THE UNIVERSITY of EDINBURGH
School of Biological Sciences

Bio2B: Genetics and Evolution

Practical guide 2023/24

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Practical sessions

The practicals have been designed around scenarios that might be encountered in the scientific world. You will be asked to think carefully about experimental design and data interpretation (via questions included in this handbook). This approach will help you to gain a deeper understanding on how experiments address scientific questions; the experimental design involved; the data analysis required; and the conclusions you can draw based on all the previous steps. There is a lot more to gain from this approach, compared to simply following several different protocols. Practical skills are important to have for any researcher but the intellectual component in experimental science is just as necessary. It might feel slightly different from what you have experienced before (and quite possibly more challenging), but we are here to help you.

You will be assigned to a group for your practicals and will see this appear on your MyTimetable. You should attend one practical session each week.

Please bring a physical copy of this Practical Guide and a notepad to every Lab Practical. A notepad is required as there will be limited space in the Guide for you to make notes.

Make sure you read the relevant section in this guide BEFORE attending your practical session. This will ensure that you can make the most of your lab time. You should not be reading the protocol for the first time during the lab.

	Field trip	
Week 2		Zoo visit
	Microbial practicals	
Week 3	1	Inducible gene expression in *Escherichia coli*
Week 4	2	Gene expression in *Schizosaccharomyces pombe* (Part 1) Genetic interactions in *Saccharomyces cerevisiae* (Part 1)
Week 5	3	Gene expression in *S. pombe* (Part 2) Genetic interactions in *S. cerevisiae* (Part 2)
	Plant practicals	
Week 7	1	DNA extraction from *Arabidopsis thaliana*
Week 8	2	PCR for insertion lines (and Portfolio activity)
Week 9	3	Gel electrophoresis and segregation ratios

PRACTICAL 1

Inducible gene expression in *Escherichia coli*

Learning objectives

1. describe what **reporter genes** are and how they can be used to monitor gene expression in living organisms
2. design different types of reporters suitable for different purposes/contexts
3. perform a **beta-galactosidase** assay to monitor inducible gene expression in *E. coli*
4. provide (and justify) a scientific recommendation based on collected data

There will be an initial short presentation delivered by the floor leader. Listen to it carefully and take some notes on the organism we will be using in the practical, and the concept of reporter genes. You will need this information to carry out the rest of the practical.

Reporter construct design

1	Native promoter of gene of interest
2	Inducible promoter
3	Cell-type specific promoter
4	Foreign promoter of gene coding for enzyme/florescent protein
5	Coding sequence of an enzyme
6	Coding sequence of a fluorescent protein
7	Coding sequence of protein of interest

You have been provided with a series of functional DNA units (shown above). Together with your lab partners, use these components to create the following reporter constructs (promoter+ protein coding sequences):

- A reporter to check IF a gene of interest is expressed in a specific cell-type. You will monitor the reporter using a microscope:
- Two reporters to check WHERE a native protein localises within a cell. You will monitor the reporters using a microscope. Please note that to build these 2 reporters you should use the same DNA components.

Prepare your choices and check your answers with a demonstrator.

Scenario

We have been contacted by the industry representatives who want to use *E. coli* to produce a new top-secret drug. They want the bacteria to trigger the production of this compound only under specific conditions, not all the time. This is because making the compound has a negative impact on the growth of the bacteria. Production of the drug should only be triggered when the bacteria have proliferated enough to reach a critical density (to maximise the compound production). The industry representatives are seeking our advice to build a suitable genetic construct for the drug production

Experimental planning

Based on the scenario presented, consider the following questions:

- What type of promoter could control the gene expression in the way described by the industry representatives? You can revisit the card options provided in the previous exercise.
- What type of reporters would you design to test different promoters for this type of response? Would they be transcriptional or translational reporters? Ask a demonstrator if you are unsure about the difference (and revisit the first exercise). Imagine that you monitor the reporter using a biochemical assay.

Check your answers with a demonstrator.

There will be a short presentation delivered by the floor leader. Listen to it carefully and take some notes on the β-galactosidase enzyme. You will need this information for the next activity.

Strains

You will be working in groups of 4 students and you will be provided with 3 *E. coli* strains (“a”, “b” and “c”). In these strains, the coding sequence for β-galactosidase (the *lacZ* coding sequence from *E. coli*) has been placed under the control of one of three different promoters (“A”, “B” or “C”) (Figure 1). You will test if the transcription from these promoters can be induced by heat or osmotic changes. Each group will test the three strains under one of the two conditions (heat shock OR osmotic stress).

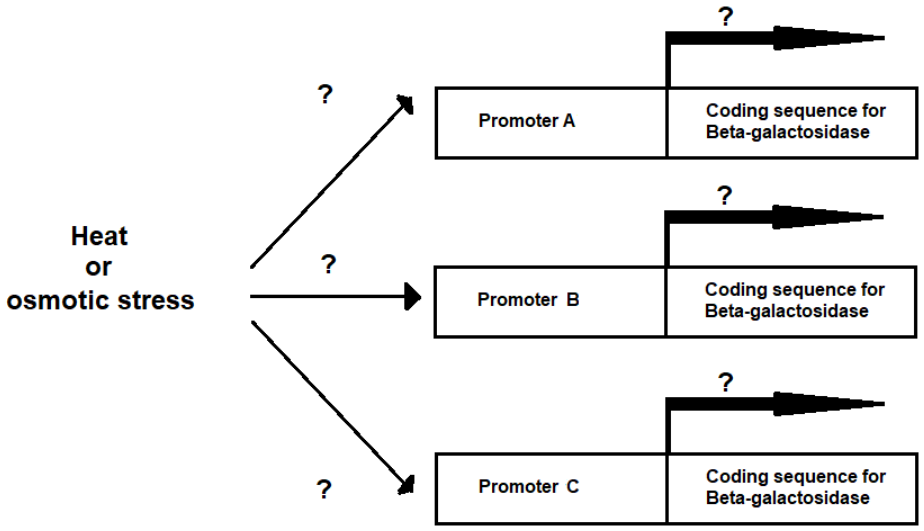


Figure 1. Reporter genes being used in our practical

- What condition have you been assigned? Look for a card on your bench

<u>Setting up</u>

You are provided with the following:

- A full set of pipettes and tips
- A microcentrifuge tube rack
- 5M sodium chloride solution (for osmotic shock experiment only)
- A container with 1.5 ml microcentrifuge tubes
  Please DO NOT use the round bottomed 2 ml tubes which are for later use.
- 3 x 1.5 ml microcentrifuge tubes with white label and punctured lid.
- An ice bucket
- *E. coli* cultures a, b and c grown to logarithmic phase, provided in 30 ml vials.
  These are held in the 30ºC incubator keeping them warm

Thermomixers are positioned on the side benches in the lab and are set to a temperature of 30ºC for the osmotic shock experiment and 43ºC for the heat shock experiment.
You will measure the β- galactosidase activity of the cultures as they are incubated in thermomixers over a 1-hour time-period. You will take samples for β-galactosidase measurement at the time points t=0 minutes, t=20 minutes, t=40 minutes and t=60 minutes for each of the cultures (a, b and c).

The collected samples will be placed on ice to halt cell growth.

After completion of the 1-hour incubation, you will then carry out a β-galactosidase assay on each of the samples collected (12 in total).

The 3 x 1.5 ml microcentrifuge tubes with the white label are for your cultures which you will

label a, b and c. Also label these tubes with your own group number so you can identify them in the thermomixer.

The other 1.5 ml microcentrifuge tubes are for your time point samples t=0, t=20, t=40 and t=60. **Label these before you start your experiment**. One set for each of the cultures a, b and c. Label these A0, A20, A40, A60 etc.

When you have labelled your tubes, collect a vial of each of the *E. coli* cultures a, b and c from the incubator.

Osmotic or heat shock

If your experiment is **osmotic shock,** pipette 60 µl of 5M Sodium Chloride solution into the 3x white labelled microcentrifuge tubes.

If your experiment is **heat shock,** then the sodium chloride is not required.

Pipette 1 ml of each of the cultures a, b and c into the corresponding white labelled microcentrifuge tubes. Mix the contents by pipetting up and down a few times. This is important to mix in the sodium chloride if this is used.

Immediately take the first time point t=0 sample by transferring 100 µl from each white labelled tube a, b and c into its corresponding t=0 tube, placing these t=0 tubes on ice.

For the **osmotic shock** experiment, take your 3x white labelled culture tubes to a **30ºC thermomixer** for incubation, then start a timer.

For the **heat shock** experiment, take your 3x white labelled culture tubes to a **43ºC thermomixer** for incubation and start a timer.

Incubate your tubes with shaking at 1,000 rpm for 20 minutes.

After the incubation period, retrieve your tubes and collect the t=20 samples, placing these samples on ice.

Immediately return your culture tubes to the thermomixer and again incubate these for a further 20 minutes. Repeat the process until you have collected all 4 samples for each of the 3 strains (t=0 min, t=20, t=40 and t=60 min). You will have 12 tubes in total by the end of the experiment

Forward planning

During your 20-minute incubations and sampling, read the following section on the β-galactosidase assay. Familiarise yourself with the procedure and begin labelling the tubes and cuvettes you require for the assay.

β-galactosidase assay

You are provided with the following:

- A microcentrifuge tube rack
- A cuvette rack
- A floating microcentrifuge tube rack in a 37ºC water bath
- A vortex mixer
- Semi-micro cuvettes
- 13 x 2 ml microcentrifuge tubes

Make sure you use the 2 ml microcentrifuge tubes and NOT the 1.5 ml tubes

- A 30 ml Plastic vial containing Z-buffer
- A 7 ml plastic bijou containing ONPG solution
- A 30 ml plastic vial containing Sodium Carbonate stop buffer
- Distilled water in a glass bottle
- Waste liquid container containing Virkon

Spare items and consumables will be positioned on the side benches Spectrophotometers are available on each bench.

You will be setting up a β-galactosidase assay for each of the samples you took during the incubation experiment.

Take 13 x 2 ml microcentrifuge tubes and label one tube for each of your culture time point samples. Also label one 2 ml tube for a blank sample.

To every 2 ml tube, add 750 µl of Z buffer. To the tube labelled Blank, also add 50 µl of distilled water.

Vortex all of your culture samples to make sure they are fully homogenised. Add 50 µl of each culture sample to its corresponding labelled 2 ml tube.

Close all tubes, then mix the contents by inverting the tubes a few times. Place the tubes in a floating rack in the 37ºC water bath, incubating them for 10 minutes to lyse the cells.

After incubation, remove the tubes from the water bath. Add 200 µl of ONPG solution to each tube to start the reaction.

Close all the tubes and again mix the contents. Incubate for a further 15 minutes in the floating rack in the water bath.

During this incubation period, label up a set of cuvettes, one for each tube.

After incubation, remove the tubes from the water bath.

Add 500 µl of sodium carbonate buffer to each tube to stop the reaction. Close all the tubes and again mix the contents

Transfer 1000 µl from each tube into the corresponding labelled cuvette

Read the absorbance of each cuvette using the spectrophotometer set at 420 nM. Use the Blank sample to blank the spectrophotometer first and then run all the culture samples to obtain the readings for the experiment.

Fill in your readings on the table below.

Culture	Shock type	ABS t=0 min	ABS t=20 min	ABS t=40 min	ABS t=60min
a					
b					
c					

The OD_{420} values provide an ***indication*** of the β-galactosidase activity in each sample.

- What trends can you observe in your data? Are these consistent with an induction of gene expression?

Please share the measurements with a demonstrator: they will input the data in the class sheet.

Data analysis

Look at the class data shown on the screen and complete the following table

Reporter	Starting levels of expression (low/high)	Heat inducible? (yes/no)	Osmotically inducible? (yes/no)
β-galactosidase driven from promoter A			
β-galactosidase driven from promoter B			
β-galactosidase driven from promoter C			

Address the following questions:

- Which promoter AND induction strategy would you recommend to the industry representatives? Consider the initial scenario carefully.
- Should we have measured something else during our practical? Those of you who attended the Life 1 Practical might remember this. Consider that the population doubling time for *E. coli* is around 30 min; would this influence our values?

Check your answers with a demonstrator.

PRACTICAL 2

Gene expression in *Schizosaccharomyces pombe* (Part 1)

Learning objectives (for Part 1 and Part 2)

1. discuss the role of **chromatin** in the regulation of gene expression in eukaryotes
2. forecast different **gene expression** scenarios and their impact on the organism of interest
3. **plate** *S. pombe* on suitable growth media and score its appearance
4. analyse a phenotype and identify the most likely cause for it

Comparing organisms

1	Circular chromosomes
2	Linear chromosomes
3	Semi-conservative DNA replication
4	Single origin of replication
5	Multiple origins of replication
6	Nucleus
7	Coupled transcription-translation
8	Gene exons
9	Gene introns
10	Splicing
11	Operons
12	Nucleosomes
13	Post-translational modifications
14	Non-chromosomal DNA

You have been provided with a series of cellular and genetic features (shown above). Together with your lab partner, assign them to prokaryotes, eukaryotes or both. Create a table in your notepad (like the one shown below). Don't worry if you are unsure about some of the features, discuss them with your demonstrator. You can also think back to some of the lectures in this and in previous courses.

Prokaryotes	Eukaryotes

Check your answers with a demonstrator.

Scenario

S. pombe is used in traditional brewing across Africa to produce Bantu beer (Pombe means beer in Swahili). A local brewing company has reached out to you because they are experiencing problems: the quality of their fermentation is not consistent. Some batches of beer are very good while others have a low alcohol content. They suspect that this issue has something to do with their yeast strain. You have been tasked to investigate the expression of the alcohol dehydrogenase gene (important for fermentation).

There will be a short presentation delivered by the floor leader. Listen to it carefully and take some notes on the two organisms we will be using in the practical and on the *ADE6* reporter system in *S. pombe* (Figure 2). You will need this information for the next activity.

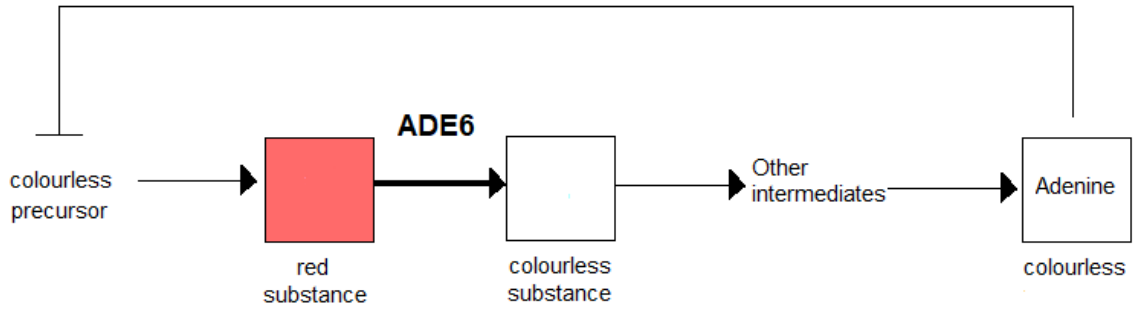


Figure 2. Pathway for adenine production in *S. pombe*

Construct design

1	Promoter of the gene involved in alcohol fermentation
2	*ADE6* promoter
3	Coding sequence of the gene involved in alcohol fermentation
4	Coding sequence of *ADE6*
5	Coding sequence of fluorescent protein

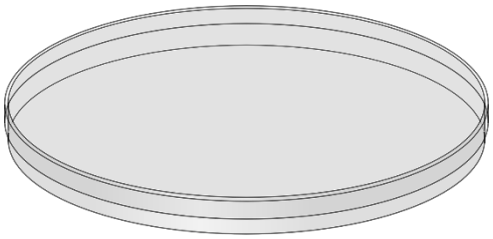
You are provided with a series of DNA components (shown above). Together with your lab partner, use the components to create a reporter construct to visually monitor fermentation potential in yeast

Check your design with a demonstrator.

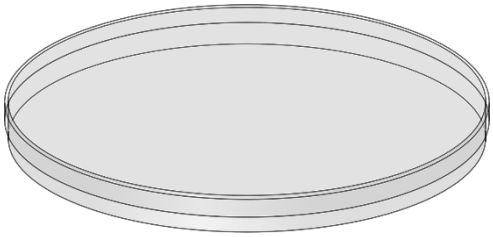
Experimental planning

Consider the following questions:

- Can we introduce the reporter directly into the strain provided by the brewing company? Would we be able to monitor if the alcohol dehydrogenase promoter is active? *ADE6* is a gene normally present in yeast. What colour of colonies would we get (irrespective of the presence and transcription of the reporter construct)? Draw your expectations using the coloured pens/pencils available



- What other genetic background might we need? What would be the appearance of this strain before introducing the reporter? Draw your expectations



- Imagine we have introduced the reporter into this latter strain. On what media should we plate the yeast strain? One with no, low or high adenine? Motivate your choice. Consider Figure 2 and the biological function of adenine.

Discuss with your demonstrator.

Set up

You are provided with the following:

- Pipettes and sterile tips
- Sterile spreaders
- 3 x agar plates of YES agar
- One microcentrifuge tube containing ***S. pombe* WT strain** labelled WT_1
- One microcentrifuge tube containing ***S. pombe ade6* strain** labelled ade6
- One microcentrifuge tube containing ***S. pombe* Reporter Construct strain** labelled RC

The cultures have already been diluted in sterile phosphate buffered saline and should produce countable numbers of colonies when plated as instructed.

Plating

You will be working in pairs. Label your agar plates with your pair number; your initials; and the strain you will plate. Ensure you do this, or you won't be able to collect your plates next week.

Vortex your microcentrifuge tubes just before plating (to thoroughly resuspend the yeast cells).

Plate 100µl of each strain onto the appropriate plate and use a sterile spreader to spread the culture over the plate surface.

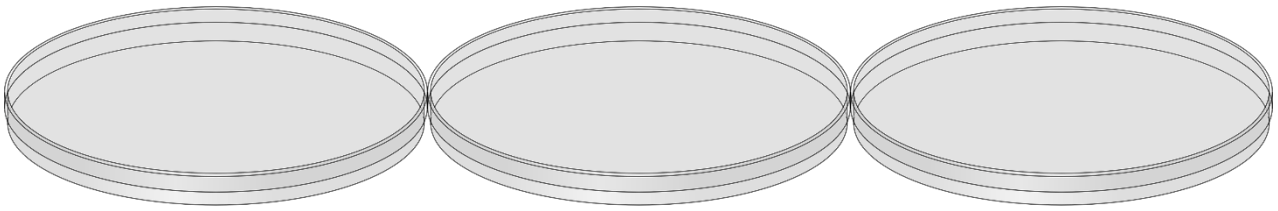
Leave the plates until the liquid has soaked into the agar surface, then turn the plates lid side down and place in the incubation rack provided.

Hypothesis generation

You already forecasted the appearance of yeast colonies from the WT and *ade6* strains. What will colonies from your reporter strain look like next week? Consider situations in which your reporter gene is:

- expressed at high levels
- expressed at medium levels
- not expressed/expressed at low levels

Reporter construct



High Medium No/Low

What would be the alcohol content of the beer produced in these three situations? Remember that the reporter we are using acts as a visual proxy for the expression of the alcohol dehydrogenase gene.

Check your answers with a demonstrator.

Genetic interactions in *S. cerevisiae* (Part 1)

Learning objectives (for Part 1 and Part 2):

1. discuss the role of genome instability in **cancer** development (and treatment)
2. make serial dilutions of *S. cerevisiae* strains and plate them on suitable growth media. Score the number colonies grown on each plate.
3. calculate and compare **mutation rates** in different strain backgrounds
4. identify **genetic interactions** between different mutations and use them to place the gene functions in a hypothetical genetic pathway.

Properties of cancer

1	No or tightly controlled cell proliferation	2	Uncontrolled high-rate cell proliferation
3	De-differentiated	4	Functional differentiation is maintained
5	Cells stay within their tissue	6	Cells migrate throughout the body
7	Unstable genome	8	Stable genome
9	Low mutation rate	10	High mutation rate
11	Mutations in the DNA repair genes are common	12	Mutations in DNA repair genes are rare

You will be provided with a series of properties (shown above). Together with your lab partner, assign them to healthy or cancerous cells. Create a table in your notepad (like the one shown below). Don't worry if you are unsure about some of the features, discuss them with your demonstrator.

Healthy	Cancerous

Address the following questions:

- What core cellular processes influence the mutation rate?
- Do you know any well-known mutations contributing to the genetic instability found in cancer? You might have heard about them in conjunction with the actress Angelina Jolie
- Consider how mutations may help cancer cells "adapting" to a therapy drug. Suggest one scenario

Check your answers with a demonstrator.

Scenario

You have been contacted by the pathology department of a major hospital. They have sequenced a wide range of tumour tissues and noted that mutations in three genes (labelled *C1, C2,* and *C3*) appeared frequently in cancer cells, but not the surrounding healthy tissues. More than one of these genes is often mutated in each tumour sample. The pathology researchers hypothesised that these genes might all contribute to carcinogenesis by increasing the DNA mutation rate. Increased mutation rates can also influence anti-cancer therapy as they lead to a higher likelihood of therapy-resistant tumours. Potentially, mutations in more than one gene could result in cumulative effects on the mutation rates. To be able to better forecast the prognosis of the therapy, the researchers are interested in knowing the genetic relationships between these mutations. You will be using *S. cerevisiae* with the *C1, C2* and *C3* homologous genes knocked out to address this question

- Explain why yeast is a suitable (and more convenient) model system for this study.

- Can you envisage 3 potential scenarios describing different genetic interactions between mutations in any 2 genes? What could be the mutation rate in the double mutant relative to the corresponding single mutants in each of the scenarios?

Check your answers with a demonstrator.

Experimental planning

To assay the mutation rates in yeast we will use the system based on cell resistance to the toxic drug canavanine. Plants produce this compound to protect themselves from herbivores.

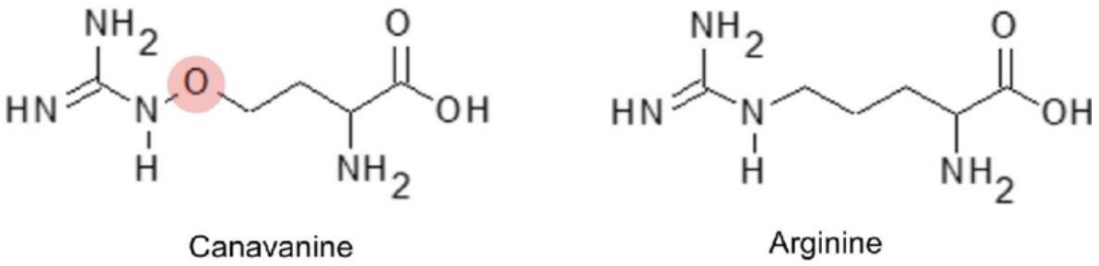


Figure 3. Chemical structure of canavanine and arginine

- Compare the structure of canavanine (CAN) with that of arginine (shown in Figure 3). In what type of macromolecules is arginine normally incorporated? Can you make an educated guess on the mechanism of CAN toxicity?

At some frequency CAN-resistant yeast can arise because of spontaneous mutations. The higher the mutation rate, the more frequent the mutations are and the higher the fraction of the canavanine-resistant cells in the population is.

- How might CAN be taken up by cells? Mutations in what type of cellular components could enable cells to survive in presence of the drug in growth media?

- Should we use a growth medium with or without arginine for our experiment? Explain why.

The mutation rate (MR) can be calculated as following:

$$MR = \frac{\textit{number of CAN resistant cells}}{\textit{number of all cells}}$$

- To find the number of all cells (resistant/non-resistant) what type of media would we need to use?

Because CAN resistant cells are rare (as mutations are rare in general) we must plate a high number of cells on a plate with CAN, to get just a few resistant ones. Most of the plated cells will die in the presence of CAN, but the mutants (shown in black in Figure 4) will survive and each will grow into a colony. On a plate without CAN, all the cells (both in black and in yellow in Figure 4) would be able to grow into colonies.

- Looking at Figure 4, can you explain why we need to serially dilute the culture multiple times before plating on the plate which has no CAN?

We will consider these different dilutions in the next practical.

Check your answers with a demonstrator.

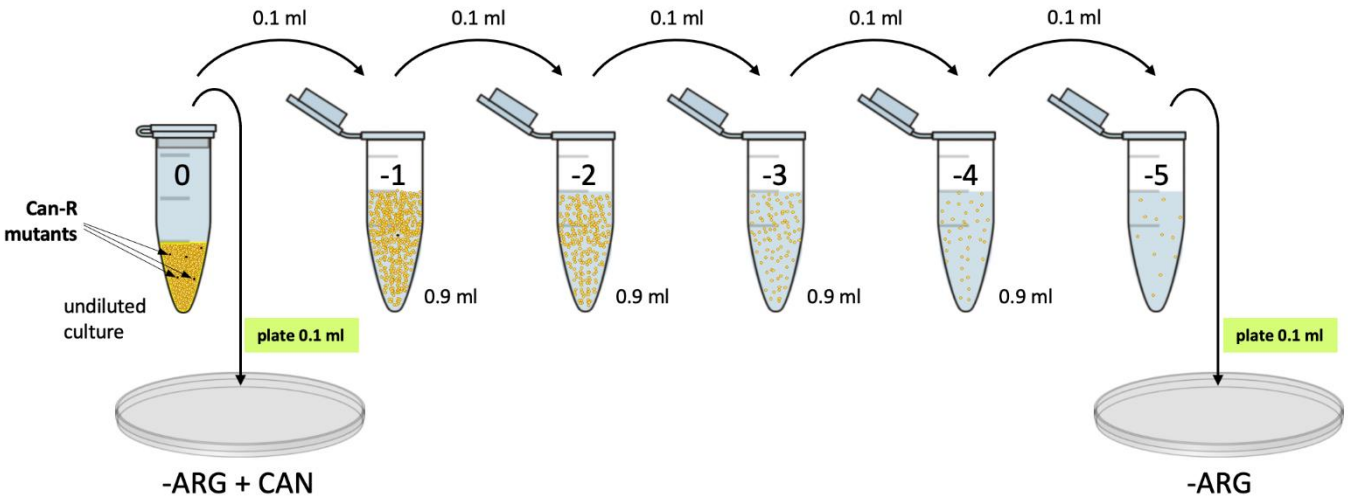


Figure 4. Serial dilution and plating approach. Each step provides a 10-fold dilution of a culture.

Set up

You are provided with the following:

- A set of pipettes and sterile tips
- Sterile Microcentrifuge tubes
- Sterile spreaders
- 4 plates with –ARG agar medium
- 4 plates with –ARG +CAN agar medium
- 4 bijou bottles containing your *S. cerevisiae* strains
- A vial of sterile water

You will be working in groups of 4 students. Each group will be given 4 different yeast strains: a WT, two single mutants (two of C1, C2 and C3) and one double mutant containing both mutations you have in the two single mutants. Please note down which strains you have been given.

Serial dilutions and plating

Follow this protocol (work aseptically all the time to avoid any contamination):

Label the plates on the back (the part containing agar), as lids can be swapped while the bases are physically linked to the medium. First, label all the plates with your group number (this is written on your plate rack)

Organise the plates into 4 pairs, one –ARG plate and one -ARG+CAN plate per pair. Label each pair of plates with the corresponding strain name.

Write the dilution of the culture you will be plating on each plate: 0 (undiluted culture) - for all the plates with canavanine (-ARG+CAN) and –5 for the plates without the drug (-ARG).

Aliquot 0.9 ml of sterile water in 20 sterile microcentrifuge tubes and organise them into 4 sets of 5 tubes for your series of dilutions.

Label the tubes in each set with the strain name and the dilution factor from –1 to –5, as shown on the schematic (Figure 4). You should have 5 tubes labelled WT, –1 to –5, and the same for all your other strains.

Make the dilutions for each strain by pipetting 0.1 ml from one tube to the next, starting from the undiluted culture. Remember to vortex the tubes before transferring any cells into the next tube to ensure complete mixing.

Each strain will be plated onto one –ARG plate and one –ARG+CAN plate. You will only be using the undiluted culture and the –5 diluted culture for your plates. Vortex the undiluted and the –5 diluted cultures right before plating.

For the undiluted strain, vortex the tube then pipette 100 ul onto the –ARG+CAN plate. Use a sterile spreader to spread the culture evenly over the agar surface.

For the –5 diluted strain, vortex the tube then pipette 100 ul onto the –ARG plate. Use a sterile spreader to spread the culture evenly over the agar surface.

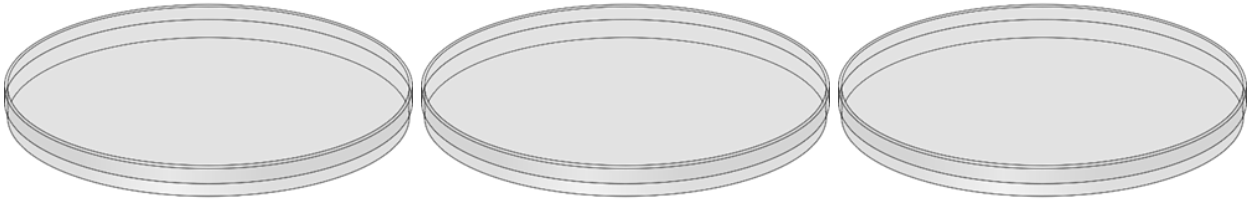
Repeat these last 2 steps for all the remaining strains

When the culture has completely absorbed into the agar, turn the plates lid side down and place them in the incubation rack provided. These will be incubated for 3 days at 30°C.

PRACTICAL 3
Gene expression in *Schizosaccharomyces pombe* (Part 2)

Analysis of plates

Collect you plates and draw their appearances.



WT *ade6* *Reporter construct*

Address the following questions:

- Is this what you expected to see? Compare the plates (and the drawings you just made) to the sketches you created in the last practical.

- Can you envisage an explanation for the difference(s) you might be observing? When thinking about potential biological explanations consider differences between prokaryotic and eukaryotic organisms and recent lectures.

- How is the appearance of the line carrying the reporter construct consistent with the situation reported by the brewing company?

Discuss with a demonstrator.

Follow-up experiments

To test your hypothesis, you can only do 1 extra experiment: the company is under a lot of pressure, and they can't delay beer production for much longer. In groups of 4 students (along one side of the bench), consider each of the following hypothetical experiments.

What would they tell you? If you are unfamiliar with a specific technique, ask your demonstrator.

a. Grow new cultures from a few red and a few while colonies (from the reporter construct plate) and plate them again on the same kind of agar plates to see the colour of the colonies.
b. Pick a few white and a few red colonies (from the reporter construct plate) and knock out a gene involved in transcription. Plate this newly generated strains on the same media to see the colony colour.
c. Run a PCR (Polymerase chain reaction) with primers for the *ADE6* gene in a few red and in a few white colonies.

d. Perform a Northern blot using a probe against *ADE6* mRNA on a few red and a few white colonies. In Northern blots mRNA derived from an organism is exposed to a specific synthetic probe. The probe can bind to complementary sequences and often has fluorescent groups attached for detection.
e. Pick a few white and a few red colonies and knock out a gene involved in methylation. Plate this newly generated strains on the same media to see the colony colour.
f. A different experiment you thought of. In this case, please speak to the floor leader.

When you have made your decision, ask the demonstrator to give you the envelope containing the results for your chosen experiment. Read the results and determine if they support (or challenge) your hypothesis. Note that some experiments might not be at all informative.

Proposed explanation

Have a brief chat with the group on the other side of your bench, did you pick the same experiment? If not, share your results, this will help you gain more information! Briefly write your proposed final explanation on the supplied piece of paper. Please hand it over to your demonstrator that will collect them.

There will be a presentation delivered by the floor leader. Listen to it carefully and take notes.

Chromatin conformations

Three potential chromatin conformations around the alcohol dehydrogenase gene will be shown on the screen. Analyse them and complete the following table:

	Picture 1	Picture 2	Picture 3
Brief description			
Expression level of the alcohol dehydrogenase gene			
Alcoholic content of the beer			
Likely nature of the histone modification(s)			

- Which conformation would be most ideal, from the point of view of the brewing industry?
- How could you genetically intervene to favour this conformation? Would this create some regulatory issues for the use of the strain in the food & beverage industry?

Check your answers with a demonstrator.

Genetic interactions in *S. cerevisiae* (Part 2)

Analysis of plates

Collect your plates. Each group member can count the number of colonies on one plate. To do this, look at the plate face up and take a note of any contaminants growing. Their colonies will look different from yeast. Flip the plate and mark the contaminating colonies with X marks. Score the remaining colonies by placing a dot against the ones you have already counted and write the total number of yeast colonies on the plate.

Using the following table, write down the yeast strain in column 1 (WT has already been added). Add the total number of colonies for each strain (and type of plate) in columns 3 and 6.

Strains	-ARG + CAN plates			-ARG plates			Mutation rate (MR)
	dilution	Number of colonies	Can resistant cell titre, ml^{-1}	dilution	Number of colonies	Total cell titre, ml^{-1}	
(1)		(3)	(4)		(6)	(7)	(8)
WT	none			-5			
	none			-5			
	none			-5			
	none			-5			

You can now calculate the total number of cells (the “cell titre”) in the original 1 ml cultures (columns 4 and 7).

- Start from the total cell titre (column 7). You plated *0.1 ml* of the –5 dilution and have ….. colonies (column 6). How many cells did you have in *1 ml* of the –5 dilution?

- How many cells did you have in *1 ml* of the –4 dilution? -3? -2? -1? 0? The number you get for the 0 dilution is your total cell titre, in cells per 1 ml.

- Following a similar logic, use the colony count for the CAN plate (column 3) to calculate how many CAN resistant cells you had in *1 ml* of the same culture (column 4). Pay attention to the different dilution, compared to plates that did not contain CAN. (Figure 4)

You can now calculate the mutation rate - MR (column 8).

- Remember that MR = number of CAN resistant cells/number of all cells. Calculate this for all the different strains. Be careful manipulating the powers of 10.

Check your calculations with a demonstrator. The demonstrator will then add your data to the

class spreadsheet.

- Return to your own data to find out if the mutations in your two genes do indeed affect the mutation rate. That was the assumption from the pathology researchers. Have a look at column 8.

Scientific recommendation

Using the class data, address the following questions:

- What are the genetic relationships between the different pairs of mutations?
- Tumours carrying what single mutation might be more adaptable to anti-cancer therapy?
- In which scenarios acquiring a second mutation out of the 3 studied in the practical would matter for the genome stability (and potentially prognosis)?

Check your answers with a demonstrator.

Genetic pathway

Draw a (hypothetical) genetic pathway that includes the C1, C2 and C3 functions according to the genetic relationships established from the experimental data. The final process regulated by pathway should be the “Mutation rate”. Blunt arrows (⊥) can be used to indicate inhibition while sharp arrows (→) to indicate stimulation.

To help you, consider the following question:

- Are two genes acting in a synergistic/additive way more likely to be in the same branch of the overall pathway or on different branches? What about genes acting in an epistatic way?
- We have observed the mutation rates caused by loss of these genes (in yeast mutants). What must have been the original function (stimulation/inhibition) of the genes?

Check your sketch with a demonstrator.

PRACTICAL 4

DNA extraction from *Arabidopsis thaliana*

Learning objectives:

1. describe what **insertion lines** are and how they can be used in genetics
2. design and carry out a **DNA extraction protocol** using plant material
3. check **DNA quality** using a nanodrop machine and use the DNA in PCR reactions
4. appreciate the importance of **science outreach**

There will be an initial presentation delivered by the floor leader. Listen to it carefully and take some notes on *Arabidopsis thaliana*, the organism we will be using in the practical. The floor leader will also introduce T-DNA insertion lines (Figure 5) and their importance in genetics. You will need this information to carry out this and following practicals.

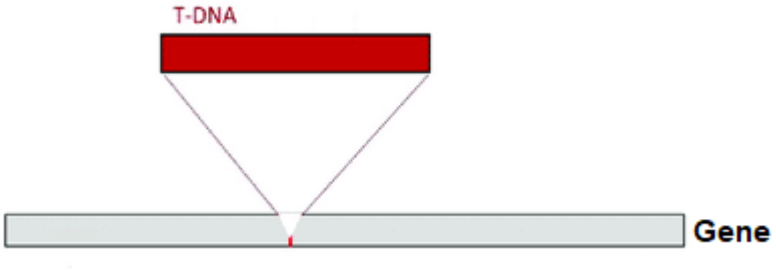


Figure 5: T-DNA insertion in a gene

Address the following question:

- T-DNA insertions at what positions, along a gene, are more likely to disrupt gene function? Sketch a gene (with introns and exons) and identify 2 types of insertions that would likely compromise the function of that gene. Explain your reasoning.

Scenario

You are interested in a family of proteins called MCTPs. To study their genetic roles, you had ordered 4 different seed lines from NASC (the *A. thaliana* seed distributor). In addition to WT seeds, you were supposed to receive three insertion lines: two single mutants (*mctp3* and *mctp4*) and one double mutant (*mctp3,4*). All these lines are homozygous for the insertion. However, an accident happened during transport: the different seeds got mixed-up. Can you work out the genetic identity of individual plants grown from the mixed-up seeds? One of those plants is on your bench.

Address the following questions:

- What does it mean that the lines are homozygous for the insertion? Draw a sketch of what the *MCTP3* and *MCTP4* loci might look like in a WT background and the three insertion lines. Remember that *A. thaliana* is a diploid organism

- Draw a similar sketch for a situation where the three lines are heterozygous for the insertion.

- Look at your plant and compare it to the ones from the other groups along the bench. Can you see some phenotypic differences between them? Do you feel you could make an educated guess about the identity of your plant?

- What molecular approach will we need to figure out the identity of the plants?

Check your drawings and answers with a demonstrator.

Protocol design

...	Grind the plant material to	...	Add isopropanol to	...	Spin the samples to .....
...	Transfer supernatant in new tube to	...	Air-dry the pellet to	...	Spin the samples to .....
...	Incubate at room temperature to	...	Add water to	...	Discard supernatant to .....
...	Add ethanol to	...	Vortex to	...	Discard supernatant to .....
...	Add extraction buffer to	...	Spin the samples to		

You have been provided with a series of steps involved in DNA extraction (shown above). They have been provided in a random order. Together with your lab partners, order these components (assigning numbers from 1 to 14) to create a logical protocol that will successfully extract DNA from your plant. Try to identify the function of each step.

Check your design with a demonstrator.

Setting up

For this experiment you will be working in pairs. You are provided with the following:
- A vial of DNA extraction buffer
- A vial of Isopropanol (on ice)
- Vial of 70% Ethanol
- One microcentrifuge tube containing Nuclease free water also known as double distilled water (ddH_2O)
- Pre-labelled microcentrifuge tubes (for your final DNA extraction). These will identify your samples for the next practical (add your initials to the tubes as well).
- Pestle for grinding up your leaf material. **Pestles are not disposable. They are washed and re-used.**

The Arabidopsis plants will be positioned on the end of your bench.
There will be 2 plants from each seed line, labelled “J”, “K”, “L” and “M” (as mentioned, we don’t know the genotype of these plants so there are just provisional labels).

Each pair of students will select one plant and note down its *provisional* label. Each student will then prepare 1 x DNA extraction from their selected plant (2 extractions per pair)

DNA extraction

Take some leaf material from the plant. Please avoid damaging the plant more than necessary, as we are hoping to re-use some of the plants for the next class. Either part of a large leaf or a few small leaves. Collect enough so it would just about cover a microcentrifuge tube cap. Place these in a 1.5ml microcentrifuge tube.

Grind up the leaf for about 30 seconds using the pestle provided (keep the pestle in the tube for now)

Then add 400 µl of extraction buffer to the tube. Stir the pestle a few times to rinse off any plant pant material adhering to it.

Remove the pestle then vortex the tube for 15 seconds

Centrifuge the tube at top speed for 1 minute

Carefully pipette 300 µl of the supernatant into a fresh microcentrifuge tube. Try to avoid taking up any of the pellet material. You can centrifuge the tube again if you disturb the pellet too much.

Add 300 µl of chilled isopropanol to the supernatant and mix the tube by inverting it a few times. Allow this to incubate at room temp for 5 minutes

Centrifuge the tube at full speed for 5 minutes

Carefully pipette off and discard the supernatant leaving the small pellet intact

Add 500 µl of 70% ethanol as final wash step.

Vortex the tube for 15 seconds

Centrifuge the tube again at full speed for 5 minutes

Carefully pipette off and discard the supernatant, leaving the small pellet intact **(if the pellet breaks up, then try to remove as much of the supernatant as possible without removing the pellet material. This may be made easier using the P200 pipette)**

Air dry the pellet for 10 minutes

Add 100 µl of sterile double distilled water and vortex for 30 seconds to dissolve the extracted DNA.

At this point there may be insoluble material remaining, or the solution looks cloudy. Centrifuge the tube again for 3 minutes at max speed.

Pipette off the supernatant, transferring it into your pre-labelled microcentrifuge tube. This is your DNA solution.

DNA quality control

A demonstrator will now take individual student groups to a nanodrop machine (to avoid congestion). They will show you how to use this instrument to check the amount and quality of the DNA you extracted. Work through these questions while you wait for your turn.

- What macromolecules might have been co-purified with DNA during the extraction? What could be their impact on DNA or applications involving the same?

- What other “contaminants” (also linked to the reagents we used in the protocol) might be present? What could be their impact on DNA or applications involving the same?

Pick the best sample (out of the two that you have) to use in the PCR reaction.
Please do not worry if you don’t get to check the DNA quality of your samples during the session (due to time constraints), there will be another opportunity next week.

Your DNA will be stored frozen and returned to you in the next practical.

Science outreach

There will be a presentation delivered by the floor leader. We will discuss together the

importance of science outreach. You will be provided with an outreach activity suggestion. Make some plans with your lab partner and other groups along the bench:

- Who will be your target audience? How will you introduce DNA as a concept to them?
- Do you have all the items to carry out the outreach activity? Is somebody happy to share and work together with you?
- When are you planning to carry out this activity?
- Could you reach a wider audience using social media?

Let the demonstrators and floor leaders know some of your plans, we are here to help!

PRACTICAL 5

PCR for insertion lines (and Portfolio activity)

Learning objectives:

1. compare and contrast **PCR** with DNA replication
2. identify features of PCR **primers** important for successful DNA amplification
3. check **DNA quality** using a nanodrop machine and use the DNA in PCR reactions
4. design a suitable PCR **thermocycling protocol**

There will be an initial short presentation delivered by the floor leader. Listen to it carefully and take some notes on Polymerase Chain Reaction (PCR) and its importance for molecular (and evolutionary) biology. You will need this information to carry out the rest of the practical.

- Consider some of the differences between PCR and *in vivo* DNA replication. You may want to think about temperature and enzymes involved in the process; as well as the final products of these reactions.

Discuss your ideas with a demonstrator

If you didn't get a chance to check the DNA quality of your samples last week, please let your demonstrator know, otherwise move to the primer design exercise.

Primer design

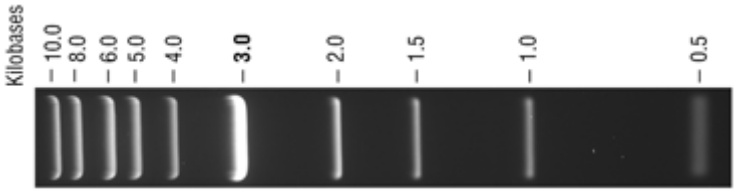
The presentation delivered by the floor leader will now focus on PCR approaches to genotype T-DNA insertion lines. Listen to it carefully and take some notes.

You will then be provided with the DNA sequence of a portion of the *MCTP3* gene (as an example). The T-DNA insertion is within this region (the precise position is not critical for this exercise). *Potential* PCR primers for the gene are colour coded on the sequence and listed in the table below.

Your task is to select an appropriate pair of primers to amplify the WT version of the *MCTP3* gene. You might want to consider the following question:

- Primer annealing is based on DNA complementarity; what is the impact of the length of a primer on the process?

- What would be an ideal PCR product size? Have a quick look at the standard DNA ladder that is normally run alongside PCR products on a gel.



- To amplify a specific DNA region, we need a *pair* of (suitable) primers. Do we need to consider the properties of the individual primers in the set?

Fill the table with your considerations and select a primer pair.

	N	Primer sequence and properties	Type of primer? (LP/RP)	Issues? Intrinsic to the primer or in pairing it to others
	1	GAGAGAAGAAG (Tm 23; GC 45%)		
	2	TATTATGGTCGATGGAGAGAAGAAG (Tm 64; GC 40%)		
	3	TTCAGGATTATGGGAGTTTTGAGTG (Tm 66; GC 40%)		
	4	CGAACTCTTCATCTAGCTCGTCGGG (Tm 72; GC 56%)		
	5	ACCTGTCATAC (Tm 24, GC 45%)		
	6A	GTATGACAGGTTGAGGAGTATTGCT (Tm 64; GC 44%)		
	6B	AGCAATACTCCTCAACCTGTCATAC (Tm 64; GC 44%)		

- Which primer will you then use in combination with the LB primer (annealing on T-DNA) to test for the presence of the insertion?

Check your comments and choices with a demonstrator

<u>DNA quality control</u>

If you didn't get a chance to check the quality of your DNA last week, you will be able to do it now. The protocol is described in Practical week 4.

PCR protocol design

...	Start cycle	...	Heat the lid of the PCR machine to 95°C to ….....	...	72°C for 5 minutes to ….....	...	94°C for 2 minutes to ….....
...	End cycle	...	Repeat cycle 35 times to ….....	...	72°C for 1minute/kb to ….....		
...	Hold reaction at 10°C for ….....	...	50-60°C for 30 seconds to ….....	...	94°C for 30 seconds to ….....		

You have been provided with a series of temperature steps involved in PCR (shown above). They have been provided in a random order. Together with your lab partners, order these components (assigning numbers from 1 to 9) to create a programme that will successfully amplify your DNA of interest. Identify the function of each step.

Check your design with a demonstrator

PCR

For this practical you will be working in pair

You will be provided with the following:
- P200, P20 pipettes and tips
- PCR strip tube pre-labelled with your pair number
- DNA preps from the previous practical (These are in boxes on the side bench)
- Ice bucket with microcentrifuge tubes containing PCR Master mix A, B, C and D. **Note that these are supposed to be shared between 2 pairs.**

The mixes contain the following primer combinations:

Master mix (MM)	Primers	Purpose (complete this)
A	MCTP3 LP + RP	
B	MCTP3 **LB** + RP	
C	MCTP4 LP + RP	
D	MCTP4 **LB** + RP	

Collect your DNA preps from the side bench

Take your pre-labelled PCR strip tube. This will be labelled with your pair number. You must also label each of the 4 tubes a, b, c, and d. **(Please do-not cut the strip up into individual tubes! It is important they are kept joined to keep your samples together)**

Aliquot 48 µl of each of the PCR master mixes A, B, C, D into the corresponding PCR tube a, b, c, d. Use a fresh pipette tip for each aliquot so as not to cross contaminate the PCR master mixes. Remember, the master mix microcentrifuge tubes are to be shared with 2 student pairs so make sure your pipetting is accurate.

Select one of the DNA preps you collected. This will be the prep that you had chosen last week/earlier today when you measured the quality of the DNA sample on the nanodrop spectrophotometer.

Take your chosen DNA prep and vortex this for about 10 sec.

Aliquot 2 µl of the DNA prep into each of your PCR tubes with your 48 µl of Master mix. Again, use a fresh tip for each aliquot.

Close each tube **(make sure the lids fully click into place !)** then flick your PCR strip tube a few times to mix the DNA prep in with the master mix.

Spin the PCR tubes in the SciSpin centrifuge for a few seconds to bring down the liquid (The SciSpin has the coloured domed lid)

Take your PCR tubes to a waiting PCR machine. This will be set on ICE BUCKET mode to keep your samples cool until the cycle is started.

A technician will start the PCR machine when all samples have been submitted. After the cycle is complete, the tubes will be stored in the freezer and will be returned to you for the next part of the practical the following week.

The rest of this practical will consist in activities linked to the Portfolio

PRACTICAL 6

Gel electrophoresis and segregation ratios

Learning objectives:

1. identify plant lines based on distinctive patterns of PCR bands on a gel (**genotyping**)
2. use **segregation in the F2 generation** to determine if genes are linked or unlinked
3. understand the concept of **genetic redundancy**
4. appreciate the importance of **troubleshooting** in molecular biology

Set up

Your PCR mixtures will have gone through thermocycling since last week. You will find your PCR strip on your bench. A demonstrator will take you to load the gel (to avoid congestion). **While you wait for your turn, start the exercise on F2 segregation.**

You will then follow this protocol to load your samples on a pre-stained agarose gel.

For this procedure you will be working in pairs

You are provided with:
- P20 pipette and tips
- 1.5 ml microcentrifuge tube Labelled 1kb+. This contains your DNA ladder.
- Your four PCR reactions from the previous week

Gel tanks are positioned at the ends of the benches on either side. **Please ensure you only use the gel tank on your side of the bench. If you use any other gel tank, then it will make it more difficult for the technicians to return your correct gel photograph.**

There are two spare gel tanks on the side bench which can be used in case of mistakes where samples are loaded incorrectly. Consult a demonstrator if you need to use one of these.

Beside each gel tank is a loading plan which you will complete when you load your samples.

Loading gels

Each pair of students will load 4 lanes on the gel as follows.
First, demonstrators will load 1 lane with 10 µl of the ladder.
Then, load each of the next 4 lanes with 10 µl of your four PCR reactions (a, b, c, d).

The next pair of students will do the same, but do not leave any gaps in between. Please see the arrangement shown below.

L	1a	1b	1c	1d	L	2a	2b	2c	2d	L	3a	3b	3c	3d	L	4a	4b	4c	4d
	1st pair					2nd pair					3rd pair					4th pair			

All of your 4 PCR samples + ladder (L) will need to be on the same gel. If there is not enough room due to mistakes made while loading, then speak to a demonstrator who will arrange for you to use one of the spare gels.

Please fill in the gel plan next to each gel tank. You will need to record which lanes you have used and also the number of the gel you have used.

Once the gel has been fully loaded, a technician will start the gel running. When the gels have been run, they will be removed and photographed using the gel documentation camera.

Photographs of your gel will be returned to you before the end of the practical for you to examine. Results will also be placed on Learn

Once you return to your bench address these quick questions:

- What is the principle of gel electrophoresis?

- Why do we need to "stain" a gel before imaging it? What is the staining agent doing?

<u>F2 segregation</u>

Consider the following questions

- Some of the plants in our practical should be double (recessive) mutants. How was the double mutant generated in the first place? Note that *A. thaliana* normally self-fertilises but it can be experimentally outcrossed (Figure 6). What lines could have been crossed to produce the double mutant?

- What does it involve, in practice, crossing two plant lines? Think about flowers (Figure 6).

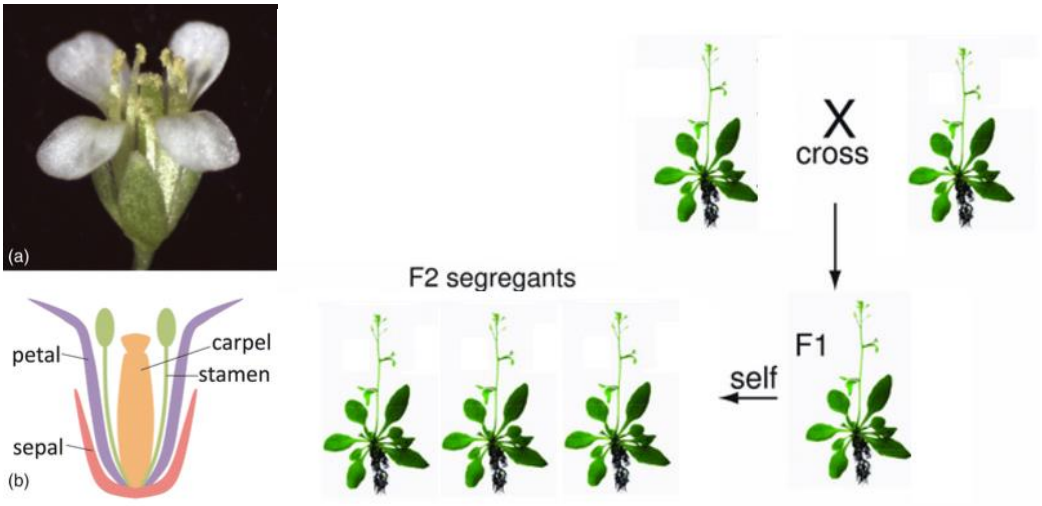


Figure 6: *A. thaliana* flowers and crossing scheme

- What would the genotype of the F1 generation of this cross be? Use “M” as a shortcut for “MCTP”; capital letters for WT versions of the genes (e.g., M3; M4); lower-case letters for the mutant versions (e.g., m3; m4). Separate the two allele for each gene using a dash (/). Lastly, use a semicolon (;) to distinguish the two loci. WT plants would be written as M3/M3; M4/M4

- The F1 generation is then allowed to self-fertilise (Figure 6). Draw a punnet square to identify the different genotypes of the offspring (F2 generation). Write the gametes produced by the F1 plant in the first row and column (boxes shaded in grey). <u>Assume that the two genes are unlinked (this will be our null hypothesis)</u>.

Punnet Square:

- Using the table below, write down how many individuals of each genotype you would expect to see in a population of 16 individuals (same as the punnet square). You will complete the “Observed (o)” column later.

Count:

Genotype	Expected (e)	Observed (o)

16 plants in the F2 generation were genotyped (in the same way as you did for your plants) and the gels have already been imaged. You will be provided with a handout with the pattern of PCR bands obtained. Remember that LP+RP amplify the WT version of the gene while LB+RP the T-DNA insert. Assign a genotype to each plant using the table below.

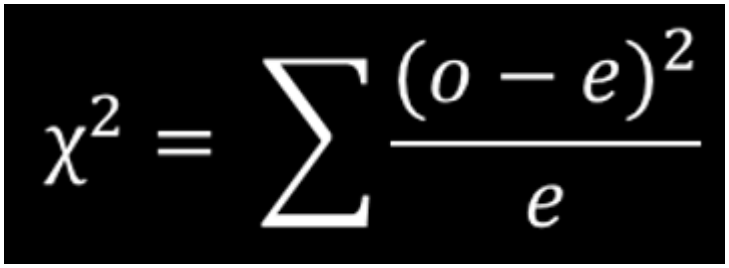
	MCTP3 (M3)		*MCTP4 (M4)*		
N	LP+RP (+/-)	LB+RP (+/-)	LP+RP (+/-)	LB+RP (+/-)	Genotype
#1					
#2					
#3					
#4					
#5					
#6					
#7					
#8					
#9					
#10					
#11					
#12					
#13					
#14					
#15					
#16					

- Fill the number of observed individuals for each genotype in the Count table.

Check your answers with a demonstrator

Statistical testing

There will be a short presentation delivered by the floor leader on statistical testing. They will specifically introduce Chi-square tests (Figure 7) and how they can be applied to genetics problems. Pay attention and take some notes.



d.f.	.05
1	3.84
2	5.99
3	7.81
4	9.49
5	11.07
6	12.59
7	14.07
8	15.51
9	16.92
10	18.31

Figure 7: Chi-squared formula: "Σ" stands for sum, "o" for observed, "e" for expected. A table with threshold values for significance ($p<0.05$) based on the number of degrees of freedom (d.f.) is also provided

- Use a Chi-squared test (Figure 7) to assess if our observations reject the (null) hypothesis that two genes are unlinked.

Check your answer and workings with a demonstrator.

Sample identification

You will now be provided with images of the gels you loaded earlier. Locate the lanes of your samples. Based on the pattern of PCR bands, identity the genotype of your *A. thaliana* plant.

- Was it a WT, a single mutant or a double mutant plant?

- Revisit your notes on the appearances of plants along your bench (Practical 4). Now correlate those with genotypes (you will need to speak to the other groups for this). Are you surprised by your genotyping results? Is there some correlation between genotypes and phenotypes?

- What do the results suggest about the role of MCTP3 and MCTP4 in rosette growth?

Check your answers with a demonstrator.

Troubleshooting

Not everything in science works the first time round. For example, your gel lanes might not show a band pattern compatible with a WT, single mutant or double mutant plant. It is important to "troubleshoot" and work out what went wrong. In a professional context, you would need to repeat the experiment, so you don't want to make the same mistake(s) twice.

Even if your genotyping experiment worked fine, address the following scenarios:

- Your lanes didn't display any band. What could have happened?

- Your lanes displayed too many bands (in addition to the intended ones). What could have happened?

Check your answers with a demonstrator.