SLE254
Genetics and Genomics

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Practical Manual
2024

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Preface

This manual has been designed as a guide to the practical components of the course and encompasses two broad tasks. Specifically, we will look at the epistatic interactions between genes that attribute to mammalian (cat) coat colour in Practical 1, and the genetic approaches to distinguishing the sex of a species (using domestic chickens as an example) in Practicals 2-4.

In T2 2024, these practicals will be delivered face to face on campus. These will run in Weeks 2, 4, 6 and 8 at Burwood and the Waurn Ponds campus. You will be expected to attend these practicals at your campus.

It is important that you attend these sessions as these practicals will form the basis of two specific assessment items: 1) a series of assessable online questions as part of Practical 1 (worth 8% of your final mark); and 2) a practical report written up as a scientific poster encompassing Practicals 2-4 (worth 32% of your final mark). All of the results required for the development of the associated assessment items will be made available to you throughout the sessions or on the Unit Cloud site.

For further information regarding the practical rules and laboratory safety instructions, please see Appendix 1 and 2 and the end of this manual.

Contents

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Practical 1 Epistatic interaction between genes: the genetics of mammalian coat colours

Felis catus

There are no hazardous chemicals or equipment used in this practical.

Introduction

Mammals, unlike worms and insects, contain a restricted number of pigments. One group of these pigments is melanin, the most important producer of coat colour in mammals.

In mammals, melanins exist in two distinct forms:

a. eumelanin (brown or black)
b. phaeomelanin (yellow or reddish, also called orange)

These melanins occur as minute pigment granules in the cortex and medulla of hair, in the epidermis, and other sites such as the iris. The genetics of coat colour in mammals is mainly about genes that affect pigment granules either by altering their number, shape or arrangement, or by substituting one type of pigment for another.

We will consider the domestic cat, *Felis catus* (2n = 38), as an exemplar of mammalian coat colour genetics as these animals display genetic polymorphisms that are expressed in the readily visible variations in coat colour, coat pattern, and coat composition

Aims

1. To understand the role of melanins in coat colour polymorphisms in mammals.
2. To investigate the major genes, and their alleles, that affect the nature and organisation of melanins associated with coat colour in mammals.
3. To understand the relationship between the primary control by alleles of a gene and how their action may be perceived in a phenotype.
4. To examine special genic effects such as epistasis.

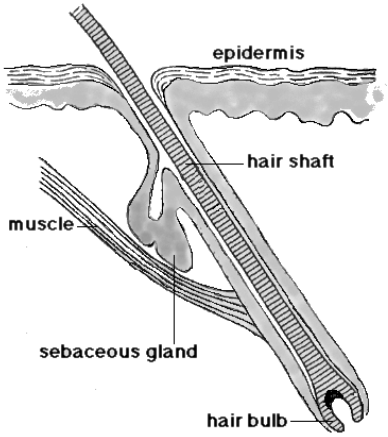
Fur (hair) pigmentation in mammals

The development of colour or pigmentation in the coat of a cat is typical of the situation for all mammals. It is a complex process involving many steps that can be influenced by both genetic and environmental factors. To best understand how some of the different colours and patterns arise, it is useful to first consider the cells responsible for the manufacture of pigment. Melanoblasts are presumptive pigment-producing cells that form during embryonic development in the region of the neural crest that lies close to the site of formation of the spinal cord. During embryonic growth, the melanoblasts

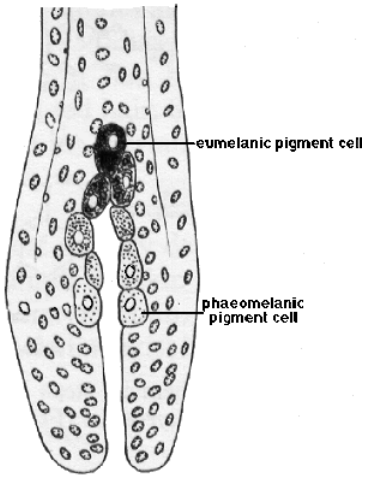
migrate from their site of formation in the neural crest to the skin where they lodge at the base of developing hair follicles where they become known as melanocytes.

Hair, also called fur, growing in regions where pigment producing cells have lodged could be expected to be pigmented; fur growing in regions where pigment producing cells have failed to migrate could be expected to be unpigmented, that is, white.

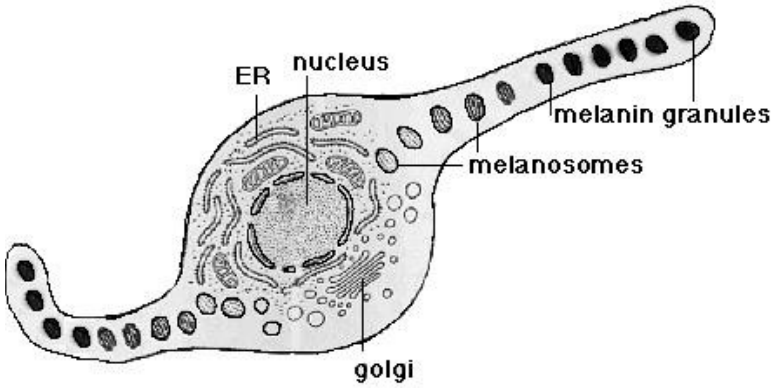
Look at the figures below. The structure of a hair follicle is shown in **a**. Note the bulb area of the hair. This is the site of active melanocytes as detailed in **b**. Figure **c** shows the structure of an active melanocyte. Note the different stages of development of the pigment granules (melanosomes) within the melanocyte. The pigment granules are transferred from melanocytes into the hair.



a. Hair follicle



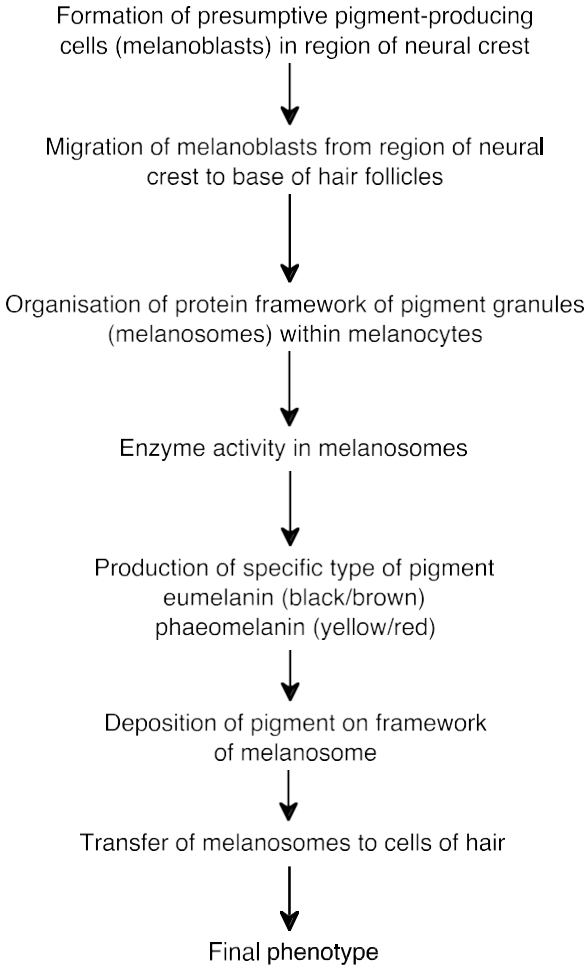
b. Detail of agouti bulb region showing location of melanocytes



c. Melanocyte containing melanosomes and melanin granules

The development of melanocytes, their migration, and pigmentation can be summarised in the flow chart below: Refer to the chart as you work through the exercise.

What is the characteristic actually controlled at each step and in what ways are the particular actions of the alleles of the genes involved perceived in the phenotypes of the animals.



Procedure

You will have cards representing **10 'stations'** of photographic posters related to mammalian coat colour and patterns (sometimes more than one card for a station). Examine each of these, make notes if appropriate and answer the questions below related to each display. These questions must be considered in the context of material in the practical manual, demonstration material provided in class, and material discussed in lectures.

As you work through the examples, recall the relationship between gene action and the ultimate phenotype that we are able to observe. In which cases does epistasis occur? What is the end result of variation in migration rates of cells, production of pigment, its packing into granules and their distribution within hair?

Binomial theorem activity

At one of the stations (Station 4), you will be required to use the binomial expression to determine the probabilities of particular combinations of phenotypes that would arise from parents with specific genotypes. You will also be going through the binomial

theorem in lectures! Make sure you are familiar with how to apply this theorem for solving phenotype probability.

The Binomial Theorem

The **binomial theorem** can be used to determine the *probability that any group of F_2 (2^{nd} generation) individuals will have a particular combination of phenotype*

n = total number of events (offspring)

x = number of times event A (***dilution***) occurs

(n – x) = number of times event B (***non-dilution***) occurs

occurs n! = factorial n = n(n - 1)(n – 2) …….1

p = Probability of ***dilute*** phenotype

q = Probability of ***non-dilute*** phenotype (1 - P)

$$\text{Probability of phenotype} = \frac{n!}{x!\,(n-x)!}\, p^x q^{(n-x)}$$

Remember FACTORIALS (!):
If there are 6 kittens:
6! = 6x5x4x3x2x1

Mother's genotype \ Father's genotype	D	d
D	DD	Dd
d	Dd	dd

Offspring genotype possibilities:

Non-dilute (DD or Dd): 3/4

Dilute (dd): 1/4

Assessment

Work through the questions asked in relation to each station and make sure to take notes and complete ALL questions (including the special problems) related to the stations. Once you have all of your answers ready, log into the Unit Cloud site and complete the Cat prac worksheet under the Assessments/Quizzes folder. Each student will be allocated random questions, thus make sure that you have completed all stations and have all of your notes/answers ready from the practical session.

You will have 2 hrs to complete the worksheet**, after 120 minutes you will be prevented from making further changes. ONCE YOU START YOU MUST FINISH (you cannot log out of the test, then log back in, the timer is continuous).**

This is worth 8 % of your final mark.

Colour polymorphisms and their genes

Migration of melanocytes — Station 1

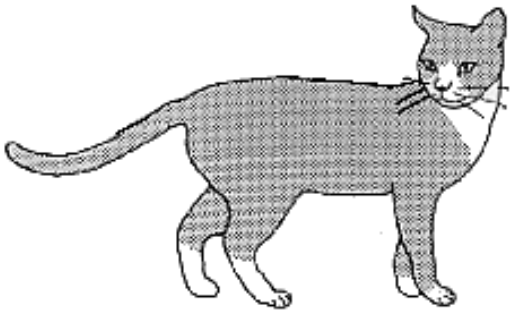
The migration of melanocytes is under the control of a single gene which has the alternative alleles

S : melanocyte migration incomplete → white spotting

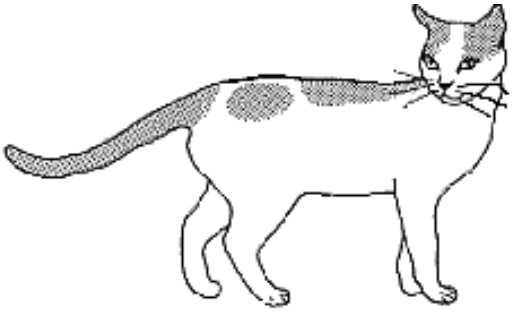
s : melanocyte migration complete → no white spotting

The presence of white spotting is dominant to their absence.

The migration of melanocytes in cats that are homozygous **SS** or heterozygous **Ss** is restricted so that melanocytes do not lodge in some areas of the skin. As a consequence, some cats have more or less extensive areas of unpigmented (that is, white) fur. Note that this trait does not refer to the few scattered white hairs that one can sometimes find on a cat. There is evidence to support the view that homozygous cats, **SS**, have more extensive white areas than heterozygotes, **Ss**, so that these cats appear as follows:



Heterozygous for spotting **Ss**



Homozygous for spotting **SS**

You will see examples of these and the range of variations on your poster sheets.

Examine poster 1 related to cats.

Q1. *(1 mark) What is the genotype, with respect to the **'S'** gene locus, of the cat*

- ***a.*** *with some white?*
- ***b.*** *with extensive white?*

Q2. *(3 marks) Consider the actual action of the **'S'** gene and offer an explanation for the fact that cats with the same genotype with respect to this gene can show a difference in the distribution of non-pigmented areas.*

Synthesis of pigment — Station 2

The synthesis of pigment occurs in the melanocytes and is a complex process involving many steps. A simplified pathway is as follows:

Tyrosine → Dopa → Dopa Quinone → → → → → Melanin (pigment)

The speed at which each step in this series of reactions proceeds is influenced by enzymes. For example, the enzyme tyrosinase catalyses the first and second steps shown above. The nature of this enzyme is controlled by a gene which has multiple alternative alleles

C	:	normal enzyme produced	→	full colour
c^B	:	less active enzyme produced	→	Burmese dilution
c^s	:	temperature-dependent enzyme produced	→	Siamese dilution

Full colour is dominant to Burmese dilution which in turn is dominant to Siamese dilution. The effect of Burmese dilution when present in the homozygote (**c^B c^B**) or heterozygote (**c^B c^s**) is to reduce the colour of a potentially black animal to brown.

When the Siamese dilution is present in the homozygous condition (**c^s c^s**), it restricts pigment production to those areas of the body where the temperature is below a certain level. In effect, pigment appears only on cooler areas of the body, namely feet, tail, ears and mask. This case also demonstrates that the environment can also influence the expression of a phenotype

Examine poster 2

Q3. *(1 mark) What is the genotype of the Blue Burmese cat with respect to the **'C'** gene locus? The kittens in the photograph, taken at a cat show, are from the same litter. Note the ribbons around the necks. This 'code', pink for female and blue for male, is used by breeders to indicate the sex of kittens they may have for sale. At least one of the kittens has been mis-sexed. (Recall from lectures that the **'O'** gene is on the **X** chromosome – refer to station 5.)*

Q4. *(4 marks) Explain which kitten has been mis-sexed.*

Q5. *(2 marks) What colour is the father of the litter? What colour is the mother of the litter?*

Q6. *A Siamese cat has an operation in the abdominal region. During this operation a patch of fur is shaved off. When the fur regrows, it is much darker than the fur in the surrounding area.*

a. *(3 marks) Suggest an explanation for this observation.*

b. *(3 marks) Explain whether you believe the patch of fur would remain dark permanently or lighten over time.*

Packing of pigment in pigment granules Station 3

Pigment produced by melanocytes is packaged into small parcels called melanosomes or pigment granules. These granules have a protein framework which forms first, then the pigment is laid down on the framework.

A gene influences the organisation of the protein framework in the pigment granules. This gene has the alternative alleles

B : protein framework which allows dense packing of pigment inside granule→ black granules

b : protein framework allows less dense packing of pigment inside granule → brown granule

Black is dominant to brown so that cats **BB** or **Bb** will be black and cats **bb** brown.

This gene also exists in dogs except that the **bb** phenotype is called 'liver' rather than brown.

Examine poster 3 - cats showing a mother cat with her litter of seven kittens.

Q7.

- ***a.*** *(1 mark) What is the genotype of the mother at the **'B'** gene locus?*
- ***b.*** *(1 mark) What is the genotype of the mother at the **'C'** gene locus?*
- ***c.*** *(1 mark) What is the genotype of the mother at the **'S'** gene locus?*

Examine poster 3 showing a brown tabby cat.

Q8. *(1 mark) What is the genotype of the brown tabby cat at the **'B'** gene locus?*

Distribution of pigment granules in hair — Station 4

Pigment produced in cells which are located at the base of growing hairs is packed into granules which are then transferred to the growing hair shaft. The gene controlling this transfer of pigment granules to growing hair has the alternative alleles

D : uniform distribution of pigment granules in hair → intense colour
d : uneven distribution of pigment granules in hair → dilute colour

The colour of cats homozygous **dd**, is diluted as shown below

Non Dilute		Dilute
black	**dd** →	blue
brown	**dd** →	lavender
orange	**dd** →	cream

The colour of cats either **DD** or **Dd** is intense, or non-dilute. Note that alternative names for colours are sometimes used. Seal for black, grey for blue, chocolate for brown, lilac for lavender and red or yellow for orange. Genetically, there is no difference between the particular alternatives.

Examine poster 4 – cats.

Q9. *(3 marks) What is the genotype, with respect to the **'B'** and **'D'** loci of a*

- ***a.*** *blue point kitten*
- ***b.*** *lavender point kitten*
- ***c.*** *brown point kitten*

Q10. ***a.*** *(1 mark) What is the genotype of the blue cat at the '**B**' and '**D**' loci?*

b. *(2 marks) Is this identical to any of the kittens in the litter shown? If so, at which gene locus does it differ from the kittens to give such a difference in appearance of colour distribution?*

Binomial theorem activity *(see pages 7-8 for details)*

Q11. *(4 marks) Use the binomial expression to calculate the chance of two heterozygous parents (Dd) generating the following combinations of kittens:*

- *5 dilute kittens in a litter of 6 kittens*
- *4 dilute kittens in a litter of 6 kittens*
- *3 dilute kittens in a litter of 6 kittens*
- *2 dilute kittens in a litter of 6 kittens*
- *1 dilute kitten in a litter of 6 kittens*

What kind of pigment? Station 5

The specific type of pigment produced by a cat is under the control of an X-linked gene with the alternative alleles

O^+ : non-orange
O^o : phaeomelanin pigment produced → orange

In contrast, all other cat genes discussed in this exercise are located on autosomes. Since female cats have two X chromosomes, they may have one of three different combinations of the alleles, namely

O^+O^+ : non-orange, colour determined by other genes
O^+O^o : tortoiseshell
O^oO^o : orange

Male cats contain only one X chromosome, and hence can only be

O^+Y : non-orange
O^oY : orange

The two colours (orange hairs and non-orange hairs) of a tortoiseshell cat relates to the phenomenon of X-inactivation.

Examine posters 5 and 5X.

Q12. *Look at the photographs of Spadgie and Emily. These two cats have the same genotype at the '**O**' locus.*
- ***a.*** *(0.5 marks) On which chromosome is the **'O'** locus?*
- ***b.*** *(0.5 marks) What is their genotype with regard to this locus?*
- ***c.*** *(3 marks) Explain why the distribution of black and orange fur is different in each cat in spite of the identical genotypes.*

Q13. *(2 marks) Note the photograph of the blue and cream tortie. In what way is the genotype of this cat*
- ***a.*** *similar to that of the other torties shown?*
- ***b.*** *different from that of the other torties shown?*

Examine poster 5L that show the results of reciprocal crosses between black and orange cats. Neither of these crosses show orange females and yet orange females can and do exist.

Q14. *(2 marks) Which mother and father combination(s) could produce orange female offspring?*

It is incorrectly claimed by some that orange female cats are not possible. However, they certainly appear less frequently than do orange male cats.

Q15 *(3 marks) Write an explanation for this difference in sex frequency with orange cats that could be readily understood by a non-student of genetics.*

All cats are tabby cats! Station 6

Consider stations 6 and 7 together.

The two common patterns of striping in non-pedigreed cats are mackerel and blotched and are shown in the following figure.

Mackerel tabby stripes

Blotched tabby stripes

These patterns are under the control of a gene that includes the alternative alleles

T^m : mackerel (parallel) stripes
t^b : blotched stripes

There is another allele of this gene

T^A : Abyssinian stripes

which is present in the homozygous state in pedigreed Abyssinian cats. This results in these cats having faint striping on the legs and tail.

Mackerel striping is dominant to blotched, so cats that display the blotched pattern must have the homozygous recessive genotype, **t^bt^b.** You may wish to question the heading of this section and argue that a black cat is not a tabby cat, however all cats must have two alleles for tabbying so how is a black cat possible? That question takes us to the next gene (***'A'*** gene: station 7) that in fact determines whether tabby stripes can be readily observed on a cat.

Examine poster 6. *The photographs include examples of mackerel, blotched and Abyssinian patterns of tabby markings. Each of these cats must have at least one* ***'A'*** *allele for the tabby patterns to be expressed.*

Note: *Abyssinian tabby results in faint striping only when compared with other types of tabby.*
Note: *Mackerel tabby stripes on the cat shown are 'broken'. This is not true for all mackerel tabby cats.*

Q16. *What are the genotypes of the three tabby cats with respect to the '**B**', '**T**' and '**A**' loci?*

- ***a.*** *(1 mark) Tabby cat on the left-hand side of pair photograph.*
- ***b.*** *(1 mark) Tabby cat on the right-hand side of pair photograph.*
- ***c.*** *(1 mark) The Abyssinian tabby.*

Q17. *(3 marks) Explain whether you think the pair of tabby cats could be from the same litter.*

*The black cat is also a tabby cat. However, the genetic instructions for tabby have not been expressed because the cat is homozygous recessive at the '**A**' locus.*

Q18. *(3 marks) What is the genotype of the black cat with respect to the '**B**', '**T**' and '**A**' loci?*

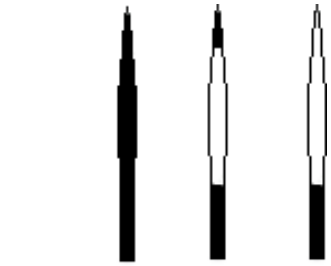
What turns a potential tabby into reality? Station 7

Although all cats have the gene and hence alleles for tabbying, the expression of this genetic information in non-orange cats is influenced by a second gene. This gene has the alternative alleles

A : agouti → tabbying
a : non-agouti → solid colour in non-orange

In non-orange cats that carry at least one **A** allele, tabbying is expressed. Although non-orange cats homozygous for non-agouti, **aa**, do not display tabby markings, in orange or cream cats, tabbying is expressed regardless of whether the cat is carrying the **A** allele or not. Why this is so is not fully understood. Similarly, the control that leads to stripes of agouti combined with stripes of solid colour that typify a tabby cat is unknown.

The agouti locus controls the regional distribution of eumelanin and phaeomelanin pigments in the hair. Agouti hairs have one or more terminal or sub-terminal bands of yellow, due to phaeomelanin pigment granules, the rest of the hair showing the black or brown of eumelanin pigment. Variations in the distribution of hair pigment to show the agouti pattern include



Examine poster 7

Q19 *(1 mark) What is the term given to the genetic phenomenon that exists between the '**T**' and '**A**' genes?*

Examine poster 7.

Note the agouti ticking at the ends of some of the hairs.

Q20. *(1 mark) What changes in kind of pigment and distribution within hairs cause the agouti pattern?*

Inhibition of pigment Station 8

The lack of yellow pigment in the agouti hairs of a tabby cat results in a colour known as silver tabby. Such a cat has black stripes that stand out on a white background instead of the tawny background typical of most tabby cats.

This characteristic is controlled by a gene with the alleles

I : inhibition of pigment
i : full pigment

This gene also affects non-agouti cats. In orange cats, it gives rise to a colour called 'cameo' and in non-agouti, black cats gives a colour called 'smoke'. A range of other colours also occur when the **I** allele is present in cats that are genetically brown, lavender or blue.

Examine poster 8.
*Note the effect of the '**I**' allele on the three lighter kittens. Compare these with the three darker kittens.*

Q21. *(3 mark) What is the genotype, with respect to the '**I**', '**T**' and '**A**' genes, of the centre light kitten?*

Epistasis - genes interacting

Epistasis refers to non-allelic gene interaction that impacts on a particular trait of an organism. That is, the alleles at one gene locus affect the outcome of the alleles at another gene locus. The interaction between the **A** and **T** genes as indicated above is a good example of epistasis. A black cat that is **aa** will not reveal its potential tabby striping with regard the **T** gene.

Another example of epistasis in cats is the masking of colours by dominant white.

Dominant white - what lies underneath? Station 9

One gene in cats that masks the expression of other genes has the alleles

W : all-white
w : non-white or not all-white

Cats **WW** or **Ww** are all white and all other genes affecting coat colour and pattern fail to be expressed. This is an example of *dominant epistasis*. It is only from the information gained from breeding records, or experiments, that the genetic make-up of gene loci other that the 'white' locus can be determined.

***Examine poster 9 and the two special problem posters associated with this gene locus**. You are provided with images of various litters prodcued by two white cats mating.*

***Remember:** White is epistatic to all other colours and markings. Whatever the genotype at other gene loci, the colours and markings fail to be expressed in cats homozygous or heterozygous for the **'W'** allele.*

The procedure for generating the litters was the same in both cases.
A pair of white parents was generated at random within a computer for Special Problem One. These were mated for a number of times and litters were generated.
A different pair of white parents was used to generate the litters for Special Problem Two. The sexes of the kittens are not given.

***Q22.** (1 mark) Were the parents in each problem homozygous or heterozygous at the W locus? How do you know?*

***Q23.** (3 marks) Analyse the data on both of the special problems poster. Use the information given to establish the genotype of the parents at the B, D, S & T loci, for each of the special problems.*

Bes Bes – examine coat, predict her genotype Station 10

Examine the phenotype of Bes Bes in Poster 10

Q24. *(5 marks) Predict her genotype at each of the following gene loci:*

S, C, B, D, O, T, A, I, W

Generate your answers in the form of a table with three columns with the headings:

Gene
Phenotype
Possible genotypes

Practicals 2-4: Determining the sex of the domestic chicken

Gallus gallus

Some hazardous chemicals & equipment are used in these practicals. Please make sure to consult the laboratory safety instructions beforehand in Appendix 2.

Introduction

In sexually dimorphic species, females and males can easily be distinguished by differences in their phenotype. However, there are many species that are sexually monomorphic and cannot easily be distinguished. The availability of molecular markers which can determine the sex of an individual has become an extremely valuable tool, enabling us to genetically identify the sexes. In mammals, females carry two identical sex chromosomes (i.e. XX – the homogametic sex) and males carry two different sex chromosomes (XY – the heterogametic sex). However, in birds females are the heterogametic sex, carrying one copy each of the Z and W sex chromosomes, while males are homogametic (ZZ). Many bird species and most chicks are sexually monomorphic (i.e. it is difficult to distinguish males and females from their phenotype). Universal genetic markers now exist which are able to reliably sex almost all species of birds. These molecular markers were designed by targeting highly conserved primer flanking regions within the chromo-helicase-DNA binding gene 1 (CHD1), carried on the sex chromosome of birds. The sex of an individual is determined by detecting a size difference between CHD1 on the W chromosome (CHD1W) (female) and the CHD1 variant the Z chromosome (CDH1Z) (male). The sex of an individual can be determined by extracting genomic DNA from a small sample and then using PCR to amplify the CHD1 genes and visualizing the resulting PCR product using gel electrophoresis. If the individual is a male (ZZ) we will observe one band, if the individual is female (WZ) we will typically observe two bands on the gel.

Your Task

Your task is to extract DNA from one of three tissue types and use that DNA to determine the sex of domestic chicken (*Gallus gallus*). You will be allocated one of three tissue types for an individual – 1) feather, 2) muscle, or 3) preserved blood.
This experiment will be run over three practical sessions:

- Practical 2 DNA extraction
- Practical 3 PCR
- Practical 4 Gel electrophoresis

Aims

1. Pipette user's tutorial (Prac – 2, Part A).
2. Extract DNA from muscle, blood or feather of *Gallus gallus* (Prac – 2, Part B).
3. Visualise your extracted DNA product on an agarose gel and estimate the concentration of the extracted DNA (Prac - 3 [Part A]).
4. Amplify the CHD1 gene using PCR (Prac - 3 [Part B]).
5. Visualise your PCR product on an agarose gel (Prac - 4).
6. Analyse the gel to determine the sex of your sample (Prac - 4).

Assessment

1 x scientific report in poster format worth **22%** of the total mark for this unit. Please consult the poster instructions and accompanying rubric (which indicates how the marks will be allocated) within the assessments folder available on the Unit Cloud site.

Practical 2 (Part A) Pipette user's tutorial: an essential skill for molecular biology

Knowing how to pipette properly is an important skill in molecular biology. Pipetting errors are one of the most common reasons for experiments to fail in molecular biology classes (not reading the lab manual carefully would have to be the 1st). Pipette volumes generally range from 10 mL down to 0.1 µL (microlitres). Most often, the volumes you will need to dispense with pipettes will be less than 1 mL or 1000 µL. It is helpful to start thinking of a µL as an everyday unit.

Q1. If there are 1000 µL in 1 mL, how many µL are there in 1L?

Q2. How many µL are there in 0.225 mL?

Q3. How many µL are there in 0.001 mL?

Q4. If 1 ml of water weights 1 gram, then how much do the following weigh in grams:

- 500 µl
- 1000 µl
- 1 µl
- 1000 nl (nanolitres)

Get to know your pipettor

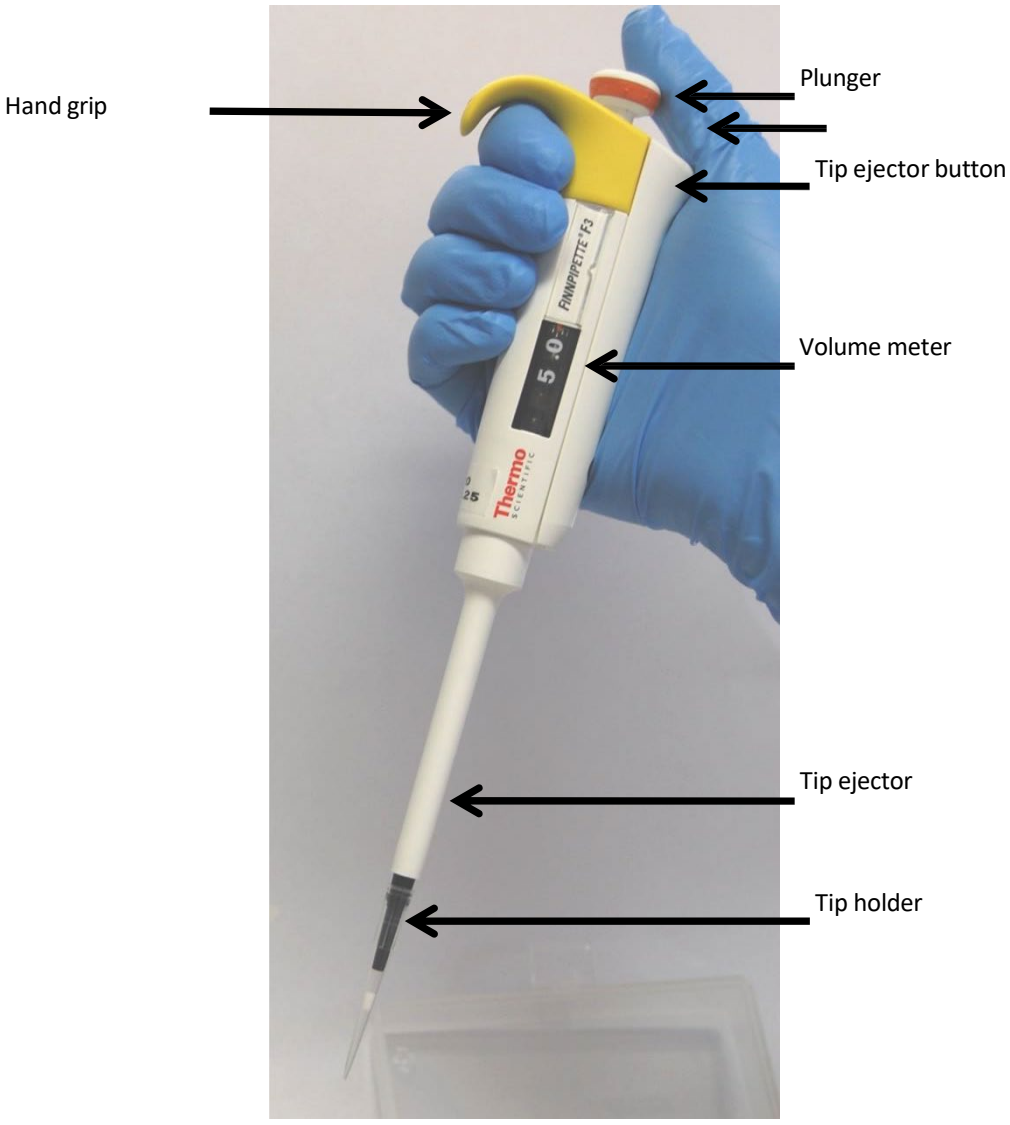


Image source: Original by Stella Loke, Deakin University 2012

Using a pipette: a quick reference guide

1. Select the correct pipette for the volume you need to dispense. The volume range of the pipette is printed on the side.

2. Set the pipette to the correct volume by turning the knob at the top of the plunger.

3. Select the correct tip for the pipette.

4. Push the tip firmly onto the tip holder.

5. Gently depress the plunger to the 1st stop with your thumb.

6. Keeping the plunger depressed, lower the tip into the liquid just below the surface.

   Where possible hold the pipette and the container at eye level so that you can watch the liquid as you draw it up and dispense it.

7. Slowly, and with control, draw up the correct amount of liquid. Keep the pipette upright. Hold the tip in the liquid for a count of two before pulling it out.

   Important! Make sure you do not release the plunger too quickly. Maintain a slow release on the way up. This stops splash-back and air-bubble formation.

8. To dispense the liquid, hold the vessel you wish to dispense into at an angle, place the end of the tip near the bottom of the tube. Slowly depress the plunger, allowing the liquid to run down the side. Make sure you do not release the plunger quickly. Maintain a slow release on the way up.

9. When dispensing solutions, try to pipette the solution to the bottom of the container. If you are dispensing more than one solution into the same container, dispense each solution onto the previous one. Allow surface tension to draw the solution out of the tip.

10. If any solution remains in the tip depress the plunger past the 1st stop to the 2nd stop. This will force extra air out. Only release the plunger once you have taken your tip out of the solution, otherwise you will draw the solution back into the tip.

11. Depress the tip eject button to discard the tip.

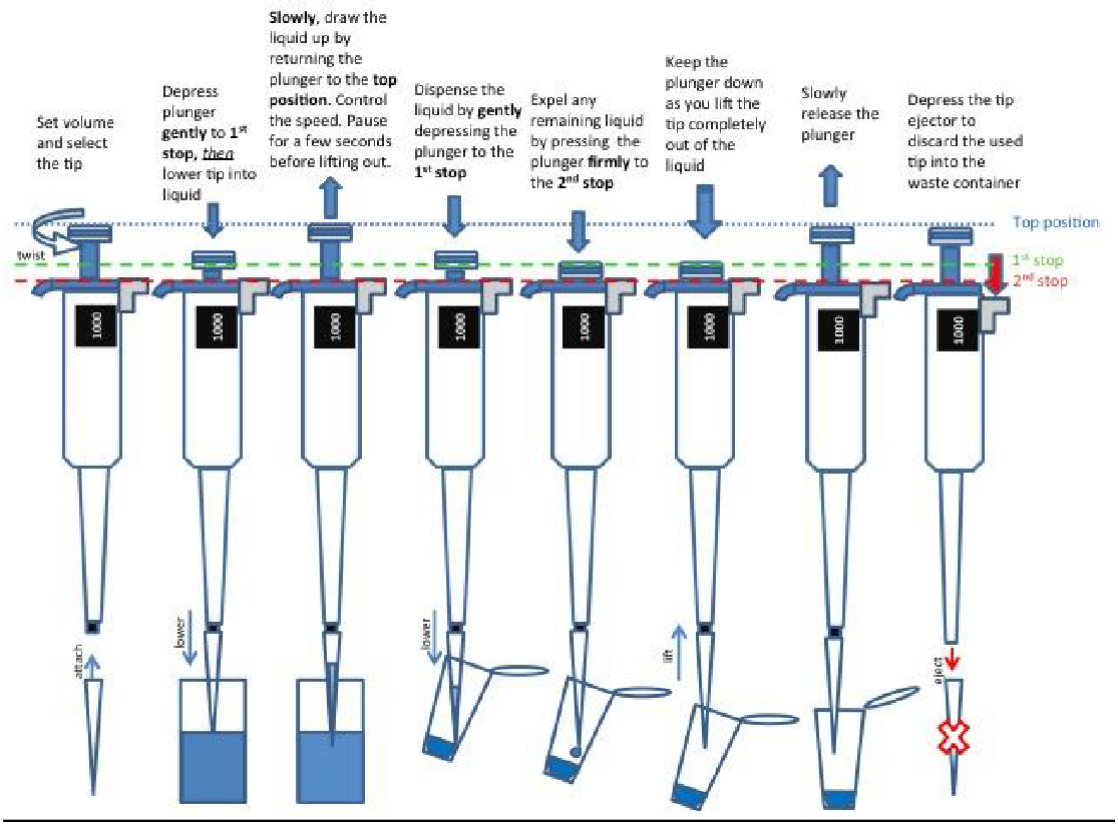


Image source: Original by Stella Loke, Deakin University 2012

Pipette trouble-shooting

Problem: Liquid is leaking from the tip

Reason: If the liquid is dripping out, air is leaking out somewhere. Have you selected the correct tip? Does the tip fit snugly on the tip holder? Is the tip holder damaged?

Solution: Replace the tip with a fresh one. Make sure the tip is pressed on the tip holder firmly. If leaking still occurs ask your demonstrator to assist you. The leak could be internal (within the actual pipette housing) or the tip holder may be damaged. Some liquids with low viscosity such as ethanol will leak out more easily than others. When pipetting a low-viscosity solution try to transfer your liquid the transfer vessel quickly.

Problem: I keep getting air bubbles

Reason: Air is getting in!

Solution: Replace the tip. Make sure you depress the plunger before you lower the tip into the solution. Watch the liquid as it is drawn up into the tip. Make sure your pipetting action (i.e. how you release the plunger with your thumb) is slow and steady enough for the liquid that you are pipetting. Make sure the end of your tip stays below the surface of the liquid while you are drawing it up. Viscous liquids can take longer to pipette. Make sure you pause for a few seconds before taking the pipette tip out of the liquid to allow time for the full amount of liquid to enter the tip. The more viscous the liquid the longer you may have to wait.

Problem: There is still liquid in my tip after I have dispensed the solution

Reason There are a few of reasons this could happen. Here are a few common reasons:

A) Too much liquid may have been drawn into the tip. Did you accidentally push the plunger down to the second stop before you drew up the solution? This will cause you to take up more than the desired volume.
B) There may be air leaking in. Is the tip still fitting snugly? Did you press the tip eject button accidentally after you drew up the solution or as you were dispensing the solution?
C) Did you release the plunger too early?

Solutions

A) Start again with a fresh tip. Unfortunately, you may have to discard the reaction mixture that you were preparing because you cannot be sure if it contains the correct concentration of all ingredients. Ask your demonstrator.
B) If the tip ejector was knocked and the tip has come loose, you can try to push it back into place with your fingers (avoid touching the tip below the bevelled edge) and dispense the remaining solution. Ask you demonstrator if this is OK. It is best to start again with a clean tip if you can.
C) If the plunger was released too early you may have drawn solution back into the tip. If this is the case, try dispensing the liquid again. Remember…..dispense the liquid by pushing the plunger down. Keep the plunger down until you have lifted the tip clear from the solution, then release the plunger. This way you will only draw air back into the tip.

Problem: I was pipetting a small volume. There is nothing in my tube

Reason: Either you did not draw up the solution in the first place or the volume you are pipetting is difficult to see.

Solution: If you are pipetting less than 10µL the droplet can sometimes be very difficult to see. Briefly spin the tube in the microcentrifuge to collect the solution at the bottom. Hold the tube up to the light to see if your droplet is there. If no solution was in fact dispensed, start again with a fresh tip.

Problem: There is liquid on the outside of the tip.

Reason: The solution is adhering to the outside of the tip either because the tip has been put too far down into the solution when taking it up or there are droplets of solution on the walls of the solution you are dispensing from. Viscous solutions are more likely to be problematic.

Solution: Either tap or centrifuge the vessel you are dispensing from to any collect any liquid clinging to the sides of the container to the bottom. When pipetting, keep the tip just below surface of the liquid you are drawing up. Wipe any droplets from the outside of the tip with a clean Kimwipe before dispensing the liquid.

Practical 2 (Part B) Extraction and purification of DNA from different tissue types

Introduction

In this practical you will be using a commercial DNA purification kit (Qiagen DNeasy Blood & Tissue Kit) to purify DNA from chicken blood, muscle and feathers. The amount and quality of DNA extracted will often vary between different tissue types.

The DNA you extract in Practical 2 will be used in Practical 3 to perform a PCR (polymerase chain reaction). In order for your PCR to work you will need to be careful and follow the manufacturer's instructions closely. PCR can be inhibited by contaminants that are carried over from your DNA extraction if the procedure is not performed correctly.

The DNeasy Blood & Tissue procedures are outlined in the flowchart below. The cells of your tissue sample are first lysed (broken apart) using the enzyme proteinase K. This releases the DNA into solution. The lysate is then loaded onto the DNeasy Mini spin column. During centrifugation, DNA is selectively bound to the membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors are removed in two wash steps and the DNA is then eluted into water or buffer, ready for use.

Each pair will be provided with one tissue type (blood, muscle or feather) to extract DNA from. Three protocols are provided in your notes. You will need to follow the appropriate DNA extraction protocol for your tissue type.

Important: Centrifugation steps need to be carried out at the correct speed. Check each step carefully.

Protocol 2a: Purification of genomic DNA from preserved blood

Materials

Equipment

Pipettors (P20, P200 & P1000)
Sterile 1.5 mL microfuge tubes
Heat block or hybrid oven (56 °C)
Vortex mixer
DNeasy spin columns
Collection tubes
Microfuge (variable speed)
Marker pens
Yellow tips(sterile)
Blue tips(sterile)
Tip discard
Liquid waste container
Lid locks
Sterile forceps
Sterile petri-dish
Sterile scalpel blade

Reagents

Chicken blood sample
Proteinase K (20 mg mL^{-1})
RNase A (100 mg mL^{-1})
PBS (Phosphate Buffered Saline)
Buffer AL (Lysis buffer)
95% ethanol (AR grade)
Buffer AW1 (Wash buffer 1)
Buffer AW2 (Wash buffer 2)
Buffer AE (Elution buffer)

My tissue code is:

Procedure for nucleated red blood cells (wear gloves throughout the procedure)

Before starting any procedures, make sure to spin down all your reagents to collect the liquid on the bottom of the test tubes.

1. Pipette 20 µL of **proteinase K** into a sterile1.5 mL microcentrifuge tube. **NOTE:** the microcentrifuge tube is different from the spin column tube and collection tube.

Microcentrifuge tube.

2. Add 166 µL of **PBS** to your tube.

3. Add 4 µL **RNase A** (100 mg mL^{-1}) – this enzyme degrades RNA so that you are only left with DNA at the end of the extraction.

4. Cut one hole-punch-sized sample of the preserved blood card containing the chicken blood sample in quarters. Transfer the card to the tube using sterile forceps.

5. Incubate for 5 min at room temperature before continuing with step 6.

6. Add 200 µL of **Buffer AL**. Mix thoroughly by vortexing for 15 s.

7. Incubate your tube at 56°C for 30min.

   * The cells should now have lysed.

8. Add 200 µL 95% ethanol (AR grade) to the sample, and mix thoroughly by vortexing for 15s.

   * Your DNA has now precipitated and is ready to be bound to the membrane.

9. Remove your DNeasy spin column/collection tube from the sterile bubble packaging. Label the lid with your unique sample code.


DNeasy spin column/collection tube.

10. Pipette the all of the liquid from your tube into the DNeasy Mini spin column, being careful not to touch the white membrane at the base of the column. Perforating this column will cause you to lose your DNA.

11. Centrifuge the spin column unit at 6000 x g (8000 rpm) for 1 min.

    * Your DNA is now bound to the membrane in the spin column and ready for washing.

12. Discard the flow-through liquid and collection tube. Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL **Buffer AW1**.

13. Centrifuge for 1 min at 6000 x g (8000 rpm). Discard the flow-through liquid into the hazardous waste container provided and the collection tube into the tip discard.

14. Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL **Buffer AW2**, and centrifuge for 3 min at maximum speed (13 -14,000 rpm) to dry the DNeasy membrane.

15. Remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through liquid. Empty the collection tube into the hazardous waste container, then place the spin column back into the collection tube and centrifuge for 1 min at maximum speed (13,000- 14,000 rpm).

    * This step should remove any residual ethanol. Your DNA is now ready to be eluted from the membrane.

16. Discard the collection tube and place the DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube labelled with your unique sample code and your practical group number (G1, G2, G3 or G4).

17. Pipette 100 µL **Buffer AE** directly onto the DNeasy centre of the membrane. Do not touch the membrane with the tip in case you damage it.

18. Incubate the column at room temperature for 1 min, then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

    *Your DNA is now in the solution at the bottom of your tube.

19. Discard the column.

20. Place your tube containing your DNA into the esky at the front of the lab. All samples will be stored at -20°C until the next practical.

Protocol 2b: Purification of genomic DNA from muscle tissue

Materials

Equipment	Reagents
Auto pipettors (P20, P200 & P1000)	Chicken muscle
Sterile 1.5 mL microfuge tubes	Proteinase K
Heat block (56 °C)	RNase A (100 mg mL^{-1})
Vortex mixer	Buffer AL (Lysis buffer)
DNeasy spin columns	Buffer ATL (Tissue lysis buffer)
Collection tubes	96-100% ethanol (AR grade)
Microfuge	Buffer AW1 (Wash buffer 1)
Marker pens	Buffer AW2 (Wash buffer 2)
Yellow tips	Buffer AE (Elution buffer)
Blue tips	
Tip discard	
Liquid waste container	**My tissue code is:**
Lid locks	
Sterile petri dish	
Sterile pointed forceps	
Sterile scalpel blade	
Sharps discard	
Sterile petri dish	

Before starting any procedures, make sure to spin down all your reagents to collect the liquid on the bottom of the test tubes.

Procedure for muscle tissue (wear gloves throughout)

1. With a sterile razorblade, use a chopping action to macerate approximately 20 mg of tissue (approximately match head size) in a sterile petri-dish (slicing the petri dish heavily will just remove plastic). Make sure that no large tissue pieces remain before transferring the tissue into a sterile microcentrifuge tube.

   NOTE: the microcentrifuge tube is different from the spin column tube and collection tube.

Microcentrifuge tube.

Scalpel blades need to be discarded in the yellow sharps container provided.

2. Add 180 µL of **Buffer ATL** to the tissue and vortex for 15s.
3. Add 20 µL **proteinase K**. Mix thoroughly by vortexing for 15 s, and incubate at 56°C for 30 mins.

4. After incubation, add 4 µL **RNase A** (100 mg/mL^{-1}), mix by vortexing for 15 s, and incubate for 2 min at room temperature before proceeding to step 5.

   * The cells should now have lysed.

5. Vortex for 15 s. Add 200 µL **Buffer AL** to the sample, and mix thoroughly by vortexing for 15 s. Then add 200 µL **ethanol** (96–100%, analytical grade), and mix again thoroughly by vortexing for 15 s.

   It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetteting to yield a homogeneous solution.

   * Your DNA has now precipitated and is ready to be bound to the membrane.

6. Remove your DNeasy spin column/collection tube from the sterile bubble packaging. Label the lid with your unique sample code.


DNeasy spin column/collection tube.

7. Pipette the mixture into the DNeasy Mini spin column unit.

8. Centrifuge the DNeasy Mini spin column unit at 6000 x g (8000 rpm) for 1 min. Discard the flow-through liquid into the hazardous waste container provided. Place the collection tube into the tip discard container.

9. Put the mini spin column in a new 2 mL collection tube, add 500 µL of **Buffer AW1**, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard the flow-through liquid into the hazardous waste container provided and the collection tube into the collection tube into the tip discard.

10. Put the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL **Buffer AW2**, and centrifuge for 3 min at maximum speed (13-14,000 rpm) to dry the DNeasy membrane.

11. Remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through liquid. Empty the collection tube, then place the spin column back into the collection tube. Centrifuge the column a second time for 1 min at maximum speed to remove any residual ethanol.

    * This step should remove any residual ethanol. Your DNA is now ready to be eluted from the membrane.

12. Discard the collection tube into the container provided and place the DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube labelled with your unique sample code and your practical group number (G1, G2, G3 or G4). Pipette 100

µL **Buffer AE** directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

13. Add a further 100 µL **Buffer AE** to the membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute. In this step you will combine the eluates. You should end up with 200 µL of DNA.

14. Discard the column.

15. Place your tube containing your DNA into the esky at the front of the lab. All samples will be stored at -20°C until the next practical.

Protocol 2c: Purification of genomic DNA from feathers

Materials

Equipment

Auto pipettors (P20, P200 & P1000)
Sterile 1.5 mL microfuge tubes
Heat block (56 °C)
Vortex mixer
DNeasy spin columns
Collection tubes
Microfuge
Marker pens
Yellow tips
Blue tips
Tip discard
Liquid waste container
Lid locks
Sterile petri dish
Pointed forceps
Sterile scalpel blade
Sharps discard

Reagents

Whole Feather: Tip (FT) or Blood Spot (FS)
Proteinase K

Buffer AL (Lysis buffer)
Buffer ATL (Tissue lysis buffer)
96-100% ethanol (AR grade)
Buffer AW1 (Wash buffer 1)
Buffer AW2 (Wash buffer 2)
Buffer AE (Elution buffer)

My tissue code is:

Before starting any procedures, make sure to spin down all your reagents to collect the liquid on the bottom of the test tubes.

Procedure for feathers

1. With a sharp razor blade or sterile scissors cut a small section from the feather shaft (see additional notes). Slice your section of feather into small pieces.

2. Using sterile forceps, add your feather pieces to a sterile 1.5 mL microfuge tube containing 180 µL of **Buffer ATL**. NOTE: the microcentrifuge tube is different from the spin column tube and collection tube.

Microcentrifuge tube.

Scalpel blades need to be discarded in the yellow sharps container provided. DO NOT place scissors/forceps in this bin. Leave scissors/forceps on bench.

3. Add 20 µL **proteinase K**. Mix thoroughly by vortexing for 15 s, and incubate at 56°C for 30 mins.

* The cells should now have lysed.

4. Vortex for 15 s. Add 200 µL **Buffer AL** to the sample, and mix thoroughly by vortexing for 15 s. Then add 200 µL **ethanol** (96–100%), and mix again thoroughly by vortexing for 15 s.

It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

* Your DNA has now precipitated and is ready to be bound to the membrane.

5. Remove your DNeasy spin column/collection tube from the sterile bubble packaging. Label the lid with your unique sample code.


DNeasy spin column/collection tube.

6. Pipette the mixture into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard the flow-through liquid into the container provided and the collection tube into the collection tube discard.

7. Place the mini spin column in a new 2 mL collection tube, add 500 µL of **Buffer AW1**, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard the flow-through liquid into the container provided and the collection tube into the collection tube discard.

8. Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL **Buffer AW2**, and centrifuge for 3 min at maximum speed (13-17,000 rpm) to dry the DNeasy membrane.

9. Remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through. Empty the collection tube, then place the spin column back into the collection tube. Centrifuge the column a second time for 1 min maximum speed (13-17,000 rpm) to remove any residual ethanol.

10. Discard the collection tube in the collection tube discard container and place the DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube labelled with your unique sample code and your practical group number (G1, G2, G3 or G4).

11. Pipette 50 µL **Buffer AE** directly onto the DNeasy membrane. Incubate at room temperature for 3 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

12. Discard the column.

13. Place your tube containing your DNA into the esky at the front of the lab. All samples will be stored at -20°C until the next practical.

Additional notes on DNA extraction from feathers

Not all regions of a feather are suitable for DNA extraction. DNA can be extracted from the tip of plucked or shed feathers, however, the quality and quantity of the DNA is often poor. A richer source of DNA can be found in the blood spot which is located at the superior end of the calamus. If your feather has a visible blood spot (see Figure 1.) cut this section out with a sharp scalpel blade on a petri dish, holding the feather firm with forceps. If your feather doesn't have a visible blood spot or your feather is small (e.g. contour or down feather) cut 2-5 mm of the tip of the feather slicing it into several pieces before digestion.

Take care that the feather sample actually goes into the tube.

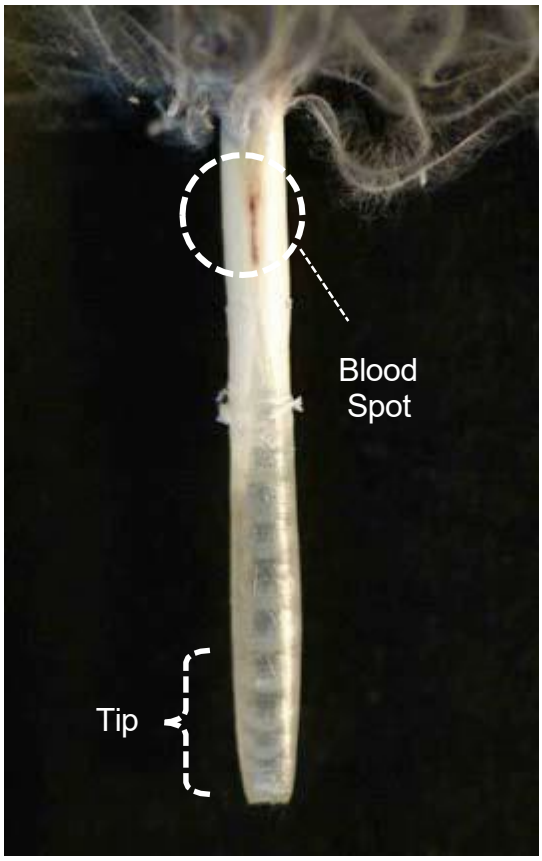


Figure 1. Feather morphology
Original Photo by Dr. Fiona Hogan, Deakin 2006

Practical 2 – Questions

All questions need to be completed before leaving the practical class and this page needs to be signed by the demonstrator.

1. What charge does DNA have?

2. Why does the DNA migrate through the agarose gel?

3. DNA fragments migrate through the agarose gel based on their _____?

a. Weight

b. Fluorescence intensity

c. Size

d. Shape

Demonstrator Name: _____

Signature: _____ **Date:** _____

Practical 3 (Part A) Gel electrophoresis of extracted DNA

Introduction

Gel electrophoresis is a technique used to separate DNA molecules on a gel matrix using an electric current. The gel is prepared by pouring molten gel into a casting tray with a comb inserted into one end. When the gel sets the comb is removed. The fingers of the comb leave behind small wells. The gel is then placed into an electrophoresis tank and the reservoir is filled with an electrophoresis buffer. Before loading samples into the wells the DNA is mixed with a loading dye. The loading dye is a dense solution which makes the DNA sink down into the well and contains coloured indicator dyes. The dyes travel at a predictable rate through the gel, enabling the progress of the electrophoresis to be monitored.

When the electric current is applied to the gel the negatively charged DNA molecules move through the gel matrix toward the positively charged anode (red cables) and away from the negative charge of the cathode (black cables). Smaller DNA molecules migrate through the gel (towards the anode) at a faster rate than larger DNA molecules.

DNA is visualised by the addition of a small amount (0.5µg mL^{-1}) of the chemical Ethidium Bromide/ SYBR Safe to the gel. These stains intercalates with DNA molecules and fluoresces under UV light, therefore DNA can be visualised as fluorescent bands in each of the lanes in a UV transilluminator.

NB: Ethidium Bromide/ SYBR Safe binds to DNA and therefore may be a potential carcinogen. Do not handle the gel.

The size and concentration of a DNA fragment can be estimated by including a DNA size / concentration standard, also called a molecular weight marker, in one of the wells. The size of the band is estimated by comparing the DNA band of your sample to a band of known size in the standard. The concentration of DNA can be predicted by matching the brightness of your DNA sample band to the brightness of a band in the standard.

Gel electrophoresis of genomic DNA

Materials

Equipment	Reagents
Auto pipettors (P20)	1 x TAE buffer
Microfuge	150mL 1% TAE agarose gel
Large gel electrophoresis tank	SYBER Safe (Invitrogen)
Power pack	DNA sample
Yellow tips	Molecular weight marker in loading dye
Tip discard	6 x DNA loading dye
Gel casting tray	
Gel casting gates	
20 well gel comb	

Procedure for gel preparation

Steps 1 & 2 will either be demonstrated or prepared for you in advance by technical staff.

DO NOT ATTEMPT TO POUR YOUR OWN GEL!

1. Prepare a 1% agarose (w/v) in 1 X TAE electrophoresis buffer by boiling until dissolved in microwave, and allow to the molten gel to cool to 50°C in a water bath.

2. Add **SYBR Safe** (10,000x) solution to 150 mL of gel. (**Danger**: **SYBR Safe** is a potential mutagen, toxic and suspected carcinogen. Use gloves. Risk: low)

3. Insert the casting tray into the gel tank and place the black casting gates at each end of the casting tray. Pour gel into casting tray, and the insert comb. The gel takes 30 min to set room temperature.

Q1. How many grams of agarose would you need to prepare 150 mL of a 1% gel?

Before starting any procedures, make sure to spin down all your reagents to collect the liquid on the bottom of the test tubes.

Sample Preparation

1. Label your tube with your unique DNA code. To prepare your genomic DNA for electrophoresis, mix 10µl of genomic DNA with 2µL of 6x loading-dye in a clean microfuge tube. You will be advised how to load onto the gel by your demonstrator.

Electrophoresis

1. Carefully remove the black casting plates and the gel comb. Slowly add 1X TAE buffer until the buffer covers the whole gel by about 5 mm. (**Danger:** SYBR Safe is a mutagen, toxic and suspected carcinogen. Use gloves. Risk: low)

2. Pipette 5 µL (1.0 µg) of the molecular weight marker (1 kb DNA ladder) into the first and last wells.

3. Pipette your DNA sample into an empty well and record the well position of your sample on the gel loading guide provided.

4. Apply the cover to the electrophoresis unit and connect to the power unit. Run at 100 V for 60 min. DNA will run from the negative cathode (black cable) to the positive anode (red cable). (**Caution**. Electrical hazard. Mop-up spills. Risk: Low).

5. When the gel has finished running, turn off the power unit and disconnect the leads. Carefully remove the gel tray from tank and transfer the gel to a plastic container**.**

6. Visualise the gel using the Gel Doc system. ***Gel photos will be placed on the SLE254 CloudDeakin site.***

Agarose gel analysis protocols

Materials

Gel doc apparatus
Molecular marker reference page
Molecular Weight Markers
6 x Loading dye
1x TAE buffer
Molecular biology grade agarose
SYBR Safe

DNA visualisation on an agarose gel

1. Place the gel on the transilluminator box of the Gel Doc apparatus (wear gloves). Close the door.

2. Under white light, adjust the focus so that the gel and the wells can clearly be seen.

3. Switch on the UV light.

4. Adjust the exposure so that the samples and the marker can clearly be seen.

5. Freeze the image and save a copy for analysis.

**A copy of the class results will be placed on DSO by the end of the week.*

DNA concentration determination

1. DNA concentration will be determined using a spectrophotometer. The exact type and model may vary from campus to campus, but typically a Nano Drop machine or a plate reader will be used to quantify the amount of DNA in your sample. You will be shown by your demonstrator how to use the machine in your laboratory class.

A Nano Drop machine.

2. How does it work?

DNA absorbs ultraviolet light in a specific pattern. In a spectrophotometer, a sample is exposed to ultraviolet light at 260 - 280 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. Note that other contaminants such as proteins can affect your readings.

Practical 3 (Part B) Polymerase Chain Reaction (PCR) of DNA

Introduction

Polymerase chain reaction is an enzyme-based method used to amplify fragments of DNA ranging from 100's to 1000's of base pairs in length. The reaction is performed by thermostable DNA polymerases. One of the most commonly used polymerases and one that we will use in this practical is called *Taq* polymerase. This enzyme was originally isolated from *Thermus aquaticus,* a thermophilic bacterium native to hot springs and thermal vents.

In order to copy specific regions of DNA, specially designed oligonucleotides (short pieces of DNA) called PCR primers are added to the reaction. These primers bind to complementary sequences on the template DNA at either end of the region of interest. The *Taq* polymerase then synthesises copies of the DNA by adding dideoxynucleotide triphosphates (dNTP's) to the end of each primer sequence. That way, both strands of DNA are duplicated.

For a visual demonstration of PCR see the following YouTube video: *http://www.youtube.com/watch?v=_YgXcJ4n-kQ* or follow the link on DSO.

Reading

A Simple and Universal Method for Molecular Sexing of Non-Ratite Birds. Fridolfsson and Ellegren, Journal of Avian Biology, Vol. 30, No. 1, 1999, pp. 116-121.

PCR set up

Materials

Equipment	Reagents
Auto pipettors (P2, P20 & P200)	Sterile MilliQ H_2O
Microfuge	DNA sample
Sterile 1.5mL microfuge tubes	Control ♀ DNA
Sterile PCR tubes	Control ♂ DNA
Esky of ice	20µM 2550F primer
Tip discard	20µM 2718R primer
Tube racks	2mM dNTP solution
Filter tips	10x PCR buffer solution
Marker pens	Taq polymerase (1unit μL^{-1})
Thermo-cycler (Biorad)	

Procedure

ALL REAGENTS NEED TO BE KEPT COLD.

Keep all tubes on ice as much as possible.

Before starting any procedures, make sure to spin down all your reagents to collect the liquid on the bottom of the test tubes.

1. To prepare one tube of master mix per group of 4-8 students, first calculate how much master mix you will need for everyone on your bench plus four extra tubes for the male and female positive controls, the negative control and to account for pipetting error. Fill in the calculated volumes in the PCR setup table.

2. Select one pair in your group of 8 to prepare your master mix. Place a tick in the box as you add each reagent in the correct order.

3. Each person will need to pipette 35 µL of the PCR master mix into a 0.2 mL PCR tube then add 15 µL of your diluted DNA.

4. Nominate other members of your group to be responsible for making up the control tubes. There must be a separate tube for each control; male female & negative. The control tubes must have 35 µL of the PCR master mix (in a 0.2 mL PCR tube) plus 15 µL of male (♂) control, female (♀) control and a negative control, one of each for your group. The control DNAs have been provided to you.

   Note down the label on each tube and what you added to that tube.

5. When you are all ready, place the prepared PCR tubes into the thermocycler and run a program optimised to amplify the CHD1W and CHD1Z gene variants with the 2550F -2718R primer set.

PCR THERMOCYCLING CONDITIONS

94°C 3 min	x 1 cycle
94°C 30 sec, 48°C 30 sec, 72°C 1 min	x 40 cycles
72°C 10 min	x 1 cycle

PCR set up table

Final concentration required in 50µL reaction	**Stock Solution Concentration**	**Volume of stock (solution added per 50µL reaction)**	**Number reactions per bench (____reactions) + Controls (3) + Pipetting Error (1) = _____reactions**
1x PCR buffer	10x concentrated PCR buffer with 15mM $MgCl_2$	5 µL	Tick when added □
200 µM dNTP's	2mM each dNTP	5 µL	□
1 U Taq DNA polymerase in 1x PCR buffer	1 U/µL	1.0 µL	□
2 pmol 2550F forward primer	20 µM	1.0 µL	□
2 pmol 2718R reverse primer	20 µM	1.0 µL	□
0.5mM $MgCl_2$	25mM $MgCl_2$	5 µL	□
Sterile milliQ water		17 µL (for 35 µL final)	
Sample DNA or milliQ H_2O		15 µL	**NA**

Practical 3 – Questions

All questions need to be completed before leaving the practical class and this page needs to be signed by the demonstrator.

1. Describe how good quality, high molecular weight (HMW) DNA looks like on an agarose gel compared with low quality highly sheared (fragmented) DNA.

2. Give one pro and one con for the different types of sample collection; destructive sampling, invasive sampling and non-invasive sampling.

3. During the PCR reaction, which enzyme is responsible for replicating the DNA?

Demonstrator Name: _____

Signature: _____ **Date:** _____

Practical 4 Gel electrophoresis and visualisation of PCR products

Introduction

In this practical you will view and analyse the results from the CHD1W /CHD1Z gene PCR you carried out in the previous practical session. Based on published data (Fridolfsson and Ellegren, 1999), we expect that the PCR fragments amplified with the 2550F-2718R primer will be smaller than 1000bp. You could use a TAE, SB or TBE buffer to resolve fragments under 1000bp.

The procedure will be essentially the same as the last electrophoresis practical except that you will be running PCR products rather than genomic DNA.

NOTE: The results for the entire prac class will be used for your scientific report and it is important that you record ALL the results for your prac class.

Materials

Equipment	Reagents
Auto pipettors (P20)	1 x TAE buffer
Microfuge	150 mL 1% TAE agarose gel
Large gel electrophoresis tank	SYBR Safe
Power pack	PCR product
Yellow tips	Molecular weight marker
Tip discard	6 x Loading dye
Gel casting tray	
Gel casting gates	
20 well gel comb	
Heat proof gloves	
Gel loading guide	

Procedure for Gel Preparation

(Steps 1 & 2 have been done for you)

1. Prepare 1 % agarose gel in 1 X TAE electrophoresis buffer by boiling until dissolved in microwave, and allow to the molten gel to cool to 50°C.
2. Add 15 µL of SYBR Safe stain (10,000x) to 150 mL of TAE gel. **Danger:** SYBR Safe is a mutagen, toxic and suspected carcinogen. Use gloves.
3. Insert the casting tray into the gel tank and place the black gates at each end of the casting tray. Pour gel into casting tray, and the insert comb. The gel takes 30 min to set room temperature.

Sample Preparation

Before starting any procedures, make sure to spin down all your reagents to collect the liquid on the bottom of the test tubes.

1. To prepare your DNA from your PCR for electrophoresis, mix 15 µL of PCR product with 4 µl of 6 x loading dye. Label your tube.

Electrophoresis

1. Carefully remove the black casting plates and the gel comb. Slowly add 1 X TAE electrophoresis buffer until the buffer just covers the whole gel by about 5 mm**.** (**Danger**: ***SYBR Safe*** is a mutagen, toxic and suspected carcinogen. Use gloves.)

2. Your demonstrator will pipette 5 µL (0.5µg) of the 100 bp molecular weight marker into the first well.

3. Pipette your DNA sample into an empty well and record the well position of your sample on the gel loading guide provided.

4. Apply the cover to the electrophoresis unit and connect to the power unit. Run at 100 V for 45 min.

   DNA will run from the negative cathode (black cable) to the positive anode (red cable). (**Caution:** electrical hazard. Mop-up spills.)

5. When the gel has finished running, turn off the power unit and disconnect the leads. Carefully remove the gel tray from tank and transfer the gel to a plastic container.

6. Visualise the gel using the Gel Doc system. **** Gel photos will be placed on the SLE254 CloudDeakin site.***

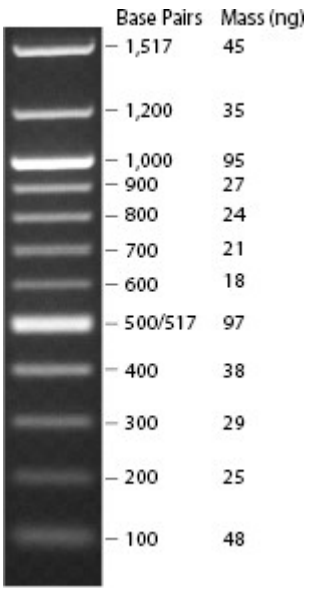
PCR product size determination

1. Compare the position of your PCR band or bands to the size standard bands in the marker.

2. Look up the size of a similar sized DNA band corresponding to one of the molecular weight markers indicated in Figure 3 or 4.

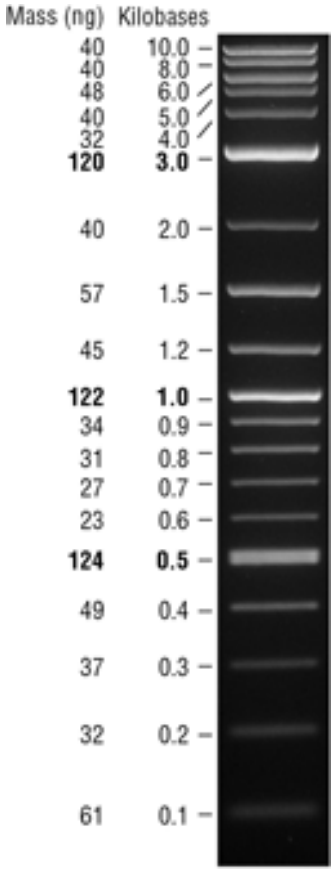
3. Determine the size (in base pairs, bp) of your PCR product/s.

4. Remember to record the results for all members of the prac class!



0.5µg 100bp marker on 1.3% TAE

Figure 3. 100 base-pair (bp) molecular weight ladder/marker (from New England Biolabs). This ladder is useful for determining the approximate size and concentration of PCR fragments.



1kb Plus DNA Ladder on 1.0% TBE

Figure 4. 1 kilobase (kb) Plus DNA Ladder (previously 2-Log DNA Ladder) molecular weight ladder/marker (from New England Biolabs).

Practical 4 – Questions

All questions need to be completed before leaving the practical class and this page needs to be signed by the demonstrator.

1. What is meant by heterogametic sex and homogametic sex?

2. In birds, which sex is the heterogametic sex?

3. List two reasons when a scientist may want to use molecular markers to sex a (non-human) organism?

Demonstrator Name: _____

Signature: _____ **Date:** _____

Appendix 1 Practical rules

1. Students must read the practical manual relevant to the practical class and do any recommended pre-reading before attending the class.

2. Attendance at all practical classes is compulsory. A 5 min lockout applies, if you are 5 minutes late, you will be turned away. If you miss a practical class through illness, you may be awarded your trimester's average mark for the missed session on the production of a medical certificate for the period. Medical certificates must be produced within a week of the missed practical class. Only one such average mark can be awarded per trimester.

3. Practical reports are to be handed in on time. Demonstrators will deduct marks for late work. Marks are deducted at the rate of 5% per day. NO marks will be given for work handed in more than 5 days after the due date.

4. A practical report cannot be submitted unless you have attended the practical sessions.

5. If you know in advance that you will be unable to attend a particular practical session you may be able to arrange to attend an alternative session providing there is one available. You must first ask the permission of the unit co-ordinator, Dr Marina Telonis-Scott (**Email: m.telonisscott@deakin.edu.au**) or campus coordinator, Dr Andrew Oxley (**Email: andrew.oxley@deakin.edu.au**). Unless prior arrangements are made for a practical class change, you will not be able to attend a different laboratory session.

6. You are required to commence the practical class on time. Each lab commences with a pre-lab tutorial. Material from the pre-lab talk is examinable so it is in your best interests to be on time.

Appendix 2 Laboratory safety

1) No eating, chewing gum, drinking or smoking in the laboratory.

2) Lab coats must be worn at all times whilst you are in the laboratory.

3) Shoes should fully enclose the foot. No thongs or open toe shoes.

4) Tie or pin back long hair and fringes while working in the laboratory.

5) Keep your bags out of the way in the shelves provided or under the side benches, not in the aisles or under your bench.

6) Upon entering the lab you are required to wash your hands with disinfectant and sanitise your work bench with the 70% alcohol provided. Try to do this as soon as you arrive so that you can start and finish on time.

7) At the end of the laboratory, wipe down your work bench with alcohol (provided on the benches) and wash your hands with disinfectant.

8) Dispose of all waste appropriately. This is extremely important, not only for your own safety, but for the safety of technical staff, general cleaning staff and other students. If you are unsure of how or where to dispose of a particular waste please check with your demonstrator.

9) Before you hand potentially dangerous chemicals, please read the material safety data sheet (MSDS). We will provide the necessary documents at the front of the laboratory during class. An MSDS for hazardous chemicals will be posted on DSO for you to refer before you work with the chemical.

Waste Disposal

General	Plastic bins with liners. General waste only. NO SHARPS OR GLASS. These bins are handled by cleaners.
Glassware	For glassware disposal only. No other waste types. No paper!! These bins are handled by staff and cleaners.
Sharps	Yellow plastic bins. For syringes, needles, blades and any blood-contaminated material. All blood and blood products are to be treated as potentially infectious. These bins are handled by staff and contractors.
Microbiological Solid Waste	"Biohazard" disposal bags will be made available for Microbiological waste and other similar solid wastes. The bags are autoclaved to render the waste "safe". These bags are handled by staff and contractors.
Microbiological Liquid Waste	Containers (glass or plastic) will be made available for liquid microbiological and cell culture waste. This waste type will be rendered safe by the addition of sodium hypochlorite before disposal, with dilution, via the drains. These containers are handled by staff.
Animal Waste	A special separate receptacle will be made available for this class of waste. These bins are handled by staff and contractors.

Solvents	For both *environmental* and *safety* reasons, chemical solvents must NOT be disposed of down the sink. What goes down the sink also ends up in the bay. Solvent wastes are segregated and separate waste bottles are provided for each type of solvent waste e.g., chlorinated and non-chlorinated organic waste.
Other wastes	Other types of waste will have containers specific for their disposal.

Hazardous signage

As part of the process of becoming a responsible scientist, each student should familiarise him/herself with the United Nations Class Labelling System that applies to hazardous substances. Briefly:

Class 1	Explosives
Class 2	Compressed Gases
Class 3	Flammable Liquids
Class 4	Spontaneously combustible materials
Class 5	Oxidising agents
Class 6	Poisons
Class 7	Radioactive goods
Class 8	Corrosive substances e.g., acids and alkalis
Class 9	Miscellaneous e.g., dry ice, asbestos

All chemicals have comprehensive labels which carry a wealth of important information. Read chemical labels carefully so that you are aware of the chemical and reagent properties, in particular the hazards associated with each chemical.

Safety equipment

Familiarise yourself with the location of safety equipment. In particular:

- eye wash stations
- safety shower
- first aid equipment
- fire exits
- fire blankets
- fire extinguisher

Accidents

All accidents must be reported and an accident report form filled out if there is any injury. Tell your demonstrator who will initiate getting further aid as required; ie. first aid or medical treatment.

Many staff members are qualified first or second level first aiders, and all security staff are qualified second level first aiders. To call security phone 222, state name, location and problem. Security staff can organise an ambulance or any other emergency service if required.

Accidents with Chemicals

If chemicals are spilled on skin or in the eye find the nearest source of **water**. Wash with copious amounts of water. If the chemical is in the eye, find the nearest tap and wash. DO NOT waste valuable time attempting to find the eye wash station. After the initial panic has settled, and with people to help you, the eye wash facility can be used for more thorough washing. Similarly, for chemicals spilled on skin, find the nearest tap and wash. DO NOT use any solvent other than water. Skin is impermeable to water but other solvents might permeate the skin which could facilitate and deepen the damage to the tissues. **Note:** Alkali is generally more dangerous than acid, hence, alkali in the eyes is more blinding than acid. TAKE CARE!

Injuries

Cuts and abrasions need to be promptly attended to. Broken skin is a primary route of entry for infections from contaminated surfaces. An accident report form **MUST** be filled out in the event of any injury. All necessary measures need to be taken to minimise the risk of infection. Any wound needs to be covered with a clean, dry protective dressing.