>
<td>05/10/2011</td>
<td>5:08 PM</td>
<td>105.02 ng/ul</td>
<td>2.104</td>
<td>1.112</td>
<td>1.89</td>
<td>DNA-50</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>C4</td>
<td>FTA25</td>
<td>05/10/2011</td>
<td>5:08 PM</td>
<td>95.83 ng/ul</td>
<td>1.917</td>
<td>1.011</td>
<td>1.9</td>
<td>DNA-50</td>
<td></td>
</tr>
</tbody>
</table>

**Internet resources and further information**

1. GeneJET™ PCR Purification Kit Instructions
2. BigDye Direct Cycle Sequencing Kit
3. Purification of PCR products for Sequencing
4. Protocol Online: PCR Product Purification
   [http://www.protocol-online.org/prot/Molecular_Biology/PCR/PCR_Product_Purification/index.html](http://www.protocol-online.org/prot/Molecular_Biology/PCR/PCR_Product_Purification/index.html)
5. Combination PEG precipitation and gel purification of PCR products for LiCor Sequencing
6. Exonuclease I - Shrimp Alkaline Phosphatase clean up of PCR products
9. Restriction Enzyme Digestion of purified PCR products

Introduction
Adapted from:

- http://en.wikipedia.org/wiki/Restriction_enzyme#Examples
- http://www.vivo.colostate.edu/hbooks/genetics/biotech/enzymes/renzymes.html

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts DNA at specific recognition nucleotide sequences known as restriction sites. Such enzymes, found in bacteria and archaea, are thought to have evolved to provide a defense mechanism against invading viruses. Inside a bacterial host, the restriction enzymes selectively cut up foreign DNA in a process called restriction; host DNA is methylated by a modification enzyme (a methylase) to protect it from the restriction enzyme’s activity. Collectively, these two processes form the restriction modification system. To cut the DNA, a restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. Restriction sites are typically four, six, eight, ten, or twelve nucleotides long. Because there are only so many ways to arrange the four nucleotides which compose DNA (Adenine, Thymine, Guanine and Cytosine) into a four- to twelve-nucleotide sequence, recognition sequences tend to occur by chance in any long sequence. Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially and are routinely used for DNA modification and manipulation in laboratories. Because of the huge variety of restriction enzymes available, potential "restriction sites" appear in almost any gene or locus of interest on any chromosome.

The length of restriction recognition sites varies: The enzymes EcoRI, SacI and SstI each recognize a 6 base-pair (bp) sequence of DNA, whereas NotI recognizes a sequence 8 bp in length, and the recognition site for Sau3AI is only 4 bp in length. Length of the recognition sequence dictates how frequently the enzyme will cut in a random sequence of DNA. Enzymes with a 6 bp recognition site will cut, on average, every $4^6$ or 4096 bp; a 4 bp recognition site will occur roughly every 256 bp.
Restriction enzymes (Res) have many uses in molecular biology. For example, they are used to assist insertion of genes into plasmid vectors during gene cloning. REs can also be used to distinguish gene alleles or differences between homologous genes between species by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a SNP alters the restriction site present in the allele or homologous gene. In this method, the restriction enzyme can be used to genotype a DNA sample without the need for expensive gene sequencing. The sample is first digested with the RE to generate DNA fragments, and then the different sized fragments separated by gel electrophoresis. In general, alleles with correct restriction sites will generate two visible bands of DNA on the gel, and those with altered restriction sites will not be cut and will generate only a single band. The number and sizes of bands reveals the sample subject's genotype. When combined with PCR, restriction analysis is called PCR- restriction fragment length polymorphism, or PCR-RFLP.

In the workshop we will use PCR-RFLP to analyse the CO1 and rbcl gene. Several enzymes are available for you to try. All are ‘frequent cutters’ in that they each have a four base recognition sequence. The products of digestion will be electrophoresed on a 2% agarose gel, and the relative sizes of the restriction fragments will be determined. The restriction pattern on the gel will be compared with in silico (using computer software) restriction analysis, which you will do during the Bioinformatics part of the workshop.

In the Workshop, we will use Thermo Scientific FastDigest restriction enzymes for rapid DNA digestion. All FastDigest enzymes are 100% active in a universal FastDigest Green buffer and are able to digest DNA in a few minutes. FastDigest Green Buffer includes a density reagent along with blue and yellow tracking dyes that allow for direct loading of the reaction mixtures on a gel. The blue dye of the FastDigest Green Buffer migrates with 3-5 kbp DNA fragments in a 1% agarose gel. The yellow dye of the FastDigest Green Buffer migrates with ~10 bp DNA fragments in a 1% agarose gel.

**Equipment and materials**

1. Nitrile gloves
2. Water bath at 37°C
3. Benchtop cooler
4. Ice bucket and ice
5. Pipettes and tips
6. 0.5 mL Eppendorf tubes
7. Nuclease Free Water
8. Purified PCR products
9. 10X FastDigest Green Buffer. Store at -20°C.
10. FastDigest restriction enzymes. Store at -20°C.
11. 2% agarose midi gels (gel length 10-12 cm). This gel size will give good resolution of the digestion products.
12. 0.5X TBE
13. DNA ladder

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognition sequence</th>
<th>Thermo Scientific #</th>
</tr>
</thead>
<tbody>
<tr>
<td>AluI</td>
<td>AG^CT</td>
<td># FD0014</td>
</tr>
<tr>
<td>HaellI</td>
<td>GG^CC</td>
<td>#FD0154</td>
</tr>
<tr>
<td>Hhal</td>
<td>GCG^C</td>
<td>#FD1854</td>
</tr>
<tr>
<td>HpaII</td>
<td>C^CGG</td>
<td># FD0514</td>
</tr>
<tr>
<td>Msel</td>
<td>T^TAA</td>
<td>#FD2174</td>
</tr>
<tr>
<td>Sau3Al</td>
<td>^GATC</td>
<td>#FD0784</td>
</tr>
</tbody>
</table>

**Method**

- Work as group to prepare digestion Master Mixes.
- Through consultation with your Tutor, select appropriate restriction enzymes to test.

2. Remove the restriction enzymes from the freezer, and keep in a bench top cooler. Microfuge briefly before use.
3. Prepare each Digestion Master Mix in a labelled 0.5 mL Eppendorf tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (1 reaction)</th>
<th>Master Mix (10 reactions)</th>
<th>Master Mix (20 reactions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>13.5 µL</td>
<td>135 µL</td>
<td>270 µL</td>
</tr>
<tr>
<td>10X FastDigest Green Buffer</td>
<td>2 µL</td>
<td>20 µL</td>
<td>40 µL</td>
</tr>
<tr>
<td>FastDigest restriction</td>
<td>0.5 µL</td>
<td>5 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>endonuclease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified PCR product</td>
<td>4 µL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>20 µL</td>
<td>160 µL</td>
<td>320 µL</td>
</tr>
</tbody>
</table>
4. Aliquot 16 µL of Digestion Master Mix into 0.5 mL Eppendorf tubes.
5. Add 4 µL of purified PCR product.
6. Mix by flicking the tubes a few times. Microfuge briefly.
7. Incubate the tubes in a water bath at 37°C for 30 min.
8. Load 10 µL of each directly onto a 2% agarose midi gel (gel length 10-12 cm). This gel size will give good resolution of the digestion products, some of which may be very small, and some may have very similar sizes.
9. Also load a DNA ladder in the 2 outside wells.
10. Run the gel at 50-100 V until the yellow dye has run 3/4 length of the gel.
11. Document the gel.
12. Determine the sizes of the bands and interpret the results.
13. Store the remaining digest at -20°C.
Example 2: CO1 DNA sequence showing positions of PCR primers and restriction enzyme sites

>gi|363409135|gb|JN543256.1| Birdstrike environmental sample clone BS09 cytochrome oxidase subunit I (CO1) gene, partial cds; mitochondrial

**VF1d_t1**
TCTCAACCAACCACAARGAYATYGG

**VR1d_t1**
TAGACTTCTGGGTGGCCRAARAAYCA

Degenerate positions: R= A or G; Y= C or T

Predicted restriction endonuclease sites in ‘Bird strike’ CO1 PCR product

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recognition site</th>
<th>Length (bases)</th>
<th>Overhang</th>
<th>Number of sites</th>
<th>Positions in sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AluI</td>
<td>AGCT</td>
<td>4</td>
<td>Blunt</td>
<td>4</td>
<td>79, 280, 399, 552</td>
</tr>
<tr>
<td>HaeIII</td>
<td>GGCC</td>
<td>4</td>
<td>Blunt</td>
<td>3</td>
<td>103, 414, 696</td>
</tr>
<tr>
<td>HhaI</td>
<td>GCGC</td>
<td>4</td>
<td>3'</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>HpalI</td>
<td>CCGG</td>
<td>4</td>
<td>5'</td>
<td>4</td>
<td>55, 337, 394, 646</td>
</tr>
<tr>
<td>MseI</td>
<td>TTAA</td>
<td>4</td>
<td>5'</td>
<td>2</td>
<td>36, 484</td>
</tr>
<tr>
<td>Sau3AI</td>
<td>GATC</td>
<td>4</td>
<td>5'</td>
<td>2</td>
<td>534, 611</td>
</tr>
</tbody>
</table>
Example 3: *Zea mays* rbcL PCR product sequence showing positions of the primers with M13 tails

\[
\text{rbcLa-fM13} \quad \text{TGTAAAACGACGGCCAGT} \text{ATGTCCACACAAAACAGAGACTAAAGC} \\
\text{rbcLa-revM13} \quad \text{CAGGAAAACAGCTATGAC} \text{GTAAAATCAAGTCCACRCG}
\]

**KEY**

**Primers**

rbcLa-fM13 \quad \text{TGTAAAACGACGGCCAGT} \text{ATGTCCACACAAAACAGAGACTAAAGC}

rbcLa-revM13 \quad \text{CAGGAAAACAGCTATGAC} \text{GTAAAATCAAGTCCACRCG}

Degenerate positions: R = A or G

Sequence in **BROWN** is the M13 tail

**Predicted restriction endonuclease sites in *Zea mays* rbcL PCR product**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recognition site</th>
<th>Length (bases)</th>
<th>Overhang</th>
<th>Number of sites</th>
<th>Positions in sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AluI</td>
<td>agct</td>
<td>4</td>
<td>Blunt</td>
<td>6</td>
<td>61, 153, 181, 190, 322, 624</td>
</tr>
<tr>
<td>HaellI</td>
<td>ggcc</td>
<td>4</td>
<td>Blunt</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Hhai</td>
<td>gcgc</td>
<td>4</td>
<td>3'</td>
<td>1</td>
<td>412</td>
</tr>
<tr>
<td>HpallI</td>
<td>cccg</td>
<td>4</td>
<td>5'</td>
<td>1</td>
<td>97</td>
</tr>
<tr>
<td>Msel</td>
<td>ttaa</td>
<td>4</td>
<td>5'</td>
<td>3</td>
<td>56, 68, 539</td>
</tr>
<tr>
<td>Sau3A</td>
<td>gatc</td>
<td>4</td>
<td>5'</td>
<td>3</td>
<td>249, 300, 426</td>
</tr>
</tbody>
</table>
Example 1: 2% agarose gel electrophoresis of digested CO1 PCR products

CO1 PCR products from unknown animals (1A-5A) digested with Hpall and HaeIII. UC: uncut CO1 PCR product. M: 1Kb Plus DNA ladder;

Internet resources and further information
1. Thermo Scientific Fermentas FastDigest Restriction Enzymes
2. Restriction enzymes
3. Restriction fragment length polymorphism
4. NEB Restriction endonucleases Technical Guide
   [https://www.neb.com/~/media/NebUs/Files/Brochures/RE.pdf](https://www.neb.com/~/media/NebUs/Files/Brochures/RE.pdf)
10. NanoDrop Spectrophotometry: Assessment of Nucleic Acid Concentration and Purity

This information was taken from:
• T042-TECHNICAL BULLETIN NanoDrop Spectrophotometers Assessment of Nucleic Acid Purity. Thermo Fisher Scientific – NanoDrop Products Wilmington, Delaware USA
  Technical support: info@nanodrop.com 302-479-7707 www.nanodrop.com
• Using a spectrophotometer to quantitate DNA and RNA.
  http://www.mc.vanderbilt.edu/root/pdfs/mclaughlin_lab/dna_and_rna_with_a_spectrophotometer.pdf

NanoDrop: Basic Use for measuring double stranded DNA
1. Select ‘DNA-50’ for dsDNA on the screen.
2. To perform a blanking cycle, perform the following:
   a. Load a blank sample (the buffer, solvent, or carrier liquid used with your samples) onto the lower measurement pedestal and lower the sampling arm into the ‘down’ position.
   b. Click on the ‘Blank’ (F3) button.
   c. Open the arm and wipe the blanking buffer from both pedestals using a soft paper tissue.
3. Analyze an aliquot of water as though it were a sample. This is done using the ‘Measure’ button (F1). The result should be a spectrum with a relatively flat baseline. Wipe the blank from both measurement pedestal surfaces and repeat the process until the spectrum is flat.
4. Then measure your samples.
   a. Absorbances of the samples are represented as if measured with a conventional 10 mm path.
   b. A 260/280 nm ratio of ~1.8 is generally accepted as “pure” for DNA.
   c. The sample concentration will be calculated in ng/µL based on absorbance at 260 nm and the selected analysis constant (1 O.D. at 260 nm for double-stranded DNA = 50 ng/µL of dsDNA)
   d. The reading at 280 nm gives the amount of protein in the sample. Pure preparations of DNA and RNA have OD 260/OD280 values of 1.8 to 2.0,
respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

5. When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping prevents sample carryover in successive measurements.

6. Cleaning the Pedestals
   Wiping the sample from both the upper and lower pedestals (as shown below) upon completion of each sample measurement is usually sufficient to prevent sample carryover and avoid residue build-up.
   Although generally not necessary, 2 µL water aliquots can be used to clean the measurement surfaces after particularly high concentration samples to ensure no residual sample is retained on either pedestal. After measuring a large number of samples, however, it is recommended that the areas around the upper and lower pedestals be cleaned thoroughly. This will prevent the wiping after each measurement from carrying previous samples onto the measurement pedestals and affecting low-level measurements. A final cleaning of all surfaces with de-ionized water is also recommended after the user's last measurement.
Background information
Nucleic acids and proteins have absorbance maxima at 260 and 280 nm, respectively. Historically, the ratio of absorbances at these wavelengths has been used as a measure of purity in both nucleic acid and protein extractions. A ratio of \(~1.8\) is generally accepted as “pure” for DNA; a ratio of \(~2.0\) is generally accepted as “pure” for RNA.

Similarly, absorbance at 230 nm is accepted as being the result of other contamination; therefore the ratio of \(A_{260}/A_{230}\) is frequently also calculated. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2.

Residual chemical contamination from nucleic acids extraction procedures may result an overestimation of the nucleic acid concentration and/or negatively influence downstream analysis. Shown below (Fig. 1) are example spectra for 4 common extraction reagents which, if not properly cleaned up, will affect sample purity.

• **Contaminant Identification**

Examination of sample spectra may be useful in identifying that a problem with sample purity exists. It is recommended that the following be reviewed after each sample measurement:

- 260/230 ratio – a low ratio may be the result of a contaminant absorbing at 230 nm or less.
- 260/280 ratio – a low ratio may be the result of a contaminant absorbing at 280 nm or less.
• Wavelength of the trough in sample spectrum— this should be at ~230 nm. Absorbance by a contaminant at a low wavelength will typically shift the wavelength of the trough. Refer to Figure 2.

• Wavelength of the peak in sample spectrum— this should be at 260 nm. Absorbance by a contaminant may shift the peak absorbance wavelength. Refer to Figure 2.

• 260/230 Ratios
Some contaminants have characteristic profiles, e.g. phenol, however many contaminants present similar characteristics: absorbance at 230 nm or less. Abnormal 260/230 values may indicate a problem with the sample or with the extraction procedure, so it is important to consider both.

A low A260/A230 ratio may be the result of:
• Carbohydrate carryover (often a problem with plants).
• Residual phenol from nucleic acid extraction.
• Residual guanidine (often used in column based kits).
• Glycogen used for precipitation.

A high A260/A230 ratio may be the result of:
• Making a Blank measurement on a dirty pedestal
• Using an inappropriate solution for the Blank measurement. The blank solution should be the same pH and of a similar ionic strength as the sample solution. Example: Using water for the Blank measurement for samples dissolved in TE may result in low 260/230 ratios.

• 260/280 Ratios
Abnormal 260/280 ratios usually indicate that the sample is either contaminated by protein or a reagent such as phenol or that there was an issue with the measurement.

A low A260/A280 ratio may be caused by:
• Residual phenol or other reagent associated with the extraction protocol
• A very low concentration (< 10 ng/µL) of nucleic acid

High 260/280 purity ratios are not indicative of an issue.
Although purity ratios and spectral profiles are important indicators of sample quality, the best indicator of DNA or RNA quality is functionality in the downstream application of interest. If the purity ratio is significantly higher than expected, it is best to review the spectral profile as a primary means of troubleshooting.

It is important to note that there are occasions when the purity ratios are within expected limits, yet there is a problem with the sample.

- **Shifts in Spectral Profile**

![Shifted peaks and troughs in spectra](image)

**FIGURE 2.** Spectra of purified DNA without contamination (A), and of the same DNA sample contaminated with guanidine (B) and phenol (C).

- **Change in sample acidity:** Small changes in the pH of the solution will cause the 260/280 to vary (1). Acidic solutions will under-represent the 260/280 ratio by 0.2-0.3, while a basic solution will over-represent the ratio by 0.2-0.3.

- **Wavelength Accuracy of the Spectrophotometers**
  Although the absorbance of a nucleic acid at 260 nm is generally on a plateau, the absorbance curve at 280 nm is quite steeply sloped. A slight shift in wavelength accuracy will have a large effect on 260/280 ratios.
The micro volume capability of NanoDrop spectrophotometers allow the researcher to quickly and easily run quality control checks of nucleic acid and protein samples. In addition, the instrument’s short measurement cycle and general ease of use greatly increases the rate at which samples can be processed, making it possible to implement multiple quality control checks throughout a procedure or process.

Internet resources and further reading
2. Nanodrop homepage www.nanodrop.com
6. Using a spectrophotometer to quantitate DNA and RNA  
   http://www.mc.vanderbilt.edu/root/pdfs/mclaughlin_lab/dna_and_rna_with_a_spectrophotometer.pdf
7. NanoDrop Microvolume Quantitation of Nucleic Acids (movie)  
   http://www.jove.com/index/Details.stp?ID=2565
11. Recipes

**Chloroform: isoamyl alcohol (25:1).** To a 100 mL Duran bottle, add 96 mL chloroform and 4 mL isoamyl alcohol. Mix by gently swirling the bottle. Cover the bottle with foil and label. Store at room temperature in a flammables store.

**0.5 M EDTA, pH 8.0.** To prepare 1 litre of EDTA at 0.5 M (pH 8.0), add 186.1 g of disodium EDTA.2H₂O to 800 mL of sterile deionised water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approx. 20 g of NaOH pellets). Adjust the volume to 1 litre with water. Dispense into 200 mL aliquots in Duran bottles and sterilize by autoclaving. *The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.*

**70 % Ethanol.** Mix 70 mL ethanol and 30 mL sterile deionised water. Store in a 100 mL Duran bottle at room temperature.

**5 M NaCl.** To prepare a 5 M solution: Dissolve 292 g of NaCl in 800 mL of sterile deionised water. Adjust the volume to 1 liter with water. Dispense into 200 mL aliquots in labelled Duran bottles and sterilize by autoclaving. Store at room temperature.

**5M potassium acetate.** Dissolve 49.07 g potassium acetate in 70 mL sterile deionised water then bring up to volume to 100 mL with sterile deionised water. Sterilize by autoclaving for 15 min.

**RNase A** (20 mg/mL). Dissolve 200 mg of RNase (Sigma # R4875) in 10 mL of 10 mM Tris-HCl pH 7.5, 15 mM NaCl in a 15 mL Falcon polypropylene tube. Heat in a boiling water bath for 15 min, then remove from the water bath and allow to cool to room temperature. Mix by inverting the tube a few times, then centrifuge briefly. Dispense into 1 mL aliquots in labelled 1.5 mL Eppendorf tubes and store at -20°C.
Alternatively, purchase RNase solution (10 mg/mL; DNase and protease-free) from a biotech company. Store at -20°C.

**20 % SDS (sodium dodecyl sulphate).** Dissolve 50 g of electrophoresis-grade SDS in 200 mL of sterile deionised water. Heat to 68°C in a water bath and then stir with a magnetic stirrer to dissolve. If necessary, adjust the pH to 7.2 by adding a few drops of 1 N HCl. Adjust the volume.
to 250 mL with water. Store at room temperature. Sterilization is not necessary. Do not autoclave.

**SDS/DTT Extraction Buffer.** This recipe is for 50 mL buffer, which is sufficient for 100 samples.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>volume</th>
<th>final conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl, pH 8.0</td>
<td>5 ml</td>
<td>100 mM</td>
</tr>
<tr>
<td>0.5M EDTA, pH 8.0</td>
<td>5 ml</td>
<td>50 mM</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>5 ml</td>
<td>500 mM</td>
</tr>
<tr>
<td>20% SDS</td>
<td>2.5 ml</td>
<td>1%</td>
</tr>
</tbody>
</table>

In a 100 mL Duran bottle mix together the reagents above, make up to 47.5 mL with sterile de-ionized water and adjust to pH 8.0 using concentrated HCl. This can be stored at room temperature for up to 1 month. On the day of use, add 1 g PVP (40,000 MW) and 500 µL of 1M Dithiothreitol (DTT)* in fume hood. Mix using a sterile magnetic bar. Then make up to 50 mL with sterile de-ionized water.

*Obtained from Sigma. Store at 4°C.

**3 M Sodium acetate pH 8.0.** Dissolve 204.12 g of sodium acetate.3H₂O in 400 mL of sterile deionised water. Adjust the pH to 8 with glacial acetic acid. Adjust the volume to 500 mL with sterile deionised water. Dispense into 100 mL aliquots in 100 mL Duran bottles and sterilize by autoclaving.

**10X TBE Buffer (pH 8).** Prepare 1 litre of a 10X stock solution: In 800 mL of sterile deionised water, dissolve 108 g of Tris base, 55 g of boric acid, and 9.3 g EDTA. Stir with a magnetic stirrer to dissolve. Make up the volume to 1 litre with sterile deionised water and mix. Transfer to a sterile 1 litre Duran bottle. Store at room temperature. The pH of the concentrated stock buffer should be approx. 8.3. Dilute the concentrated stock buffer to 0.5X with deionised water before use.
**TE-1 buffer** (Low salt Tris-EDTA buffer) (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). To make 100 mL of TE-1, mix 1 mL of 1 M Tris-HCl pH 8.0 and 20 µL of 0.5 M EDTA pH 8.0 and sterile deionised water to 100 mL. Autoclave to sterilise. Dispense 10 mL aliquots in labelled universal tubes\(^1\) and store at room temperature.

\(^1\)Universal tubes e.g. 30 mL polystyrene tube from Sterilin (# 128B):

![Universal tube](https://example.com/universal-tube.png)

**1 M Tris-HCl (pH 8.0).** Dissolve 121.1 g of Tris base in 800 mL of deionised water. Adjust the pH to 8.0 by adding approximately 42 mL of concentrated HCl. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with deionised water. Dispense into 4 x 250 mL aliquots in clean 250 mL Duran bottles and sterilize by autoclaving. *If the 1 M solution has a yellow colour, discard it and obtain Tris of better quality. Note: the pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.*

**Water**

There are different grades of water used in the Workshop.

- Tap water
- Deionised Water: water deionised on a Milli-Q system (see [http://en.wikipedia.org/wiki/Milli-Q](http://en.wikipedia.org/wiki/Milli-Q))
- Sterile deionised water: water deionised on a Milli-Q system, then autoclaved to sterilise.
- Nuclease Free Water (ultrapure DNase/RNase-Free water). This is supplied in many molecular biology kits, or can be purchased separately.
12. Additional information

1. Introduction to Molecular Biology
   http://learn.genetics.utah.edu/
   http://learn.genetics.utah.edu/content/begin/dna/
   http://www.web-books.com/MoBio/
3. Free Video lectures: Molecular Biology
   http://freevideolectures.com/Course/2305/Introductory-Biology/9#
4. MIT OpenCourseWare: Molecular Biology
5. Course: Molecular and Cell Biology C148, 001 - Spring 2011
   http://www.dnatube.com/courses/berkeleymolcellb
6. DNA sequencing
   http://en.wikipedia.org/wiki/DNA_sequencing
7. Introduction to Sanger sequencing
   http://en.wikipedia.org/wiki/Sequencing#Sanger_sequencing
8. 454 high throughput pysequencing
   http://www.sumanasinc.com/webcontent/animations/content/highthroughput2.html
   http://www.sumanasinc.com/webcontent/anisamples/molecularbiology/pcr.html
    http://www.dnalc.org/ddnalc/resources/electrophoresis.html
    http://www.dnalc.org/ddnalc/resources/pcr.html
12. Generation Challenge Programme (GCP) –Capacity building corner
    http://www.generationcp.org/sp5/
13. Guide to pH Measurement:
15. Sanger sequencing
16. DNA Sequencing by Capillary Electrophoresis
   http://tools.invitrogen.com/content/sfs/manuals/cms_041003.pdf
   http://www.amazon.co.uk/Bioinformatics-Dummies-Jean-Michel-Claverie/dp/0470089857
   Bioinformatics for Dummies Downloads:

18. IMMB Workshop 2013
   http://hpc.ilri.cgiar.org/beca/training/IMBB/welcome.html

20. Inqaba Biotec www.inqababiotec.co.za
22. Merck Millipore www.merckmillipore.com
23. Roche www.roche-applied-science.com
### 13. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPB</td>
<td>Bromophenol Blue</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>kbp</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PVP40</td>
<td>polyvinylpyrrolidone, molecular weight 40,000</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Taq pol</td>
<td><em>Thermus aquaticus</em> DNA polymerase</td>
</tr>
<tr>
<td>TBE</td>
<td>tris borate-EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>
14. Acknowledgements

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We also gratefully acknowledge the following companies who provided laboratory consumables used in the workshop: F&S Scientific (www.fnscientific.com), Inqaba Biotec (www.inqababiotec.co.za), Merck Millipore (www.merckmillipore.com) and Roche (www.roche-applied-science.com).

Sequence trace file generated by one of the participants at the 2011 workshop.

http://hpc.ilri.cgiar.org/beca/training/IMBB/welcome.html