Laboratory Handbook – Using Portable Equipment for the Assessment of Genetic Background in Ecotoxicology

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Introduction

In this class, we will extract DNA from the springtail, *Folsomia candida*, followed by a PCR and electrophoresis to assess the results using two portable labs.

Bento lab
(https://bento.bio/resources/manual/)

miniPCR bio™
(https://www.minipcr.com/tutorials/)

Bentolab demonstration:
https://www.youtube.com/watch?v=KrMvwW_QNIw&ab_channel=IGCiencia

Teaching Objectives

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

1) DNA extraction/isolation from Eukaryotic tissues using two different protocols (adapted)
    a) HotSHOT
    b) Dipstick
2) PCR techniques targeting a standard gene marker used in animal barcoding studies;
3) PCR product visualisation techniques (gel electrophoresis);
4) Data analysis of sequenced PCR products (already done on the first-class part of this course topic).

Particular attention should be paid to the following points:

- The manual should be a guide to assist you with the experiments rather than a complete recipe book.
- Specific safety requirements (in addition to those appropriate to everyday laboratory practice) must be adhered to (see next page). It is a requirement that you read the section on safety before commencing any work.

Experimental Overview, “DNA barcoding”

In the field of ecotoxicology, understanding the intricate relationship between organisms and their environment is paramount for assessing the impact of pollutants and contaminants. Central to this understanding is the recognition that genetic background plays a crucial role in determining an organism's response to environmental stressors. DNA barcoding, a powerful tool in molecular biology, offers a unique perspective in ecotoxicological studies by allowing researchers to precisely identify and characterize species based on their genetic background.

Edaphic fauna (such as bacteria, fungi, worms and springtails) are ideal organisms for terrestrial ecosystem monitoring. On the one hand they are significant contributors to the biomass and fertility of many temperate soils, and on the other hand, their lifestyles and body structure render them susceptible to human disturbance and poisoning.

A batch of springtails assembled for laboratory use in ecotoxicology studies has been gathered. These specimens are *in vivo*, and your challenge is to determine the genetic identity of the organisms, using standard gene barcoding techniques.

A reliable recovery of the mitochondrial marker, the cytochrome oxidase subunit 1 (COI) gene, is critical for the identification of a given sample. In this study, you can use a set of universal primers for increased efficiency of barcode recovery in springtails. You will then use the barcode sequence to recover an identification using sequence information available in an open data repository (e.g. NCBI).

Laboratory Practice and Molecular Biology

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

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

Responsibilities:
All glassware and solutions should be autoclaved before use. When work is finished, waste should be autoclaved and disposed of through the waste management system.

Gloves and lab coats should be worn at all times.

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

1) Nucleases are ubiquitous, and some are extremely stable enzymes. It is vital that these enzymes do not come in contact with your samples. Therefore, apparatus (eppendorfs and tips etc) and solutions that come in contact with your samples must be sterilised. Furthermore, always wear disposable gloves - the skin is an excellent source of nucleases, and there is enough enzyme in a fingerprint to ruin your experiments.
2) Experiments must be carried out using good laboratory practice. This means that the normal safety precautions applicable to the handling of animals' tissues apply to the experiments described in this manual. Furthermore, all media and apparatus used for handling tissues should be sterile.
3) Assume all your nucleic acid samples and the modification enzyme that are heat labile and keep them on ice unless indicated otherwise by protocols.
4) The key to many of the techniques described in this manual is the ability to pipette small volumes carefully and accurately. It is essential when pipetting small volumes to ensure that the tip of the pipette is touching the surface of the container before expelling the solution. Always use clean pipette tips to avoid cross-contamination. Pulse spin Eppendorfs to ensure that all of the added components of reaction mixtures are mixed at the bottom of the tube.
5) The transilluminator should be used for the minimum time possible as prolonged exposure of DNA samples to UV radiation causes random strand breakage - also the lamp has a finite life. Please avoid scratching the surface of the transilluminator as this will destroy the effectiveness of the filter - a new transilluminator costs more than €1500. A general point about DNA work is that reagents are extremely expensive. Please use reagents/enzymes as sparingly as possible.

Notes About Preparing Materials for Use with DNA

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

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

Responsibilities

- Good laboratory practice is needed (e.g., NO FOOD is allowed in the lab at any point).
- The working area must be clean during work and maintained accordingly. Leave the laboratory space a little better than you found it.
- No mobile phones on benches, and altogether avoid using them during practical work, if you need to record some data with your phone, ask first.

- Wear a lab coat and gloves to protect DNA from nucleases
- Waste disposables and solutions in autoclave bags

Equipment Required
Autoclave (operated by a specialized technician)

Method
Sterilisation
(this will be done for you)

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

1. DNA extraction

1.1 Introduction

DNA extraction, a fundamental procedure in molecular biology, entails isolating DNA from cells within a sample, typically derived from biological sources like blood, saliva, or tissue. This process involves cell lysis to liberate DNA from the nucleus or other DNA-containing organelles, then eliminating proteins and contaminants to purify the DNA. It is a pivotal initial step for numerous DNA analyses, including PCR and sequencing. Selecting an appropriate extraction method considers factors such as tissue type and DNA integrity. This routine procedure aims to separate nucleic acids from cell membranes, proteins, and other cellular constituents through steps including cell disruption, removal of membrane lipids, protein removal, and DNA precipitation.

1.2 HotSHOT protocol

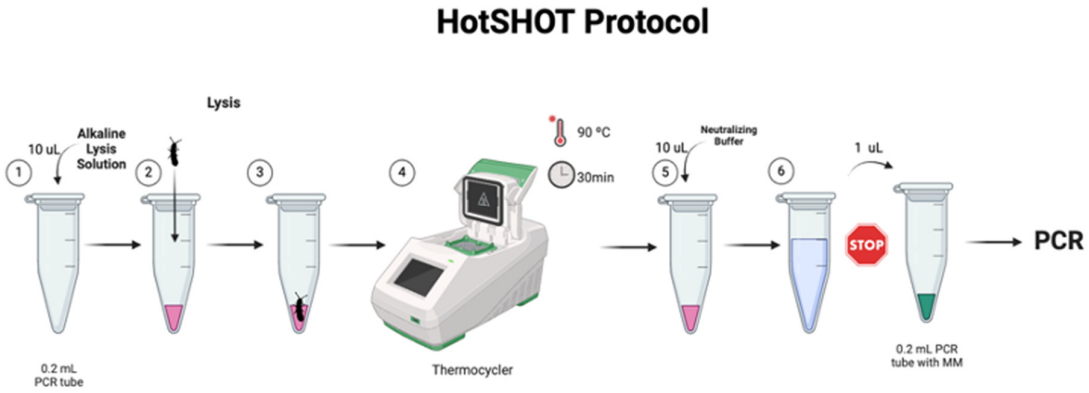
For more information: https://bento.bio/resources/datasheets/hotshot-dna-extraction-kit/
Protocol demonstration: https://youtu.be/Ej91tNYsdr8

Always wear gloves and use sterile equipment and sterile working practices. The following protocol was adapted from *HotSHOT DNA Extraction Kit*, (2021).

1. Make a numbered list of samples, and label a PCR tube per sample with the corresponding sample number using a fine permanent marker.
2. Place 10µL of Alkaline Lysis Solution in a 0.2 mL PCR tube per sample.
3. Put one springtail individual and transfer into each tube, ensuring it is immersed in the Alkaline Lysis Solution**.** Adding excess sample will likely cause the extraction and/or PCR to fail.

4. Place the tubes in a thermocycler and incubate at 95 °C for 30 minutes using the Heat Block (ab) programme. In the meantime follow the Dipstick protocol bellow.
5. Add 10 µL of Neutralising Buffer to each 0.2 mL tube.
6. Proceed to PCR protocol

Adapted from *HotSHOT DNA Extraction Kit*, (2021)



1.3 Dipstick protocol

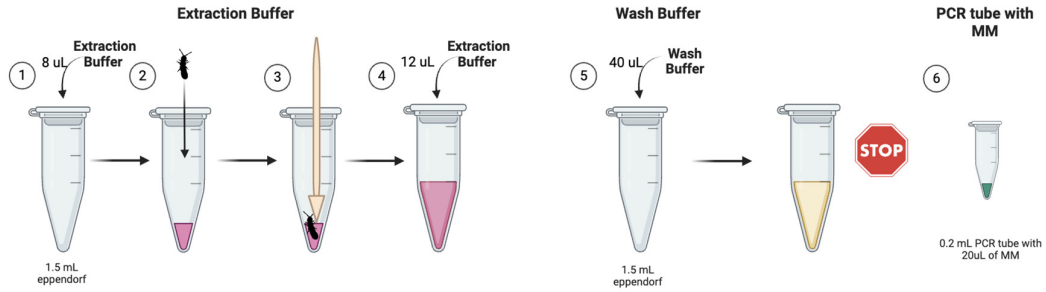
For more information: https://bento.bio/product/dipstick-dna-extraction-kit/
Protocol demonstration:
https://www.youtube.com/watch?v=i5JmZ8n13es&ab_channel=BentoLab

Always wear gloves and use sterile equipment and sterile working practices. The following protocol was adapted from *Dipstick DNA Extraction Kit*, (2021).

1. Pipette 8 µL Extraction Buffer into a 1.5 mL tube.
2. Add one springtail individual to the tube using sterile instruments.
3. Smash the individual with a sterile tip (10µL) for 10 seconds or more.
4. Add 12 µL Extraction Buffer for a total volume of 20µL, and close the tube.
5. Into a new 1.5mL tube pipette 40 µL of Wash Buffer and close the lid.
6. **Wait** and prepare the PCR master mix. Proceed to PCR protocol.

Adapted from *Dipstick DNA Extraction Kit*, (2021)

Dipstick Protocol



2. PCR

2.1 Introduction

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

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

Equipment Required:
PCR Machine (MiniPCR and Bentol Lab)

Material/ Solutions Required:
Template DNA

- Template DNA from the HotSHOT protocol
- Stick wil DNA template from DipStick protocol

2 x NZYTaq II 2 x Master Mix
COI Universal primers for invertebrates at 10 µM stock (check Table 1).
Sterile Materials:

- 1ml, 200 µl and 10 µl tips
- 1.5ml eppendorfs

- Sterile H2O
- 0.2 ml thin wall PCR tubes

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

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

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

2.2 Protocol

1. This is the PCR master mix preparation step. We are going to prepare a PCR solution enough for **3x PCR reactions**, (1 per DNA extraction protocol)l + 1 negative control (without DNA template). Keep **tubes on the ice** at all times. Add the following components to a new and labeled 1.5 ml Eppendorf tube (keep tubes in ice when not using):
    a. 13.2 µl Sterile H2O
    b. 15.8 µl 2 x NZYTaq II 2 x Master Mix
    c. 1.3 µl 10 µM mix of each Primer

    31.5 µl Total

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

2. According to the DNA extraction protocol:
    a. HotSHOT:
        i. Aliquot 9 µl of final mix into each of 2 thin-walled 0.2ml PCR tubes.
        ii. Add 1 µl of your DNA extract to the first PCR tube and label it.
        iii. To the remaining tube, add 1ul of sterile water, and this makes your negative control.

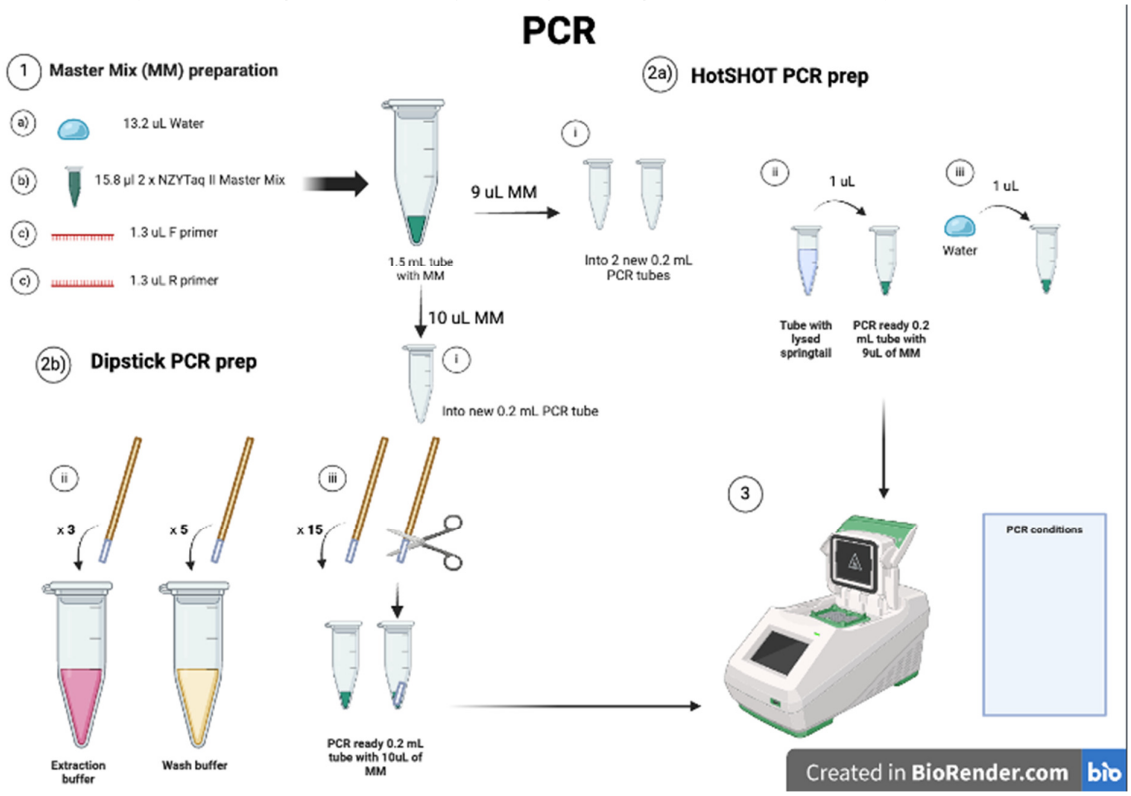
    Place your reaction on ice and give your tubes to the lecturer/demonstrator.

    b. Dipstick
        i. Aliquot 9 µl of final mix into one thin-walled 0.2ml PCR tubes.

ii. Dip the dipstick into the PCR mix up to 15 times to release the DNA. **Tip**: Push the dipstick into the bottom of the tube until the dipstick bends. This helps the liquid move through the paper, and increases the amount of DNA released. You can also cut the tip and leave it inside the PCR tube.

iii. Wipe the dipstick on the edge of the 0.2 mL tube to remove any drops of PCR mix. The PCR reaction is now ready to start.

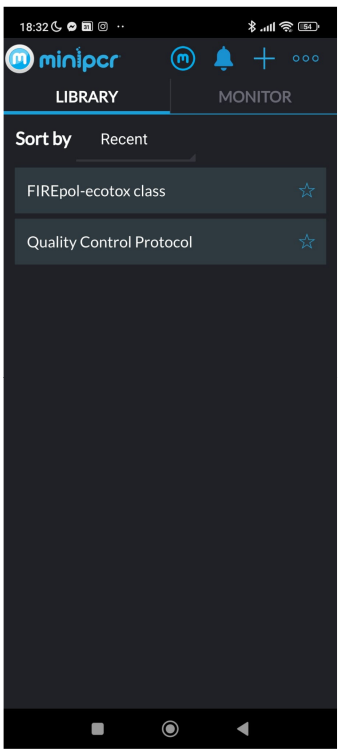
3. The tubes will be placed by the lecturer/demonstrator in a thermal cycler. Make notes of the program settings of the thermocycler. The reaction will take about 2hrs and the lecturer/demonstrator will take the tubes out. Briefly, the PCR solutions inside the tubes will pass cyclically through different temperatures at different times.
4. At the end of the cycling period, remove samples and put them in the fridge for later analysis (electrophoresis on agarose gels) or proceed immediately.

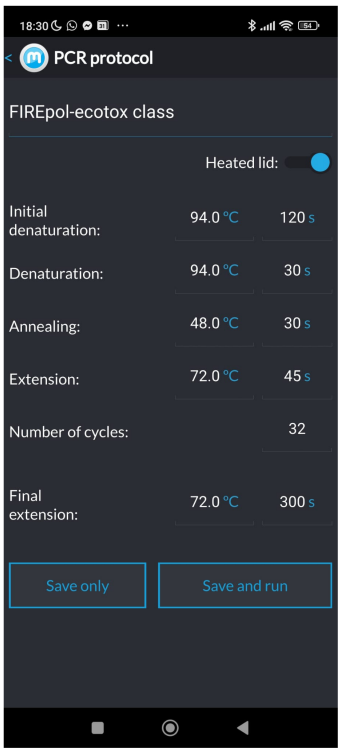


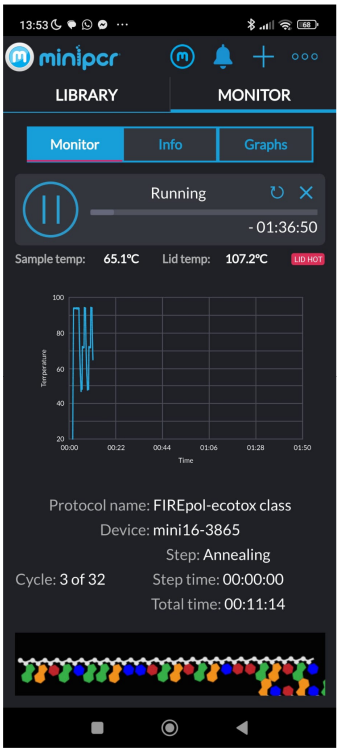
PCR steps and program (may change):

- Denaturation, separates the double strand of template DNA. It is set at 95°C for 30 sec (with an initial denaturation of 5 min)
- Annealing; during this phase, the primers attach to their complementary regions on the DNA strands. This is set at 50°C for 40 sec.
- Elongation, where the polymerase extends the attached primers. This is set at 72°C for 1 minute (primer extension). These temperature stages are set to occur cyclically 35 times. A final extension time at 72°C for 10 minutes, is included.

Example of setting up PCR conditions in







MiniPCR mobile app:

3. Agarose Gel Electrophoresis

3.1 Introduction

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

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

Equipment Required:
Transilluminator with gel doc system
55°C water bath
Bento lab electrophoresis apparatus, blueGel, Fischer miniGel

Material/ Solutions Required:
15/ 30/ x ml molten agarose (1%) in 1x TAE at 55oC
Electrophoresis buffer – 1x TAE
Sterile Materials:

- 1 ml, 200 µl and 10 µl tips
- 1.5 ml eppendorfs
- 5x Loading dye
- SafeView DNA Stain

3.2 Protocol

The following protocol is similar for both Betnlab and miniPCR blue gel electrophoresis apparatus. For demonstration of usage for each portable sets see link below:

Demonstration of the miniPCR electrophoresis with blueGel apparatus: https://www.minipcr.com/gel-electrophoresis/

1. Seal the ends of the gel holder with rubber ends (masking tape can be used if the apparatus does not contain rubber sealing ends). Also, make sure that the wells do not touch the bottom, if gel is perforated you will lose your loaded sample.
2. Place the comb in the correct orientation across the end of the gel holder.
3. Agarose will be melted in 1xTAE, in a 1% proportion, according to the size of the electrophoresis apparatus being used, in an autoclave or using a microwave, i.e. 50mL of TAE to 0.5g of Agarose. Once agarose is molten then place in a 55°C water bath (this will be performed for you in the teaching laboratory).
4. Check that agarose is no hotter than 55°C, add i.e. 2.5 µl of SafeView DNA Stain mix, (keep the proportion according to the apparatus being used) by swirling and pour into the holder taking care to avoid air bubbles. Allow to set (approx. 15 min). Do this with the gel holder on the bench - not in the electrophoresis apparatus.
5. Carefully remove the comb and seal ends and place the gel holder into the electrophoresis apparatus. Ensure that the wells are placed over the red band. Take care that the agarose gel does not slip off the plastic tray!
6. Pour 1x TAE into the apparatus until the gel is just covered. Ensure that no air remains in the wells by washing with 1x TAE.
7. Prepare your standard by the addition of an appropriate volume of 5x loading dye (i.e. 5 µl sample and 1 µl of loading dye) into a piece of parafilm. If using the 2 x NZYTaq II 2 x Master Mix, the sample can be directly loaded into the gel (i.e. 5ul, may vary according to the gel size). The Standard used is a 100 bp DNA ladder. Place the lid and connect the power supply.
8. Run the gels at 90-120 V until the dye has migrated approx. half the length of the gel (~30 min), according to the apparatus being used.
9. Switch off and disconnect the power supply.
10. If needed, remove gel on the holder and transport it to the transilluminator. Place gel on the transilluminator, ensure the door is closed and visualise fluorescence using CCD camera. If recording the picture digitally use your student number and the date as the file name. If using the BlueGEL or BentoLab, turn on the blue light.
11. Switch off the transilluminator, remove your gel and leave the equipment clean and dry.