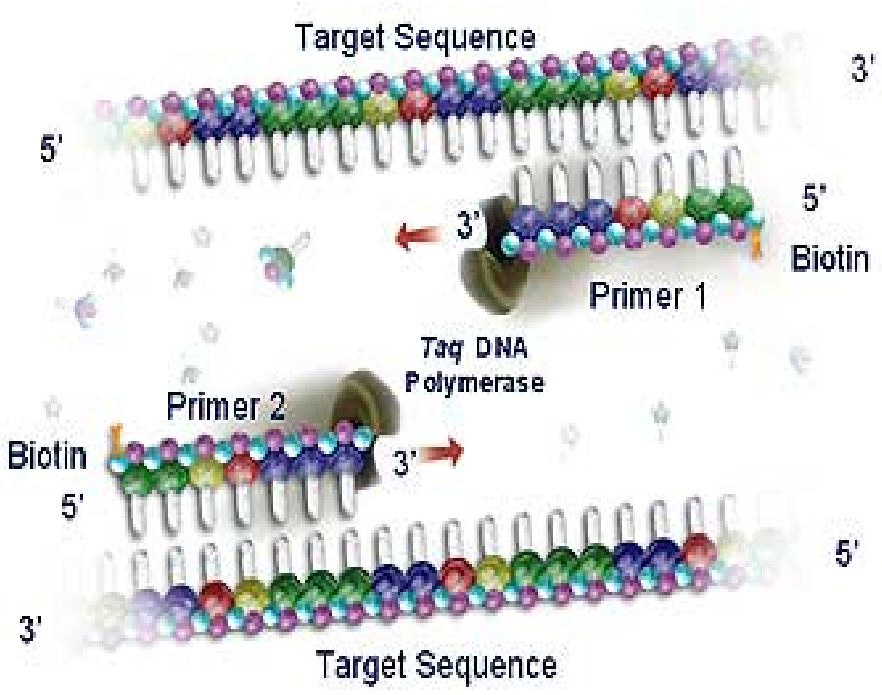
PRACTICAL: PCR and Congenics

Techniques we will cover:

- The Polymerase Chain Reaction (PCR)
- Electrophoresis of PCR products
- Microsatellite marker analysis to identify congenic strains of mice



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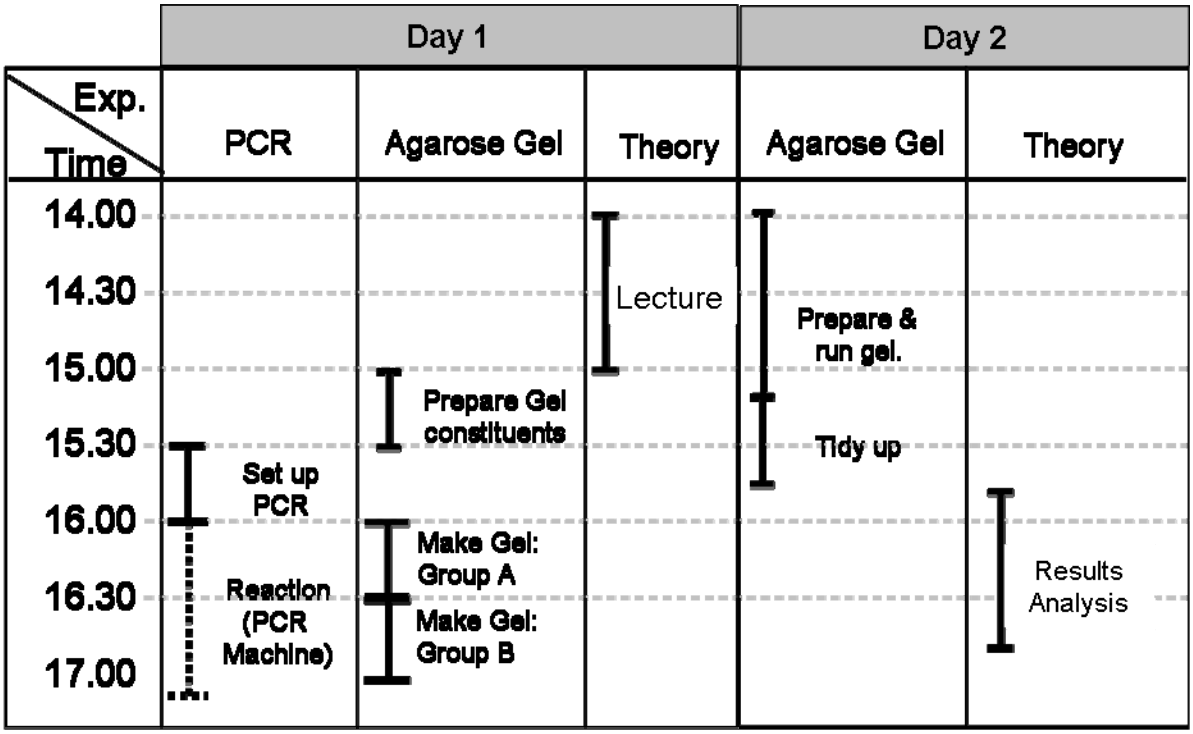
Time Plan


Day 1
Day 2
Exp.
Time
PCR
Agarose Gel
Theory
Agarose Gel
Theory
14.00
14.30
15.00
15.30
16.00
16.30
17.00
Lecture
Prepare & run gel.
Tidy up
Prepare Gel constituents
Set up PCR
Reaction (PCR Machine)
Make Gel: Group A
Make Gel: Group B
Results Analysis

Introduction

Many mouse strains have been manipulated, either genetically or by breeding, to provide tools for immunological research. Examples of mice resulting from genetic manipulation are transgenics and knock-out mice, whilst breeding strategies give rise to recombinant inbred strains, consomic and congenic mice. Some definitions and examples are listed below.

Transgenic: Mice with extra copies or altered copies of a gene in their genome.
Example: DO11.10 mice: Mice whose T cell receptors are restricted for (recognise) $Ova_{323-329}$ peptide in the context of $H2\text{-}A^{d}$.

Knock-out: Mice that have had one or both copies of a specific gene deleted or inactivated.
Example: Knock-out: IL-5 knock-out mouse does not produce any IL-5 (Kopf *et al*: Immunity **4**: 15 (1996)).

Knock-in: Mice that have had a specific gene inserted into their genome.

Inbred: A strain of mouse so highly inbred as to be genetically identical and homozygous at every locus (isogenic), except for sexual differences. Brother - sister matings have been maintained for 20 or more generations.
Example: C57BL/6, BALB/c

Recombinant Inbred: Derived by systematic inbreeding following a cross between mice of two genetically distinct inbred progenitor strains.
Example: BXSB

Consomic: A single chromosome from one inbred strain is transferred onto the genetic background of a second strain. Chromosome substitution.

Congenic: An interval (1-99%) of a single chromosome from one inbred strain is transferred onto the genetic background of a second strain.

Both consomic and congenic mice show homozygosity for the interval that has been bred in.

Microsatellites: Sometimes called Simple Sequence Repeats (SSR). Short stretches of repeated DNA found in most genomes which show exceptional variability in humans and mice. They are bordered by unique DNA sequence and can be specifically examined.

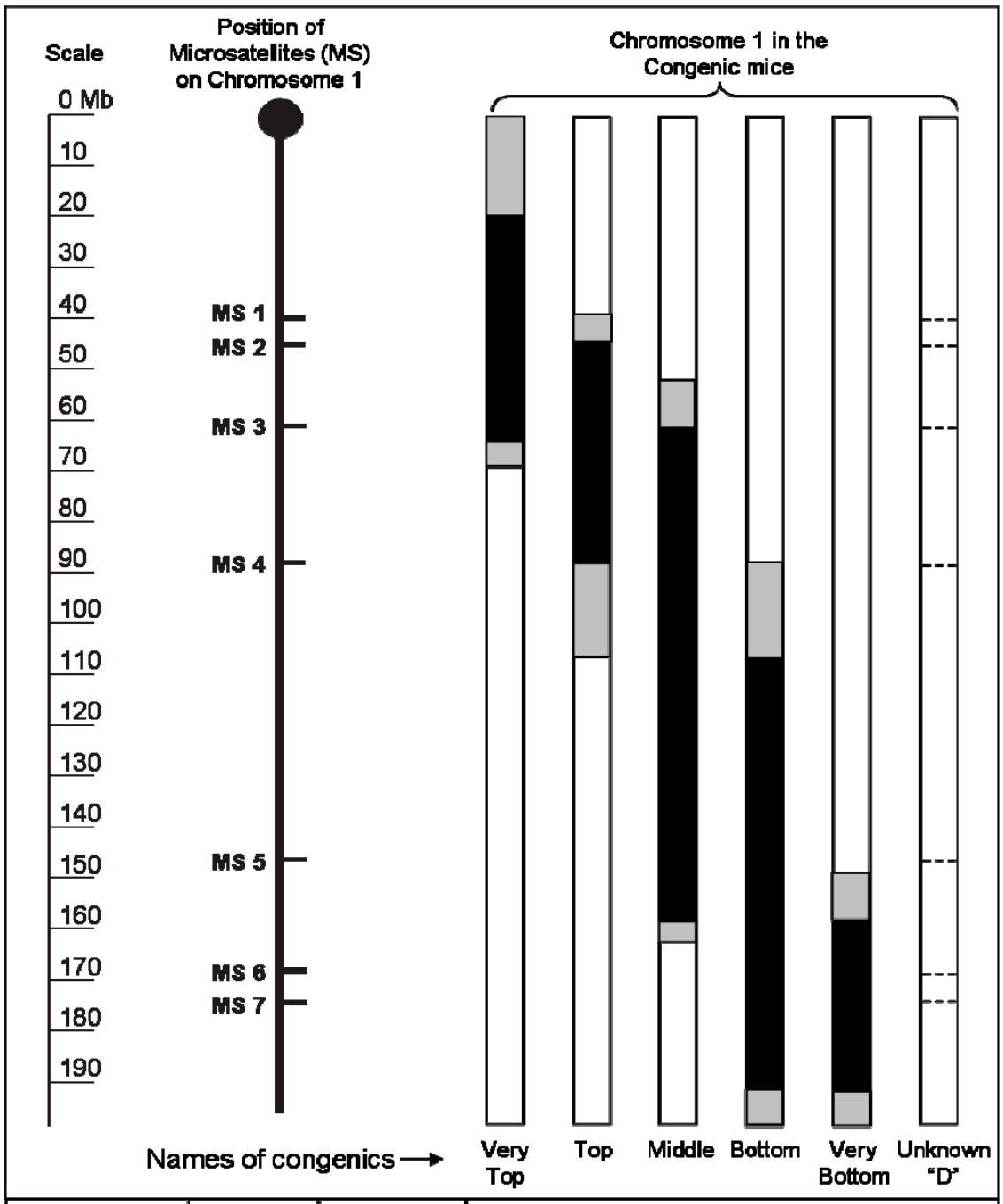
BXSB – a mouse model of SLE

Systemic Lupus Erythematosis (SLE) is a chronic autoimmune disease, the hallmark of which is the loss of tolerance, at both the T- and B- cell level, to nuclear antigens. The recombinant inbred mouse strain BXSB (C57BL/6 x SB/Le) is a model system to study the disease. It spontaneously develops a lupus-like syndrome very similar to that in humans, characterised by elevated levels of autoantibody, severe nephritis and death by 6-10 months.

Following a genome-wide linkage analysis by Dr Nicola Rogers' lab., the genetics of BXSB have been established. The lab. has 5 congenic strains that contain different intervals (ranging from 50-100 Mb) of chromosome 1. These are illustrated in Figure 1. Each congenic displays a different disease phenotype and thus these mice provide an ideal immunological tool to address the genetic causes of SLE e.g. "What causes the loss of tolerance at the T cell level in the BXSB mouse?"

For this practical, you will be provided with DNA samples that were taken from the mice during the generation and characterization of the congenics. Consequently, the samples may be from any one of the following:

- The parental control strain BXSB or B10
- F1 breeding crosses
- 1 of the 5 congenics



Microsatellite	Size in B10	Size in BXSB
MS 1	140 bp	185 bp
MS 2	150 bp	135 bp
MS 3	137 bp	125 bp
MS 4	146 bp	128 bp
MS 5	112 bp	122 bp
MS 6	160 bp	184 bp
MS 7	150 bp	124 bp

Key:
BXSB-derived
B10-derived
Derivation unconfirmed

Figure 1. Schematic representation of chromosome 1 in BXSB Congenic Mice Strains

The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a rapid method of amplifying a section of DNA using two oligonucleotide primers with sequences derived from the 5' and 3' ends of the target sequence. In a single tube the target DNA is mixed with the PCR mastermix - forward and reverse olignucleotide primers, the 4 dNTPs (dATP, dTTP, dGTP and dCTP), suitable buffer for the reaction and a thermostable DNA polymerase (e.g. *Taq* polymerase isolated from *Thermus aquaticus*). The reaction mix is placed in a thermal cycler which cycles the sample between three temperatures; denaturation, annealing and elongation (see "A Typical PCR Programme" below and Figure 2).

A Typical PCR Programme

Step	Temperature	Time	Purpose
1	95 °C	3-5 min	Initial Denaturation: Denatures dsDNA
2	95 °C	3-5 min	Denaturation: To denature dsDNA
3	Ta† = Tm‡ minus 5 to 7 °C	30 sec	Annealing: Allows primers to anneal to template DNA
4	72 °C	Fragment size and Polymerase dependent*	Elongation: The Polymerase binds to the primer:template complex and elongates the primer by replicating the template DNA
Steps 2 - 4 are repeated (cycles) 25 - 35 times (depending on the abundance of the target sequence in the template DNA and the amount of product required) to amplify the products produced in step 4. The cycles are called the Amplification phase.			
5	72 °C	3-5 min	Final Elongation: To ensure all products are full length copies

Ta†: The annealing temperature of the oligonucleotide primers (this information is provided when you buy new primers). Ta is calculated by determining the number of A's, T's, C's and G's in the primer and using a formula which multiplies the number of each nucleotide by a value which corresponds to the strength of a bond between A and T or C and G.

Tm‡: The melting temperature of an oligonucleotide primer. This is the temperature at which the oligonucleotide will dissociate from complementary DNA. Tm is approximately 5-7 °C less than Ta. Tm is a parameter that has to be determined through trial and error.

* Polymerases are capable of varying rates of replication e.g. the enzyme used in this experiment elongates the primer at a rate of 1 kbp every 15-30 seconds. Therefore elongation times depend on the length of the PCR product and the specific polymerase being used.

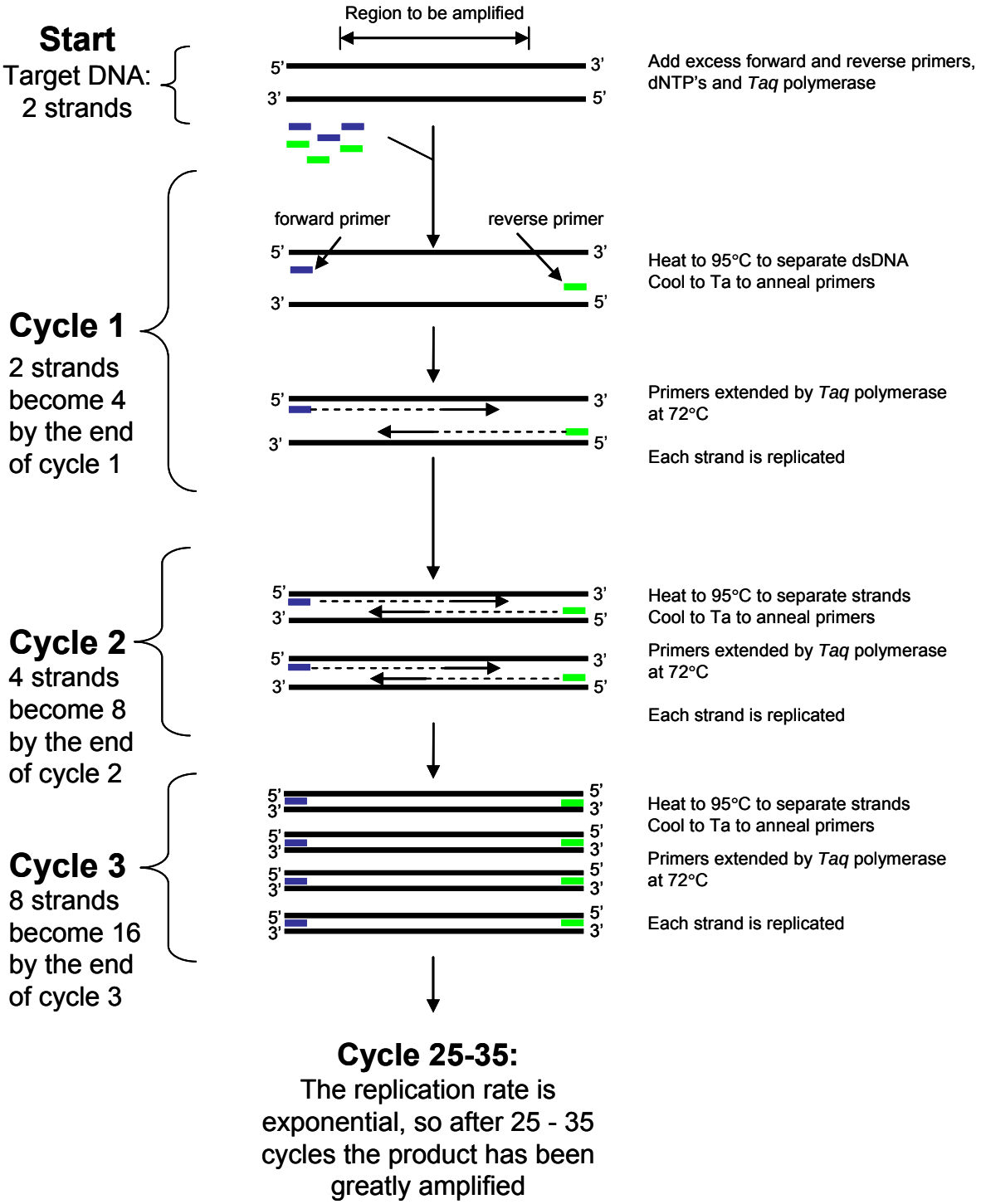


Figure 2. The Polymerase Chain Reaction (schematic)

Please refer to the core lecture on PCR to refresh your memory on PCR and its applications.

Experimental Aims

In this practical you will be analysing 4 samples of DNA (A; DNA from B10 mice, B; DNA from BXSB mice, C and D; DNA whose origin is unknown) extracted from 4 different mouse strains. As shown in Figure 1, there are 7 different microsatellite markers used to identify the different congenic strains. These markers are amplified in a PCR, using an oligonucleotide 'primer pair' specific to the marker. Each person will use one of the 7 primer pairs in a PCR to analyse the microsatellites present in their DNA samples. The 7 primer pairs will be distributed throughout the class and at the end of the end of the practical everyone's data will be pooled so that you can identify from which mouse strains samples C and D were extracted from.

Learning Objectives

By the end of this practical you will be able to:

- Explain the concept of PCR
- Interpret PCR data resulting from microsatellite analyses
- Use your data to define the different genetically modified strains of mice generated by directed breeding.

Context

PCR is an extremely sensitive method, extensively used to amplify immunologically relevant nucleic acids and to aid in the dissection of immunological responses. Reverse transcription(RT)-PCR and Real Time PCR (also known as RT-PCR) are respectively, semi-quantitative and quantitative techniques for measuring gene expression. PCR can therefore provide information that is not accessible using techniques such as ELISA, IHC and western blotting which measure output at the translational level - protein expression and production - rather than gene expression.

Experimental Considerations

Cost

The reagents that you will be using are expensive. **Please** take care. If you are not sure about anything **ASK**. We do not have extra reagents for most of this work.

Nucleases

DNA and RNA are very susceptible to degradation by nucleases. Nucleases are present on your skin, hair and saliva and they are also present in most reagents, including water. Consequently the reagents you will use for this practical have been treated, by autoclaving, to destroy any nucleases present. It is therefore important that you avoid contaminating reagents (wear gloves and take care to replace the caps on reagent containers) and that you use the reagents provided for the practical.

Lability
Taq DNA Polymerases are both expensive and unstable. To prolong the life of the enzyme you will be using, keep the enzyme and your reagents on ice and minimise the time they are out of ice e.g. have your pipette ready before using each reagent and be sure to put them back on ice after each one is used.

Contamination
You will be performing a control reaction in which you add all the reaction constituents except template DNA. This is essential to check for the presence of contaminating DNA in your reagents. To avoid contaminating your reagents make sure you **change your tip each time you take reagent from a tube**.

Safety
Read and take note of the safety instructions and COSHH. Only work when a supervisor is present. Ethidium Bromide is a powerful mutagen; wear gloves when handling anything that may be contaminated with ethidium bromide (ethidium bromide stock tube, gel apparatus and gel). Remember you have a duty of care to others so think before handling apparatus after handling ethidium bromide contaminated items. Be careful using the gel electrophoresis tanks – dry gloves before plugging in the power cables and power pack. UV light is a powerful mutagen therefore when using the UV illuminator wear appropriate personal protective equipment (PPE) (UV goggles (not safety goggles!), lab coat, gloves, and protective face masks) and do not expose any skin more than you have to.

Tidy up
For safety reasons **you** must be responsible for tidying up your work bench and correctly disposing of all waste

Materials & Reagents

4 genomic DNA samples (A – D)
Autoclaved H_20
Reaction buffer (contains *Taq* Buffer, $MgCl_2$ and dNTPs (dATP, dCTP, dGTP, dTTP))
Taq Polymerase enzyme
3.3 µM primer stocks (forward and reverse primers)
Nuclease free microfuge tubes
PCR tubes (strips)
Thermal Cycler (PCR machine)
Distilled water
10X TBE (Tris borate EDTA buffer)
Metaphor Agarose
Ethidium bromide (EtBr)
Conical Flask
200 ml measuring cylinder
Microwave
Balance and plastic weighing boats
Gel tank and accessories
DNA Molecular Weight Ladder (Hyperladder IV)
Loading buffer/dye
UV illuminator and camera

Recipes

Complete the following tables
(The chemicals can be found on the front bench):

1 L of 10X Tris Borate EDTA (TBE) in H_20

Chemical	MW	Molarity Required	Amount to add (g)
Tris			108
Boric Acid			55
EDTA			7.5

50 ml of 4 % Agarose

Reagent	Vol/Mass to Add
10 X TBE	
Metaphor agarose	
H_20	To make volume 50 ml

Protocol 1: PCR

Each member of the group has 4 DNA samples which will be analysed by PCR. You have each been given a primer pair (a forward/sense primer and a reverse/anti-sense primer) that produces a product in a PCR with DNA from B10, BXSB, B10 X BXSB F1 or any of the congenics shown on page 6.

There are 7 primer pairs in total, each primer pair anneals at a specific position on chromosome 1. The product of the PCR will allow you to identify the strain the DNA originated from (See Figure 1 for the expected sizes of PCR products in BXSB or B10 mice).

The seven primer pairs have been distributed amongst the group therefore each person will have 1 of the 7 primer pairs. Your primer pair will not necessarily be the same as your neighbour's – it is therefore important to avoid mixing up any of your reagents – if you run out of anything, please ask for more from the demonstrator.

Make a note of the primer pair you have been given:

_____F

_____R

By comparing the results from each person in the group, we will be able to work out which strains the unkown DNA samples have come from.

1. You will each set up 5 different PCRs, each containing a different DNA sample (see below) or no DNA (this acts as a negative control - water will be used instead of DNA).
2. You will each have 5 tubes containing 2 μl of DNA or 2 μl of water:

   A: B10 DNA

   B: BXSB DNA

   C: Unknown DNA

   D: Unknown DNA

   E: Water (Negative control)

3. You have 5 PCR samples to analyse so you need to prepare enough PCR reagents for 5 tubes. The best way to do this, to save time and reagents, is to prepare, in one tube, enough reagents for the 5 reactions (A "Mastermix"), and then aliquot it into your 5 PCR tubes. However, because pipettes are never 100 % accurate it is essential to make slightly more than you need to ensure you have enough for each tube, therefore you will prepare enough PCR reagent for 6 reactions.

4. You will be provided with the following reactions:
   H_20
   RB (Reaction Buffer)
   A forward primer and a reverse primer
   Taq Polymerase (Taq)

Fill in TABLE 1 below before adding the reagents to your tubes. The final volume per tube will be 18 μl of Mastermix plus 2μl of DNA or your negative control (water) – so 20 μl.:

TABLE 1:

Reagent	**1 PCR**	**Mastermix (6 PCRs)**
DNA	2 μl	DNA is different in each PCR tube therefore it is not included in the Mastermix
Autoclaved H_20	10 μl	μl
Reaction buffer*	3 μl	μl
Forward Primer	2 μl	μl
Reverse Primer	2 μl	μl
Taq Polymerase	1 μl	μl
Total	**20 μl**	**108 μl** (20 μl x 6 minus the volume of DNA)

* Reaction buffer contains *Taq* Buffer (1X), $MgCl_2$ (1.5 mM: Why do you think this is needed?) and dNTPs (200 μM) (Concentrations in brackets are the final concentrations in a 20 μl reaction).

5. Add 18 μl of your Mastermix to each of your 5 PCR strip tubes

6. Hand your tubes in to the demonstrator.

7. The tubes will be placed in a thermal cycler (PCR machine).

Questions:

1. If the stock concentration of your primers is 3.3 μM, what is the final concentration of each primer in the PCR?

2. Why do we do a negative control?

PCR programme used:

1. Initial Denaturation
Time: 3 min
Temperature: 94 ^{o}C

2. Denaturing step
Time: 1 min
Temperature: 94 ^{o}C

3. Annealing step
Time: 30 sec
Temperature: 55 ^{o}C

4. Replication/Elongation step
Time: 15 sec
Temperature: 72 ^{o}C

30 cycles of Steps 2-4

5. Final Elongation
Time: 5 min
Temperature: 72 ^{o}C

8. The PCR machine will run overnight and when the programme has finished the samples will be kept at 4^{0}C.

9. You will prepare and pour a metaphor agarose gel whilst the PCR runs. The gel will be left to set and then placed in the cold room to cool down over lunch. You will then analyse your samples by agarose gel electrophoresis in the afternoon.

Protocol 2: Agarose Gel Electrophoresis

DNA fragments are commonly separated, based upon their size, by electrophoresis through an agarose gel. The agarose forms a matrix which impedes the migration of DNA through the electric field: smaller fragments migrate through the gel quicker than bigger DNA fragments because they can move through the matrix more easily. Consequently, the % agarose of the gel influences the separation of DNA fragments – larger fragments (> 1 k bp) are resolved at lower concentrations of agarose (<1 %) than small fragments. To resolve very small fragments (< 200 bp), like the PCR products of today's experiment, a high percentage agarose gel is required: You will make a **4 % gel** from Metaphor agarose, an agarose that, when set, has very small gaps in the matrix.

STOP The demonstrator will show you how to set up the gel electrophoresis tank and make an agarose gel. Set up the electrophoresis tank and place it in the fume hood before making your agarose (label your tank by writing your name on some tape on the tank). Then make your agarose as described below and as demonstrated to you. You will work in pairs or threes. If you work in a three, make sure you pick a comb with 20 teeth, if you are working in a pair, make sure you pick a comb with 16 teeth (the demonstrator will explain this).

1. Prepare 200 ml of 1X TBE using cold distilled H_20 and cold 10X TBE stock. 60 ml will be used to make the agarose gel and 50 -100 ml will be used as electrophoresis running buffer (Store this in the bottle provided and give it to the demonstrator who will put it in the cold room to cool). The rest is spare should the gel making not go quite according to plan…

2. Aliquot 60 ml of 1X TBE into a conical flask

3. Weigh out the appropriate amount of Metaphor agarose (See table on page 11) using the balance and weighing boats, and add the agarose to the 1X TBE. Swirl the solution gently to disperse the agarose (avoid getting agarose around the mouth of the flask and too high up the flask sides – it wont dissolve later). Allow the agarose to soak for 15 mins.

4. Put a tissue in the mouth of the flask containing the gel.

Safety information:

- Agarose can become superheated in a microwave; some areas heat more rapidly than others but remain liquid. However, when the flask is swirled the agarose will boil up and bubble out of the flask. It is therefore important to swirl the agarose regularly to maintain an even heat throughout the solution but also to wear protective gloves to avoid getting burnt should it boil over.
- Metaphor is very difficult to get into solution, if you heat it too vigorously, it will burn and be useless. Please be patient and only heat the gel for 15 – 30 sec at a time!

5. Microwave on high power for 15 secs. Take out of the microwave and check that it has dissolved and melted (solution will be completely clear). If not, swirl the solution gently and return to the microwave for a further 15 secs.

6. When the agarose has melted, carefully (using protective gloves) take the agarose to the fume hood.

7. Add 25 µl of 1 mg/ml ethidium bromide (**TAKE CARE — MUTAGEN**) to the gel tank and pour the warm agarose over it. Use your gel comb to mix in the ethidium bromide, then place the comb in the comb slots and place the lid on the gel tank. When the gel has set, it will be put in the cold room to cool down. On Day 2 the gel tanks will be returned to your benches and you will perform the agarose gel electrophoresis.

8. WEARING GLOVES… remove the lid from the tank and add the remaining running buffer you made. The buffer should just cover the gel. Finally gently remove the comb and metal ends – keep these safe and on your white paper.

9. You can now load your samples into the wells. To help load your samples into the wells, add loading buffer to each sample ($1/10^{th}$ of the volume of each sample) before attempting to load the sample into the well: 20 µl sample + 2 µl of (X10) loading buffer.

   Loading buffer contains glycerol which helps the DNA sink into the well, and orange G (which makes it orange) which allows you to follow the migration of your samples through the gel. Orange G migrates at the same position as DNA approximately 100 bp in size therefore when the orange dye is close to the end of your gel you know to stop the electrophoresis.

10. Loading the gel is a bit tricky, take your time – there's no rush.

11. Load 7.5 – 10 μl of each sample
(Make a note of the order you load them onto the gel).
Remember to load 5 μl of your molecular weight ladder as well

12. When all the samples are loaded, replace the lid of the gel tank and plug the power cables into the power pack (Black to black, red to red). DNA is positively/ negatively (delete as appropriate!) charged so will migrate towards the ____ve electrode (fill in the blank!). Turn on the power pack and apply 50 V to the gel. Return 10 -15 min later to check everything is operating correctly.

13. Once the dye front is ~1/3 of the way across gel, the gel will be photographed by the demonstrator using a UV transilluminator. The size of your PCR products will be determined by comparison with the molecular weight ladder you loaded – shown in Figure 3.

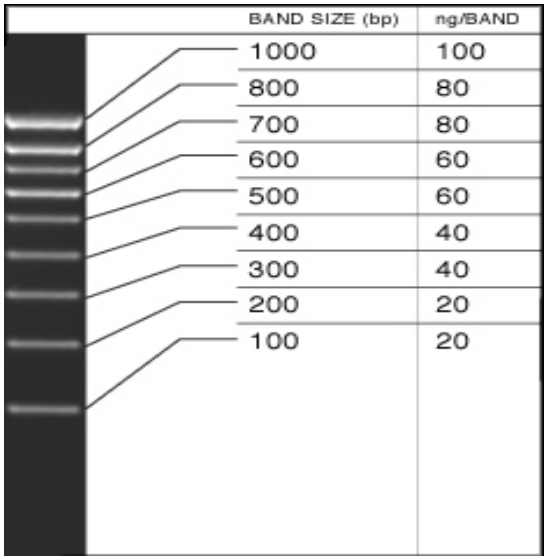


Figure 3. DNA Hyperladder IV

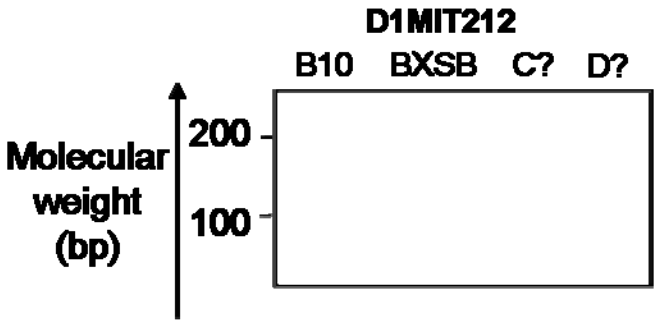
Exercise 1

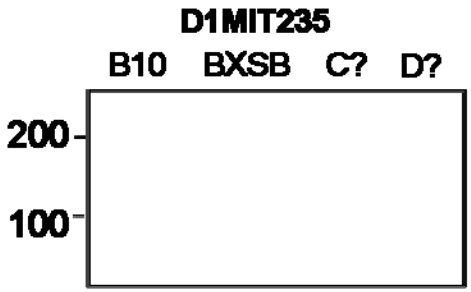
You were each provided with 4 samples of DNA which you subjected to microsatellite analysis by PCR and gel electrophoresis.

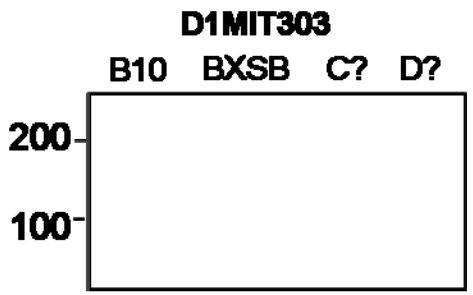
DNA samples A and B were B10 and BXSB inbred strains respectively.

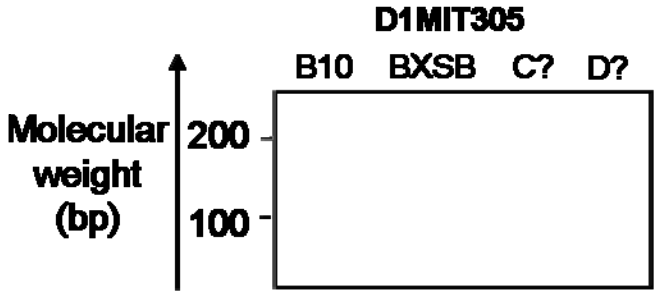
You need to identify DNA samples C and D from the DNA bands present on your gels. We will then combine the data from the whole group – in total, this will provide us with 7 microsatellite markers for the unknown mouse.

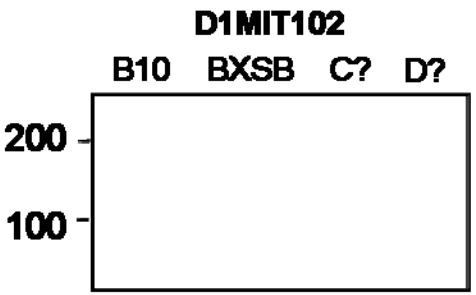
On the next page you can draw the results for each microsatellite marker and then work out the identities of DNA samples C and D.

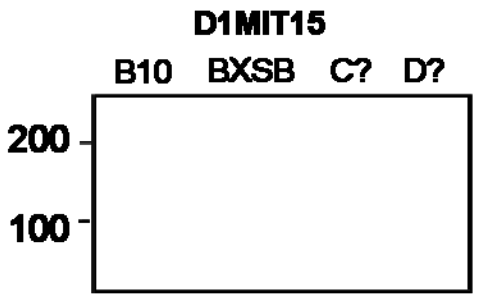


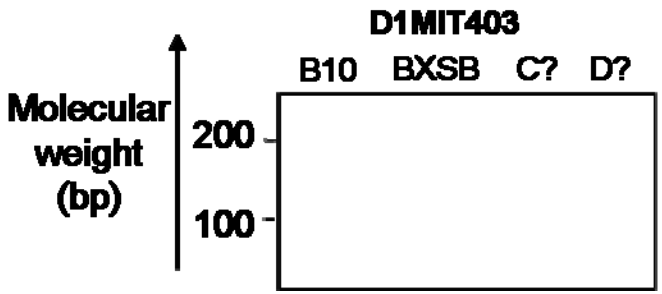












From which strains of mice were the unknown DNA samples taken from?

Sample C: _____

Sample D: _____

Abbreviations

dNTP:
dsDNA:
EtBr:
PCR:
PPE:
RT-PCR:
RT-PCR:
SSR:
SLE:
Taq:
Ta:
TBE:
Tm:

Glossary (In addition to page 4)

Annealing:

Autoclave:

Chronic:

Denaturation:

DNA Polymerase:

Elongation:

Labile:

Linkage analysis:

Microsatellite marker:

Oligonucleotide:

References

Molecular Biology is a bit like cooking; once you have learnt to do a few basic things (like making white sauce) you can do everything from a recipe book. That having been said there are a lot of mistakes that a beginner can (and will) make so it is essential to understand the concepts of molecular biology in order to set-up and troubleshoot experiments.

Methods

The classic book that is present in nearly every molecular biology lab. is "Maniatis":

- Molecular Cloning, a laboratory manual. Second Edition (Three volumes). *J. Sambrook, E.F. Fritsch, T Maniatis (1989)*. Cold Spring Harbor Laboratory Press.

This book is available in the library if you need or want to photocopy anything.

General background

- Genes VIII. *Lewin (2004)*. Prentice Hall.
  Very complete and extensive treatise on genes and genetics
- Understanding DNA and Gene Cloning. 4th Edition. *Karl Drlica (2003).* John Wiley and Sons Ltd.
  This very short book is written assuming next to no knowledge. Can be read in a long evening, and covers a lot of background. Try not to be put off by the fairly basic language!

COSHH forms

Agarose Gel

Substances:
(i) Ethidium Bromide
(ii) Gel tank
(iii) UV light
(iv) Agarose