The University Of Sheffield

The Department Of Molecular Biology & Biotechnology

LABORATORY TECHNIQUES IN MOLECULAR BIOSCIENCE HANDBOOK (MBB6011) 2019 - 20

Title of the project

Integration of a gene in yeast strain Saccharomyces cerevisiae K699 using CRISPR-Cas9 technology

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General Rules and Regulations

Working in the laboratory demands strict observance to approved instructions for personal safety to avoid laboratory accidents. Most laboratory procedures need the use of living organisms and hence part of all laboratory sessions is the **use of aseptic techniques**. You are therefore required to **maintain a rigorously clean laboratory setting to prevent contamination of experimental procedures from exogenous sources and of course the environment by microorganisms you are using**. Although the microorganisms used in the academic laboratory are non-virulent, however all microorganisms should be treated as potential pathogens. We expect you to develop aseptic techniques (free of contaminating organisms) in the preparation of pure cultures that are essential for a successful project.

The following steps should be observed at all times to reduce the contamination in the laboratory environment.

1. Never place coats, books, and other personal belongings on bench tops – use specified locations such as lockers in the corner of the lab.
2. At the beginning and termination of each laboratory session, wipe bench tops with a disinfectant solution such as 70% ethanol or 10% bleach.
3. Do not place contaminated instruments, such as inoculating loops, tips and pipettes on bench tops. Loops should be sterilized using flame, whereas tips and pipettes should be disposed of in designated bins.
4. On completion of the laboratory session, place all cultures and materials in the disposal area near the sink with appropriate disinfectant in appropriate white / green boxes.
5. Rapid and efficient manipulation of cultures is required to prevent the formation spores in the laboratory environment.

Please observe the following regulations to prevent accidental injury and infection of yourself and your colleagues

1. Always wash your hands with liquid detergent, and dry using paper towels upon entering and prior to leaving the laboratory.
2. Always use the appropriate safety equipment such as:
    a. A **laboratory coat** is compulsory while working in the laboratory. Lab coats protect clothing from contamination or accidental discoloration by staining solutions.
    b. Wear **gloves** when required as described by the instructor. Gloves protect your hands from contamination and also prevent from direct contact with stains and other reagents.
    c. **Masks** and **safety goggles** may be required to prevent materials from coming in contact with your eyes.
3. Carry cultures in a test-tube rack when moving around the department. Likewise, keep cultures in a test-tube rack on the bench tops when not in use. This serves a dual purpose: to prevent accidents and to avoid contamination of yourself and the environment.
4. Immediately cover spilled cultures or broken culture tubes with paper towels and then saturate them with disinfectant solution. Decontaminate spilled fluids with a 10% bleach, covered with paper towel and allow to react for 10 minutes before removal. After 15 minutes of reaction time, remove the towels and dispose them in orange/yellow bins.

5. Immediately report accidental cuts or burns to the laboratory in charge.
6. Pipetting is to be carried out with the help of a mechanical pipetting device only. Never pipette by mouth any broth cultures or chemical reagents. It is strictly prohibited.
7. Speak quietly and avoid unnecessary movement around the laboratory to prevent distractions that may cause accidents.
8. Tie back long hair to minimize its exposure to open flames.
9. We recommend to wear closed shoes at all times in the laboratory.
10. Never apply cosmetics or insert contact lenses in the laboratory.
11. Do not smoke, eat, or drink in the laboratory. These activities are absolutely prohibited.

I have read the above laboratory safety rules and regulations and agree to abide by them.

Name: _____

Date: _____

Please print sign and attach these pages in your laboratory note book.

Introduction

Practical work is an essential component that promotes the development of scientific attitudes such as open mindedness and objectivity. It will give you valuable insight into the scientific method, as well as supporting your learning of the background knowledge of your projects. Often there is more than one possible approach for each technique that we will cover: this handbook is prepared as a starting reference point (rather than a definitive guide) and you will be given further instructions how to proceed during your laboratory work.

The aim of the module is to give you practical experience in a range of essential molecular biology laboratory techniques so that you can confidently progress to your research project during spring semester. To accomplish this aim during this course, a mini project has been chosen that will utilise CRISPR / Cas9 gene knock out / insertion technique. This involves the construction of a plasmid with a yeast selectable marker NatMX (using sub cloning techniques in *E. coli)*. NatMX, is a dominant nourseothricin-resistance marker, and will be used as the target DNA sequence for final integration in yeast. The newly synthesised plasmid will then be used for CRISPR-Cas9 mediated NatMx gene insertion / integration in yeast strain K699. By the end of the module, you will be able to perform, and demonstrate understanding of, essential techniques in molecular bioscience such as microbiology, genetics, molecular biology and biochemistry.

You will also be able to understand the results presented in relevant scientific papers, critically analyze the material, and develop both your writing & oral communications skills.

Background

CRISPR was first discovered in 1987 by Yoshizumi Ishino when he cloned part of CRISPR together with the *iap* gene and was discovered to be a mechanism of viral immunity in bacteria. Over the next 15 years; CRISPR associated (Cas) proteins were discovered (Jansen et al., 2002), and CRISPR-Cas systems have shown potential to generate genetic modifications (Jinek et al., 2012) and be effective genome editing tools (Cong et al., 2013).

Two independent teams showed that a CRISPR-Cas9 system could be reconstituted *in vitro* (Gasiunas et al., 2012; Jinek et al., 2012) and proposed that the CRISPR-Cas9 system could be used for genome editing. For gene editing, the most commonly used CRISPR-Cas9 system uses a crRNA-tracrRNA sequence fusion known as guide RNA (gRNA). This can contain multiple crRNA sequences, with the crRNA 5' proximal 20 nucleotides having been designed to be complementary to the target DNA sequence. The gRNA complexes with Cas9 and guides it to the target sequence where it cleaves the DNA next to a PAM sequence to form a double stranded break (Figure 1). Upon cleavage the DNA is usually repaired by either the error prone non-homologous end-joining (NHEJ) pathway or the high fidelity homology-directed repair (HDR) pathway. NHEJ occurs in the absence of a repair template and causes insertion/deletions (indels) of random lengths which, if in a coding exon, can cause a frameshift and generate premature stop codons thus knocking out the gene.

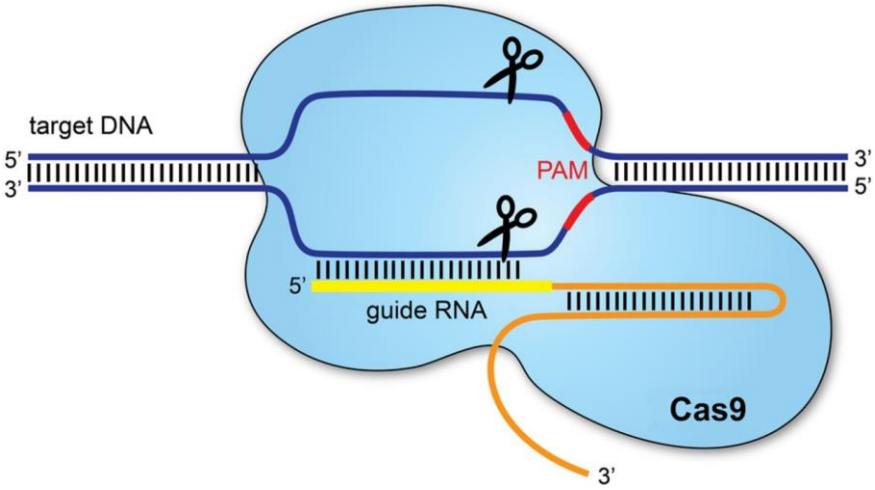

Figure 1: The CRISPR system.

A guide RNA (shown in yellow), complimentary to your chosen DNA sequence binds in the genome of the target cell.

This gRNA then targets the nuclease Cas9 to this region, where it cleaves the host DNA.

Repair of this lesion by Non-Homologous End Joining (NHEJ) can knock out the function of this gene.

There are different types of CRISPR systems which fall into two broad classes, based on the genes that encode effector modules. **Class 1** systems use multi-subunit crRNA-effector complexes such as the CRISPR-associated complex for antiviral defence (Cascade complex). Whereas **Class 2** systems use only a single protein such as Cas9 in the case of the **type II CRISPR systems** (Makarova et al., 2015).

In **type II CRISPR systems** the **tracrRNA-crRNA duplex guides the Cas9 protein to the target DNA** (Hale et al., 2009), base pairing with the homologous target DNA sequence and causing the Cas9 to cleave the DNA, creating a double stranded break in the interference step (Jinek et al., 2012). This is the technology that we will be using in this project.

In the 30 years since its discovery CRISPR has gone from being a genomic curiosity to the cutting edge of science with potential applications that could possibly change our world forever; curing diseases, aiding agriculture, and further developing understanding of the complex interactions that DNA undergoes. These advancements are surrounded by controversy but the rapid growth in CRISPR-Cas9 applications over the last decade and shifting political guidelines suggest no slowing of the pace in the near future. One thing is certain, genomic manipulation has changed forever.

Plan:

We aim to use CRISPR/Cas9 system to integrate NatMx gene into yeast genome and has been described by Peter W Daniels *et al* (2018).

Following plasmids will be used in this mini-project.

1. Plasmid **pBH750** that express the endonuclease Cas9 and a small gRNA in the yeast cell to cut the genome at the MET15 locus).

2. **pBH 756** (MET15 repair vector) plasmid which provides a repair template to repair double strand breaks (DSB) (Both plasmid were gift from Dr Bin Hu, Sheffield MBB department.

3. **pQS-NatMx-Myc9** is donor vector that provide NatMx gene with Myc9 tag at 3' end (plasmid constructed by our self). All plasmid maps are shown in appendix.

As a first step, we have to prepare plasmids DNA extraction using DNA miniprep kit and confirm these DNA preparations by restriction analysis. Then using restriction enzymes cut NAT resistance gene from plasmid pQS-NAtMx-Myc9 and sub clone into pBH 756 plasmid to generate a new plasmid that possess NAT resistance and and Met auxotrophy. The success of this will be determined by screening for the activity of a functional NatMx gene.

We have also designed and ordered primer sequences for diagnostic PCR to confirm the integration of NatmX cassette (Sequences shown in appendix).

Your task is to:

1. Learn basic microbial aseptic techniques.
2. Prepare / use competent cells to transform *E. coli* using given plasmids for amplification.
3. Plasmid DNA extraction (usually called plasmid DNA miniprep).
4. Confirmation of purified plasmids using restriction digestion followed by agarose gel electrophoresis and gel documentation.
5. Restrictive digest of NatMx Donor plasmid with restriction enzyme *Sal*I and *Nhe*I to release NatMx – Myc9 **insert** cassette (with sticky ends) (purify the DNA if needed).
6. Restrict the plasmid pBH756 with *Sal*I and *Xba*I restriction enzyme to create linearized sticky ends **vector** (purify if needed).
7. **Sub-clone** (**ligate**) NatMx insert DNA into a pBH756 linearized vector (already double digested and purified), using **T4 DNA Ligase**.
8. Transform NEB5α competent bacterial cells with ligation mixture for new construct.

9. Screening of colonies for newly synthesised construct plasmid **pMSc-MBB (NatMx-Myc9)** by restriction analysis.
10. Prepare competent cells for the yeast strain K699 (using lithium acetate / PEG method)
11. **Co-Transform yeast** Strain K699 with plasmid pBH750 and restrictive mixture of newly synthesized plasmid **pMSc-MBB (NatMx-Myc9)** to **knockout methionine** and **NatMx-Myc9 integration**.
12. Determine the genotype of yeast cells by patching and replica plating on selective plates
13. Examine the correct and successful integration via diagnostic PCR.
14. Check the expression of integration of **o**pen **r**eading **f**rame (ORF) for selected colonies by western blotting using appropriate Myc9 antibody.

Hypothesis:

Once the yeast has been transformed, the co-expression of wild type Cas9 protein and sgRNA targeted to Met15 coding sequence will take place (Figure 2).

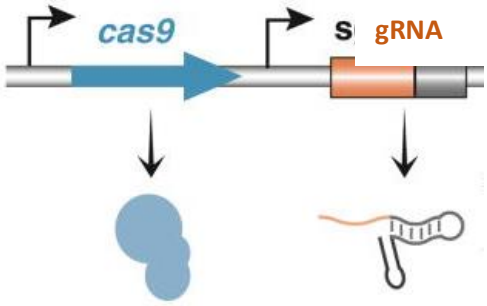

Figure 2: The CRISPR/Cas9 system to knockout Met.

When transformed into cells, the endonuclease Cas9 and the Met gRNA will be co-expressed.

The expected **protein-RNA complex assembly** of Cas9/gRNA-Met will bind the complementary sequence in the *met15* gene in the genome of cells and Cas9 will induce double strand cleavage of the met15 gene (Figure 3).

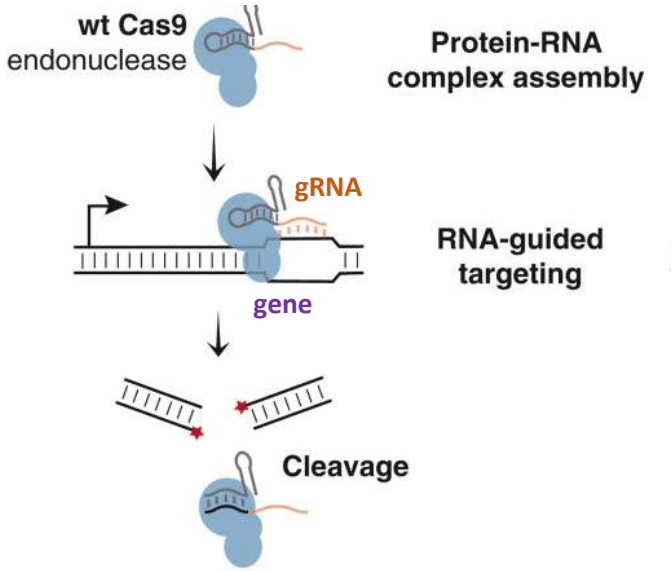

Figure 3: CRISPR/Cas9 mechanism

Formation of the Cas9/gRNA complex on DNA will cause cleavage and a double strand break. The cell with then attempt to repair this an introduce mutations that can disrupt the function of the gene.

- This double strand breakage will cause one of three consequences; **a)** cell death, **b)** repair via Homologous Recombination (HR) or **c)** repair by Non-Homologous End Joining (NHEJ).
- Survivor colonies may have undergone repair by NHEJ, with sequence alterations in the gene.
- Loss of gene activity can be monitored by genotyping using replica plating technique onto appropriate YAPD and methionine minus agar plates. Methionine deficient cells will not grow on Met- agar plates.
- For the integration of NatMx gene, we need to co-transform yeast cells together with **pBH750** and newly synthesized plasmid **pMSc-MBB (NatMx). However, it is preferred to use pre-cut newly synthesized plasmid pMSc-MBB (NatMx) with restriction enzyme *Not*I and use the restrictive mixture for co-transformation.**
- Colonies will be selected by growing on Leucine minus plates. The genetic screening of colonies will be carried out by re-streaking / patching few colonies on Leu- plates first. Then replica plate the colonies on Met- as well

as YAPD + ClonNat plates. The colonies appearing on ClonNat plates as well as Met- plates will be the one that have integrated NatMx gene at the MET15 locus.

- Colonies will also analysed for correct integration by molecular method such as diagnostic PCR using internal and external primers.
- Finally, the protein lysates prepared from yeast-transformed cells will be analysed via SDS-PAGE and Western Blot analysis using primary antibodies against Myc9 (since NatMx-Myc9 expression cassette is already integrated).

More practical details including positive and negative controls and will be set during the course.

References:

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Peter W Daniels, Anuradha Mukherjee, Alastair SH Goldman, Bin Hu (2018). A set of novel CRISPR-based integrative vectors for *Saccharomyces cerevisiae*. Wellcome Open Research, 3:72 Last updated: 27 JUL 2018. (Can be accessed via following this link https://wellcomeopenresearch.org/articles/3-72/v2)

Teaching Method

The course will be delivered through a series of laboratory sessions and tutorials, to develop the student's practical skills and experience. The module will also include written assignments to facilitate students exploring of the background literature and develop scientific communication skills.

Timetable

The module will start in the teaching laboratory (room E4 or E5) in early October. Practical work will be scheduled around your other academic commitments. Students are expected to be ***available*** to attend laboratory sessions between the hours of 9am and 5pm on weekdays during this period.

Introduction to Laboratory Work

A. Units, Conversions and Calculations Required for Laboratory Work

It is essential that at the outset of this course certain basic information is understood by all students.

Units

The official system for measuring quantities in the scientific community is the Systeme International d'Unites (SI). However scientists are a conservative group and some of the old units have been retained. The major SI units are tabulated below.

Physical Quantity	*SI Unit*	*Symbol*
length	metre	m
mass	kilogramme	kg
time	second	s
amount of substance	mole	mol
temperature	Kelvin	K

To allow very large or very small quantities to be measured prefixes are attached as shown below.

Multiple	*Prefix*	Symbol	*Multiple*	*Prefix*	Symbol
10^3	kilo	k	10^{-3}	milli	m
10^6	mega	M	10^{-6}	micro	µ
10^9	giga	G	10^{-9}	nano	n
10^{12}	tera	T	10^{-12}	pico	p
10^{15}	peta	P	10^{-15}	femto	f
10^{18}	exa	E	10^{-18}	atto	a

The exceptions to the SI system retained by many scientists are:

Temperature: SI unit is the kelvin (K), to convert from the kelvin scale to centigrade scale you deduct 273 i.e. 273 K = 0°C. Most biologists have retained the centigrade scale.

Volume: SI unit is cubic metre (m^3), the commonly used non SI unit the litre (l) is defined as being equal to a cubic decimetre (dm^3). It therefore follows that the SI unit for millilitre (ml) is the cubic centimetre (cm^3) and for microlitre (µl) it is the cubic millimetre (mm^3).

You may find some differences in the units used by different members of staff e.g. biochemists have slightly different conventions from microbiologists.

Concentration:

The non SI unit molality (m) is the number of moles of solute in 1 kg of solvent, in SI units this is 1 mol kg^{-1}. The non SI unit to express mole/unit volume is **molarity (M) i.e. amount of solute (in moles) dissolved in 1 litre of solution** i.e. 1 M = 1 mol l^{-1}. However, strictly speaking in SI system 1 M = 1 mol dm^{-3}. However, most scientists use either 1 M or 1 mol l^{-1}. One mole is defined as the number of atoms in 12g of carbon-12. This number is 6.022×10^{23} (**Avogadro Number**) and whenever we have this number of objects (atoms, molecules, ions etc.) we have 1 mole of the substance. If we have 6.022×10^{23} atoms of sodium, the weight of sodium present is 23g ∴ the molecular weight (MW) of sodium is 23. Some standard solutions have their concentrations given in units of 'normality' i.e. a 5 N solution of HCl. This is for most purposes the same as molarity, except that 0.5 N H_2SO_4 is 0.5 M H^+, so it is in fact 0.25 M H_2SO_4.

Radioactivity: The non SI unit the curie (Ci) is defined as 2.22×10^{12} radioactive disintegrations per minute, the usual basic unit is 1 μCi (= 2.22×10^6 dpm). In the SI system the curie is replaced by the bequerel (Bq), the conversion factor is 37 kBq = 1 μCi.

When expressing units the solidus is discouraged in favour of the negative index i.e. 1 mol l^{-1} instead of 1 mol/l. In any event the solidus cannot be used more than once in any unit e.g. J/K/mol is incorrect but J K^{-1} mol^{-1} is correct.

Preparing Molar Solutions

When preparing growth media or reagents for chemical tests, it is essential to understand what is meant by a molar solution. In the previous section 1 molar (M) was seen to be equal to 1 mole (mol) l^{-1}. The molecular weight of an element or of a stable combination of elements is equal to the weight of 1 mole of the substance e.g. sodium has an MW of 23 and chlorine has a MW of 35.5, therefore sodium chloride (NaCl) has a MW of 23 + 35.5 = 58.5 and thus 58.5g of NaCl is 1 mole. If we add 58.5g of NaCl to 1 litre, this is 1 mol l^{-1} = 1 M NaCl. **Therefore, if we know the molecular weight of a substance it is easy to prepare a 1 M solution in 1 litre.** However, in many cases 1 litre is too large a volume. To prepare a 1 M solution of NaCl in 50 ml we simply use proportion thus the MW of NaCl (58.5) divided by 20 i.e. 2.93g of NaCl dissolved in 50 ml of water is a 1 M solution.

Many substances are hydrated (i.e. they contain H_2O as part of their chemical structure) e.g. $CaCl_2 2H_2O$. The MW of $CaCl_2 2H_2O$ is 40 + [2 x 35.5] + [2 x 2] + [2 x 16] = 147, therefore 147g of $CaCl_2 2H_2O$ dissolved in 1 litre is 1 M. This solution contains 1 M $CaCl_2$, 1 M Ca, but 2 M Cl. Calcium chloride is also commonly available as $CaCl_2.6H_2O$, this has a MW of 219. If you add 147g of $CaCl_2 6H_2O$ to 1 litre, this is not 1 M, you need to add 219g. Also, if you are told to add 10g of $CaCl_2 2H_2O$ to 100ml of water and you only have $CaCl_2 6H_2O$ available, you take the extra water into account by multiplying 10 by 219/147 to give 14.9 i.e. 14.9g of $CaCl_2 6H_2O$ contains the equivalent amount of $CaCl_2$ as 10g of $CaCl_2 2H_2O$.

One practical difficulty with hydrated compounds is that once dissolved, the water of hydration adds to the total volume of water present. Therefore, when using compounds such as $CaCl_2.6H_2O$ or $MgSO_4.7H_20$, add the solid to a volume of water **significantly less** than the desired final volume, and then make it up to the final volume once the solid is completely dissolved. This is only usually a problem with concentrations of these hydrated compounds in excess of 1 M.

You may see amounts expressed as % weight/volume (w/v) or % volume/volume (v/v), 10% (w/v) is 10g dissolved in 100ml of solvent and 25% (v/v) is 25ml made up to 100ml with the solvent.

B Chemicals

You will find we (and everyone else except schools) refer to many chemicals by their trivial names rather than their systematic names. This is unfortunate, but you just have to get used to it. The most important ones are :

Formula	Systematic	Trivial
CH_3COCH_3	Propanone	Acetone
CH_3CO_2H	Ethanoic Acid	Acetic Acid
CH_3CHO	Ethanal	Acetaldehyde
$CH_3COCO_2^-$	2-Keto propionate	Pyruvate
$CHCl_3$	Trichloromethane	Chloroform
CH_2Cl_2	Dichloromethane	Methylene Chloride

Note that scientists tend to refer to carboxylic acids as acetate, lactate etc rather than acetic acid, lactic acid etc., because at pH 7 they are ionised.

C Calibrating automatic pipettes

It is important that your laboratory instruments are accurate, meeting the specifications of the manufacturer. To check the accuracy, carry out the procedures described below. If they are not accurate your pipette(s) will be replaced and the defective pipette(s) will be re-calibrated by the technical staff.

Procedure

To a clean 100ml. beaker add approx. 50ml. of water from the tap. Acquaint yourself with the operation of the P1000 automatic pipette (under the guidance of your demonstrator). Also consult the Appendix A, which contains instructions for the use of 'Gilson' pipettes. Fit a blue tip, securely, onto the device and set it at 1ml. Put a plastic weighing boat onto the top-pan four place balance and zero the instrument using the so-called 'tare' button. The balances are extremely sensitive, and all measurements must be taken with the windows of the balance closed, to avoid the effect of draughts. Draw up water into the pipette and dispense this into the weighing boat on the balance pan. Record the weight.

Repeat this four times so that you have five measurements of 1ml. of water. The specific gravity of water is 1.00 $g.ml^{-1}$. This means that 1ml. of water has a weight of exactly 1g. If your first five measurements do not agree within an accuracy of 0.5% (i.e. 5mg.) repeat the procedure until you have five that do. Calculate the average measurement.

Set the P1000 pipette to 200µl. and repeat the above procedure. Is the accuracy different at the two different volumes?

Now devise and carry out a calibration procedure for the P200 pipette.

Use this space to construct a Table to record your results and calculate your pipette accuracy.

The pipette manufacturer claims that the accuracy of the pipettes is within 2% at 1ml for the P1000 and at 200µl for the P200. If your devices are out by 5% or more give the pipettes to the technical staff who will replace them with functioning pipettes and recalibrate the faulty ones.

D. Making up a solution

Refer back to section A on preparing molar solutions.

Suppose you are provided with crystalline salt. To make the solution you have to work out the weight required and once you have calculated the weight required, weigh it out directly into a weighing boat (or 'tared' glass test tube placed in the small plastic beaker) (**Note**: The tare/zero is carried out with tube and beaker on the balance). Transfer it into appropriate size beaker and dispense water. (**Note**: for most compounds there is an increase in volume on solubilisation of a solute). **So always use less water than the actual volume required**. Mix the contents using the magnetic flea for stirring and mix until the contents dissolved completely. **Transfer the solution into cylinder and now make up the volume as desired. Transfer the solution to an appropriate bottle label it** (and sterilize if required) before storage.

Label need to carry all the required information. For example, typical label carries this basic information

Your name/initials	ABC
Room number	E04
Solution Name/Concentration	EDTA 0.5 M
Date of preparing solution	02-10-2018
Volume Prepared	500 ml

Microbiological Techniques

Bacterial Growth / Maintenance / Transformation of E. coli

Microorganisms can grow using many different nutrient sources and at very low nutrient concentrations; indeed, so long as sufficient nutrients are available, organisms if present can grow. This means that microorganisms are relatively easy to cultivate compared to some organisms, but it also means that contamination, with its associated risks, is also commonplace. Sterilisation, aseptic technique and careful inoculation & incubation are vital to minimising contamination. This section outlines the precautions taken for standard laboratory organisms such as *Escherichia coli.* When using organisms that require higher containment levels (i.e. *Salmonella* or *Staphylococcus* species) follow the local procedures in the lab.

Sterilisation

For sterilising media and solutions, it is commonplace to sterilise by heating in an autoclave (like a pressure cooker) at 121°C, 1 bar pressure (15 psi) for 15 minutes, which kills all living organisms, including spores. When making solutions it is worth checking if some components are incompatible with autoclaving (i.e. SDS or glucose when autoclaved under the wrong conditions). Only use the autoclaves if you have been trained in their safe use.

For smaller solution volumes (<10 ml) filter sterilisation can be used. Ensure that the solute is completely dissolved otherwise the filter will clog up. Transfer the solution into a syringe and pass through a 0.22 μm syringe filter into a sterile container. There is a risk of splashing caused by the manual pressure applied, so ensure that you filter solutions (especially those that are hazardous) carefully.

When working with bacteriophages, or when trying to eliminate ribonucleases from glassware, dry heat sterilisation can be used. Glassware is typically baked overnight at 120°C in an oven. Be careful when baking as many items commonly used in research such as blue Duran lids and the foam bungs for conical flasks will melt.

Aseptic technique

To reduce the risk of bacterial contamination it is vital to minimise the chances that bacteria will come into contact with sterile media and solutions. One of the simplest and most under looked ways of minimising bacterial exposure is the disinfection of working areas (i.e. workbench or laminar flow cabinet) before and after use using 70% industrial methylated spirit (IMS). IMS is flammable so do not use anywhere near a naked flame.

Another simple precaution when working with sterile solutions, media and agar plates is to minimise the time that they are open and exposed to air, and thus to potential contamination. When opened, maintaining an air current around an open bottle or agar plate (either using a Bunsen burner or laminar flow cabinet) will prevent bacterial entry. Flaming the neck of a bottle using a Bunsen burner produces a convection current away from the opening, and helps to prevent contamination by preventing microorganisms from entering the mouth of the vessel. **Be careful of burns when using Bunsen burners.**

Treatment of waste culture

Follow the local laboratory procedure for waste disposal. Most waste culture is treated by adding Virkon (disinfectant powder) to the flask (it turns pink) or bleach and leaving the solution overnight, before washing down the sink. Rinse the glassware with water and send it to glass wash.

Inoculation and culture

Bacteria can be introduced to a media (inoculated) by various means. When inoculating small cultures and plates with a colony, a sterile pipette tip or a sterile toothpick is used to transfer the colony across. When inoculating from a liquid culture, a heat-sterilised loop (typically 5 µl loop volume) is dipped into the inoculum culture and spread onto the surface of a (ready set) agar plate or into the new culture.

Cultures are placed in an incubator at a set temperature for growth, usually with agitation to aerate the cultures. Typically, pathogenic or human commensal organisms are grown at 37°C. Because these incubators are at an ideal temperature for growth, spillages must be cleaned up immediately.

When growing cultures in conical (Erlenmeyer) flasks, they should only be filled to 1/10 of the volume (i.e. 25 ml in a 250 ml flask) to ensure proper aeration when shaken at 250 rpm. Inoculation of these larger cultures is usually 1:100 (i.e. 250 µl into a 25 ml culture) and is carried out under a Bunsen from an overnight inoculum culture in the lab.

Storage and resuscitation of microorganisms

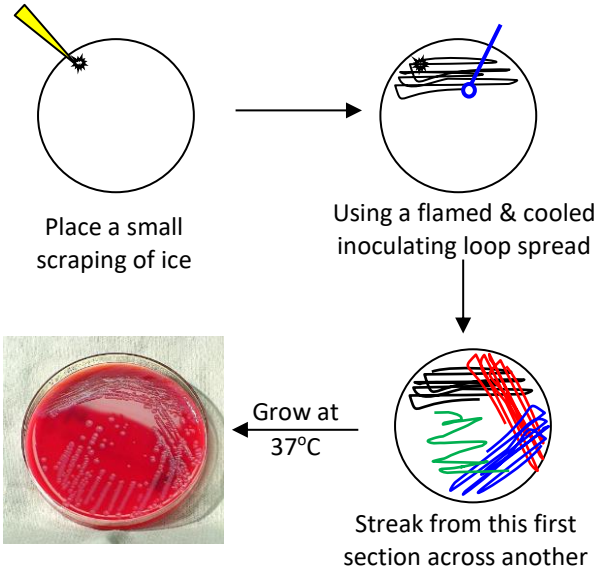
Microorganisms are stored long term (i.e. many years) as glycerol stocks at -80°C. Prepare a 50% ($^{v}/_{v}$) glycerol solution and sterilize it by autoclaving. Grow up a 5 ml overnight subculture of the strain to be stored. In a 1.5 ml micro centrifuge tube mix 500 µl culture and 500 µl 50% glycerol, mix well with a pipette and store at -80°C.

Storage and resuscitation of *E. coli*

To resuscitate a strain, remove the tube from storage and place it on ice to prevent it thawing quickly. Scrape a small flake of ice from the frozen glycerol stock with a sterile pipette tip and place on a fresh agar plate containing antibiotics as appropriate. Using a freshly flamed (and cooled) inoculating loop, streak the transferred bacteria on a section of the plate. Re-flame the loop and streak from the originally streaked area onto a fresh patch of the plate. Repeat another two times. The aim is to isolate individual colonies on the plate, which can be used to see if there is a monoculture present (the colonies should all be identical), the colonies should also look as expected (when you use a strain regularly you get used to colony size, shape and colour). Also, each colony derives from a single cell and so is clonal – this means all cells are genetically identical (barring any newly-acquired mutations) – and so are particularly suitable for use as a starting point for further experiments.

Storage and resuscitation of *S. cerevisiae*

Harvest cells from a 5ml overnight culture (15ml falcon tube) by centrifugation (3,000g, 3 min). Discard the supernatant and suspend the pellet in 2ml of 50% glycerol. Pipette duplicate aliquots (1ml) of cell suspension to screw-capped eppendorf tubes, freeze and store at -80°C. To resuscitate a strain, follow the instructions as for E. coli but using a YEPAD agar plate instead of the LB agar plate. Streak for single colonies as described in



Preparation of chemically competent *E. coli* cells (by RbCl2 method)

Outline:

Competent cells are cells that are treated in a way that allow them to take up DNA; this is of importance for cloning as it allows plasmid and ligations to be introduced into cells. Chemically competent cells are used for carrying out heat shock transformations – the alternative, electro-competent cells, require electroporation of DNA. Competent cells can be purchased; however, it is an easy and cheap job to prepare them and many scientists make their own. Following protocol uses the rubidium chloride method to prepare the cells, although some other protocols do not use $RbCl_2$.

PROCEDURE

1. Streak out cells from glycerol stock on LB plate
2. Pick an isolated colony and grow a 5 ml culture in LB (without antibiotic) overnight at 37°C with shaking.
3. Next day, dilute overnight culture 1:100 in LB (without antibiotic) (from 25 -500 ml). For example, inoculate 25 ml LB in a 250 ml flask with the 0.25 ml saturated (overnight grown) culture and incubate at 37°C with shaking. (Monitor the OD_{600} in 2 hours and every half hour thereafter until it reaches 0.5 to 0.6).
4. Harvest the cells by spinning in a centrifuge in 50 ml Falcon bottles at 3,000 rpm for 10 minutes at 4°C.
5. Discard the LB and gently re-suspend each bacterial pellet in 8 ml ice cold TFB1.
6. Keep on ice for 15 min.
7. Spin again at 3,000 rpm for 10 minutes at 4°C.
8. Discard the supernatant and gently resuspend each bacterial pellet in 2 ml ice cold TFB2 buffer.
9. Aliquot into 100 µl each and freeze.
10. Store at -80°C.
11. Use 100 µl per transformation.

Note: Usually cells obtained by this method give the transformation efficiency of $1x10^8$-$1x10^9$ cfu/mg of plasmid DNA and competent cells can be kept frozen for much longer period of time.

Transformation

1. Thaw 100 µl competent cells on ice.
2. Add 50 -100 pg DNA to tube containing 100 µl competent cells. (Move the pipette through the cells while dispensing. Gently tap the tube to mix).
3. Incubate cells on ice for 30 minutes.
4. Heat-shock cells 90 seconds in a 42°C water bath; do not shake.
5. Place the cells on ice for 2 – 3 minutes.
6. Add 0.4 ml of room temperature LB media (no antibiotic).
7. Incubate at 37°C for one hour.
8. Plate out 100 µl on an LB plate containing 100 -200 µg / ml ampicillin.
9. Incubate the plate upside down overnight at 37°C.

Notes: Work out the transformation efficiency from the transformation positive control to see how efficient your transformations are. Use a known stock of plasmid e.g. pUC18 or pUC19 to determine transformation efficiency. These are given as colonies per µg input DNA.

References: Sambrook, J. & Russell, D.W. (2001). Chapter 1: Plasmids and Their Usefulness in Molecular Cloning. In: Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp. 1.24-1.26 & pp. 1.105-1.122

High efficiency Transformation and gene knockout / integration of NatMx in Saccharomyces cerevisiae (Strain K699)

1. Yeast cells (10-15 ml culture volume in falcon tubes) were inoculated in YPD media and grown overnight at 30°C with shaking (200rpm).
2. Generally, there are $1x10^6$ cells/ml with a density of 0.1 (OD_{600}nm). When OD_{600} of the culture reached 0.4 – 2.0, cells were harvested by centrifugation (3500xg; 2 minutes). The supernatant was discarded.
3. Re-suspend the cell pellet in 1 ml of 100 mM lithium acetate and transfer into 1.5 ml tube.
4. Wash the cells twice in 100 mM lithium acetate and the pellet was re-suspended in 100 mM lithium acetate with 1-3 volume of pellets.
5. Prepare 50 µl aliquots into sterile microfuge tubes and incubated at 30°C for 30 minutes
6. Prepare the transformation mixture as below and add to each 50 µl aliquot of cells
   - 240 µl of 50% (w/v) Polyethylene Glycols (PEG)
   - 36 µl of 1.0 M lithium acetate
   - 25 µl of single stranded DNA (10 mg/ml) (sonicated, boiled at 95°C for 5 minutes and chilled on ice)
   - 50 µl DNA* **(see below)**

7. Set P1000 to 400 and the mixture was mixed gently by pipetting
8. Incubate for 30 minutes at 30°C and heat shock at 42°C for 20 minutes
9. Harvest cells by centrifugation in a microfuge (6000 rpm; 3 minutes)
10. Discard the supernatant and gently re-suspend cells in 1 ml of YPD
11. Incubate for 90 minutes at 30°C with gently shaking
11. Harvest cells by centrifugation (13, 000 rpm; 30 sec) and discard the supernatant.
12. Wash off the media with 1 ml sterile water
13. The supernatant was removed and the cells were re-suspended in 100 µl of sterile water
14. Spread the cells onto appropriate agar plates (e.g. Leu – plates) and incubate at 30°C for 2-3 days

***** 50 µl DNA:

1. For transformation of plasmid: 1 µg plasmid (typically 5 µl of miniprep) plus 45 µl of ddH_2O; 5 µl of each vector + 40 µl DNA for co-transformation.
2. For a yeast genome integration experiment:

Prepare a restrictive digestion mixture as below:

DNA miniprep 2 µg (your newly constructed plasmid)	5 µl
10x reaction buffer	3 µl
ddH_2O	20 µl
NotI (restriction enzyme)	2 µl
Total Volume	30 µl
Incubate for 30 - 60 minutes at 37° C	
Heat inactivation of NotI at 65°C for 20 minutes	
Keep on ice	
Add pBH750 (plasmid miniprep) 1 µg	2.5 – 5 µl
Add water to make up the final volume up to 50 µl	
use this mixture for yeast transformation	

3. 50 µl of ddH_2O only (as negative control)

Notes / Reference:

Gietz, R. D. & Woods, R. A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods in Enzymology Vol 350 pg 87-96

Working with DNA

Isolation of plasmid DNA (miniprep)

Outline: This protocol outlines the method used for isolating small-scale preparations of plasmid DNA (mini-prep) for subsequent cloning and analysis. Commercial kits allow quick cellular lysis and purification on a single column. For this reason, you will follow the protocol outlined in the QIAprep Spin Miniprep Kit Using a Microcentrifuge.

Inoculation

Bacterial cultures for plasmid preparations should always be grown from a single colony picked from a freshly streaked plate. Sub-culturing directly from glycerol stock or liquid cultures may lead to uneven yields or plasmid loss. Optimal results are obtained by using one single isolated colony from a freshly transformed or freshly streaked plate to inoculate an appropriate volume of starter culture containing the appropriate antibiotic, and then incubated for 12-16 at 37°C with vigorous shaking (~300 rpm; shaking incubator).
Note: Aeration is very important. The culture volume should not exceed 1/4th volume of the container.

Culture Media

The QIAprep Spin Miniprep Kit are specially designed for use with cultures grown in Luria Bertani (LB) medium. Richer broths such as TB (Terrific Broth) or 2xYT lead to high cell densities that can overload the purification system, and therefore are not recommended.
If rich media has to be used, growth times have to be optimized, and the recommended culture volumes must be reduced to match the capacity of the HiBind® DNA Mini Column.
Note: As culture ages, DNA yield may begin to decrease due to cell death and lysis within the culture.

Culture Volume and Cell Density

Do Not Exceed Maximum Recommended Culture Volumes
For optimal plasmid yields, the starting culture volume should be based on culture cell density. A bacterial density between 2.0 and 3.0 at OD600 is recommended. When using nutrient-rich media, care should be taken to ensure that the cell density does not exceed an OD600 of 3.0. Using a high-density culture outside of the recommended OD range may overload the purification system.

Materials, reagents and equipment

1. Sterile universal
2. LB medium
3. 37°C incubator
4. Colony of the bacteria that contains our plasmid of interest
5. Appropriate antibiotic
6. QIAprep Spin Miniprep Kit
7. Centrifuge that can spin 25 ml universals
8. 1.5 ml microcentrifuge tubes
9. Microcentrifuge

Protocol: QIAprep Spin Miniprep Kit Using a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations by the kit supplier.
Note: All protocol steps should be carried out at room temperature.

Materials and Equipment to be Supplied by User:

- 100% ethanol
- Microcentrifuge capable of at least 13,000 x g
- Nuclease-free 1.5 mL or 2 mL microcentrifuge tubes
- Culture tubes
- Optional: sterile deionized water

- Optional: water bath or incubator capable of 70°C
- Optional: 3M NaOH solution

Before Starting:

- Prepare DNA Wash Buffer and Solution I according to the instructions.

1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1- 5 mL LB medium containing the appropriate selective antibiotic. Incubate for ~12-16 hours at 37°C with vigorous shaking (~ 300 rpm). Use a 10-20 mL culture tube or a flask with a volume of at least 4 times the volume of the culture. It is strongly recommended that an endA negative strain of E. coli be used for routine plasmid isolation. Examples of such strains include DH5a® and JM109®.
2. Centrifuge at 10,000 x g for 1 minute at room temperature.
3. Decant or aspirate and discard the culture media.
4. Resuspend pelleted bacterial cells in 250 µl Buffer P1 (kept at 4 °C) and transfer to a microcentrifuge tube.
   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
5. Add 250 µl Buffer P2 and gently invert the tube 4–6 times to mix.
   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.
6. Add 350 µl Buffer N3 and invert the tube immediately but gently 4–6 times.
   To avoid localized precipitation, mix the solution gently but thoroughly, immediately after addition of Buffer N3. The solution should become cloudy.
7. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
   A compact white pellet will form.
8. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
9. Centrifuge for 30–60 s. Discard the flow-through.
   Spinning for 60 seconds produces good results.
10. (Optional): Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.
    This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.
    Although they call this step optional, it does not really hurt your yield and you may think you are working with an endA- strain when in reality you are not. Again for this step, spinning for 60 seconds produces good results.
11. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
    Spinning for 60 seconds produces good results.
12. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
    IMPORTANT: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions. *They are right about this.*
13. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.
    If you are concerned about the concentration of the DNA, you can alternatively add 30 µL water to the center of the column, incubate at room temperature on the bench for 5 mins and then centrifuge for 1 min. This will increase the concentration of DNA in your final sample which can be useful in some cases. See notes below for why you should elute in water rather than the Buffer EB they recommend if you plan to sequence your sample. **Even if you are not sequencing, it may be**

***beneficial to elute in water**. For instance, if you elute in buffer EB and you are using this DNA in a restriction digest, then the additional salts in your sample can affect the salt content of your digest. This may matter with some finicky enzymes.*

14. Store the eluted DNA at -20°C (freezer).

Notes:

- If you are doing more than ~10 minipreps simultaneously, it can save time to switch to the vacuum manifold version of this protocol since you eliminate having to load and unload samples into the centrifuge.
- The elution is dependent on pH, however measuring the pH of unbuffered water is difficult. However, anecdotally we have been able to get good yields using the water. Eluting in deionized autoclaved water has also produced good results.
- Passing the lysate over the column twice increases yield by about 20%.
- I sometime do two PE washes of about 300-500 µL. For the the first, I dispense the liquid from the pipette tip along the inner ledge of the spin column in a circular motion to wash off the residue there. I follow the first PE wash with a second to further de-salt the sample before the drying spin. Yes, it adds a step, but the time spend here is far less than waiting three days only to find out your sequencing didn't work.
- Heating the elution buffer to 55°C prior to loading on the column can slightly increase yields.
- Similarly, doing the elution in two steps (first a 30 µL elution and then a 20 µL dilution) can also slightly increase yields.

References:

Sambrook, J. & Russell, D.W. (2001). Chapter 1: Plasmids and Their Usefulness in Molecular Cloning. In: *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp. 1.16-1.19 & pp. 1.31-1.61.

DNA quantitation

Outline: For many techniques such as ligation reactions or restriction digests the amount of DNA present in a reaction is an important factor. Methods for measuring the concentration of DNA in a solution all rely upon spectrophotometry. Traditionally, and the way shown here, the absorbance of UV light at 260 nm by the DNA bases is measured in a 1 cm cuvette. More modern spectrophotometers with a shorter path length are now available (NanoDrop/NanoSpec) that require less sample, whilst fluorescence-based dye measurement can be used.

Materials:
1) Quartz cuvette or disposable UVette
2) Solvent (the same composition as what your DNA sample is in).
3) DNA sample
4) Pipettes

Protocol

1) Turn on the spectrophotometer to warm up.
2) If using a quartz cuvette, ensure it is clean.
3) Firstly, prepare a blank. Put 1 ml of solvent (i.e. water, TE buffer, or Buffer EB) into the cuvette and blank the spectrophotometer.
4) Remove 5 µl from the cuvette and add 5 µl of your DNA sample to be tested.
5) Mix well with a 200 µl pipette.
6) Measure the absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}).
7) A measurement of 1 A260 unit is the equivalent of 50 µg/ml double stranded DNA. So, work out the concentration of DNA in the sample using the formula:

$$\textit{concentration}\ (\text{ng}/\mu\text{l}) = A_{260} \times 50\ \text{ng}/\mu\text{l} \times \text{Dilution factor}$$

8) **Work out the A_{260}/A_{280} ratio**. A ratio of >1.8 indicates pure DNA.

Notes:

Sambrook, J. & Russell, D.W. (2001). Appendix 8: Quantitation of Nucleic Acids. In: *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp. A8.19-A8.21.

Restriction Endonuclease Digestion of DNA

Outline: Restriction endonuclease digestion is a commonly used method for the cutting up of DNA (plasmid, PCR product or genomic DNA) into fragments for purposes including cloning or for restriction enzyme mapping. The choice of restriction enzyme used typically depends upon factors including the sequence(s) that you wish to cut, the target vector that you are using for cloning and, in the case of more than one enzyme being used, and the availability of compatible buffers. Different enzymes can require different buffers and need different incubation and inactivation temperatures, so reading the information leaflet accompanying an enzyme is essential.

Materials, reagents and equipment

1. DNA to be digested
2. Restriction endonuclease
3. 10x Buffer
4. Bovine Serum Albumin (BSA) (if needed, or if not in the 10x Buffer)
5. Ice bucket
6. Water bath at a suitable temperature

Protocol:

A typical 20 µl digestion is shown here. Keep all components chilled on ice until the incubation / inactivation steps.

1) Thaw 10x restriction enzyme buffer and Bovine Serum Albumin (BSA) (if required).
2) In a sterile 1.5 ml microcentrifuge tube add:

Plasmid DNA (~0.5 ug undigested)	5-10 µl
Buffer H[1] (10x)	2 µl
BSA[2] (20x)	1 µl
Restriction enzyme (12U / µl)[3]	1 µl
ddH_2O to make up volume up to 20 µl	
Total	20 µl

3) Incubate 1 hour at 37°C[4].
4) Place at 65°C for 20 minutes to inactivate the enzyme (optional)[4]

Notes:

[1]The choice of buffer depends upon the enzyme used. Pick a buffer that allows your enzyme to have a high activity (usually the lids of the enzyme and buffer are colour co-ordinated). Avoid buffers that have a 'high star activity', which means non-specific.

[2]Some companies include BSA in their standard buffers, whilst some have it in a separate tube to add here. Read the protocol!

[3]One unit is the amount of enzyme needed to cut 1µg lambda DNA in 1 hour at 37°C. **Add the enzyme last**. Never allow to total amount of enzyme in a reaction to exceed 10% of the final volume as this causes star activity.

[4]These temperatures are very much enzyme dependent so read the product literature.

References:

https://www.promega.co.uk/resources/product-guides-and-selectors/restriction-enzyme-resource/restriction-enzyme-general-information/

https://www.promega.co.uk/~/media/files/resources/protocols/technical%20manuals/101/restriction%20enzymes%20protocol.pdf

Agarose gel electrophoresis of DNA

Outline: Agarose gel electrophoresis is used for the resolution, visualisation and quantitation of DNA fragments. The DNA that is run can be a single species (i.e. PCR product) or a complex population (i.e. digested genomic DNA). Agarose gel electrophoresis can be used for diagnostic purposes (i.e. checking the size of an insert in a plasmid), for preparative purposes (i.e. for gel extraction), or just to estimate the amount of DNA present in a single band.

We use **Midori Green Nucleic Acid Staining Solution** that is a new and safe alternative to traditional ethidium bromide (EtBr) stain for detecting dsDNA, ssDNA and RNA in agarose gels. Nearly identical to EtBr in performance and use, Midori Green is much less harmful to living organisms.

Materials:

1. 50x TAE (40 mM tris-base, 20 mM acetate, 1 mM EDTA; pH between 8.2-8.4).
2. 6x DNA loading buffer (30% glycerol with a dash of bromophenol blue to colour)
3. DNA ladder (also call DNA markers) Usually we use 1 Kb DNA ladder
4. Midori Green Nucleic Acid Staining Solution
5. Power supply
6. Gel tank, casting tray and comb
7. Masking tape
8. Agarose
9. Microwave
10. Transilluminator / Gel Documentation System

Protocol for 100 ml of 1% (w/v) agarose:

1. Prepare sufficient 1x TAE for making the gel and filling the gel tank. Dilute the 50x TAE stock solution down with distilled water to 1x.
2. Place 100 ml of 1x TAE and 1 g electrophoresis-grade agarose in a large microwaveable vessel and melt it in a microwave on a setting of around 40% until the agarose is completely dissolved. **** CAUTION – THIS IS VERY HOT ****
3. Allow the molten agarose to cool until approximately 60°C (hand-hot) in a water-bath, or at room temperature shaking regularly. Whilst cooling, tape up the casting tray and add the comb.
4. When cooled, add 6 µl Midori Green dye, and mix by swirling (try not to introduce bubbles).
5. Pour the gel into the tray. If bubbles are present, burst them using the wide end of a P200 pipette tip. Allow the gel to solidify at room temperature.
6. When cooled, place the gel in the gel tank and pour in 1x TAE until it is covered. Remove the comb (note: removing it when the gel is submerged helps prevent the wells being damaged).
7. Prepare your samples. Only DNA bands >10 ng can be visualised. For PCR products this typically 5 µl of a PCR reaction is run, whilst 5 µl of a miniprep is also run. Add water to bring each sample up to 10 µl and then add 2 µl of 6x loading dye.
8. In one well of the gel add 5 µl of DNA ladder.
9. In the other wells load the samples that you wish to analyse.
10. Put the lid on the tank, connect up the tank to the power supply and run at 100V for one hour. NB. DNA is negatively charged and runs towards the positive electrode. **** BE CAREFUL WITH MIXING WATER AND ELECTRICITY ***** Stop the gel early if you think that it has run far enough.
11. Turn the power supply off, and wearing gloves remove the gel from the tank.

12. Take the gel to the transilluminator and visualise the gel. **** UV light is mutagenic, always use a transilluminator cabinet and follow the instructions given ****
13. Sizes of bands in your samples can be estimated from the marker lanes. Because each marker band contains a known amount of DNA, comparison of the band brightness allows the quantity of DNA to be found.

Notes: Choose the concentration of agarose in the gel depending on the size of the fragments you wish to resolve.

Agarose (%)	Optimal band resolution
0.5	700 bp to 25 kbp
0.8	500 bp to 15 kbp
1.0	250 bp to 12 kbp
1.2	150 bp to 6 kbp
1.5	80 bp to 4 kbp

Different types of agarose are available, suitable for different functions; for virtually all purposes a standard electrophoresis grade agarose will suffice

References:

Sambrook, J. & Russell, D.W. (2001). Chapter 4: Gel Electrophoresis of DNA and Pulsed-field Agarose Gel Electrophoresis. In: *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp. 5.1-5.17.

Quick Yeast DNA extraction (for confirmation of integration of NatMx cassette by diagnostic PCR)

1. Prepare 1x1 cm patch on appropriate YPD plate and incubate at 30°C overnight.
2. Scratch half patch of cells using blue loop (10 ul) from plate and re-suspend the cells in 0.2 ml of SCE/Zymolase T20/2-Mercapto Ethanol mixture by vortexing.
3. Incubate 37°C with shaking for 1 hour.
4. When complete, add 0.2 ml of Lysis solution. Mix by inverting the tube 4–6 times.
5. Heat at 65°C for 5 minutes.
6. Add 0.2 ml 5 M-KOAc. Mix by inverting the tube 4–6 times
7. Leave on ice for 15-40 minutes.
8. Spin at high speed for 10 minutes. Transfer 0.35 ml supernatant into 0.8 ml ice-cold 100% ethanol.
9. Mix by inverting the tube 4–6 times
10. Spin at high speed for 10min and dispose of supernatant using water pump.
11. Add 100 µl 70% ethanol and vortex.
12. Spin at high speed for 5 minutes and remove supernatant.
13. Repeat steps 10-11 once.
14. Vacuum dry the pellet at 45°C for 5 minutes
15. Dissolve in 100-300 µl ddH_2O depending on the size of your cells pellet.
16. To fully dissolve your DNA, incubate your DNA solution at 65°C for 30minutes. Store at -20°C.
17. For a restriction digest, use 5 µl in a 20 µl reaction. For PCR, use 1µl in a 20µl reaction.

SOLUTIONS

SCE solution	1M - Sorbitol 0.1M - Na Citrate 0.06M-EDTA (autoclaved) pH7.0
SCE/Zymolase/2-ME	Mix to each ml of SCE solution add 1.5 mg zymolase 20T and 8 µl 2-ME
Lysis solution	2% SDS 0.1M-Tris/Cl pH9.0 0.05M-EDTA

Potassium acetate solution	5M-KOAc (autoclaved)

PCR amplification

Outline: PCR amplification is a method for generating specific DNA fragments for either diagnostic or cloning purposes. Each amplification reaction contains a DNA polymerase (usually from *Thermus aquaticus*), oligonucleotide primers, dNTPs and template in a suitable buffer. The reaction then undergoes repeated cycles of template denaturation, primer annealing and extension of the oligonucleotides primers to generate multiple copies of the desired sequence. Many different enzymes are available, each needing different buffers and reaction condition and so reading the product literature is essential to ensure primers are designed correctly and that the PCR is set up and run using the amplification conditions recommended for the enzyme.

Materials:

- Ice
- Polymerase enzyme (usually high fidelity polymerase such as Phusion of pfu pol)
- Reaction buffer (5x or 10x) as supplied by the company
- dNTPs Mix (10 mM each)
- Template (plasmid at a concentration between 50 pg to 5 ng/µl)
- Forward primer 5 µM (18-25 bases, Tm approximately 60°C, GC content between 30-70%)
- Reverse primer 10 µM (18-25 bases, Tm approximately 60°C, GC content between 30-70%)
- MilliQ water (autoclaved) / Molecular biology grade water
- Thin walled 200 or 500 µl PCR tubes
- Thermocycler

Protocol:

1) Thaw and keep the 10x buffer, primers, enzyme and template on ice. Spin all the tubes briefly in a microcentrifuge to collect all the liquid in the bottom of the tube.

2) Take a thin-walled PCR tube and add:

10x Standard Taq Reaction Buffer	5 µl
dNTPs (10 mM)	4 µl
Forward Primer (5 µM)	4 µl
Reverse Primer (5 µM)	4 µl
Template DNA	1-2 µl
DNA Polymerase	0.5-1 µl
Nuclease-free water (to make up volume up to 50 µl)	xx µl
TOTAL	50 µl

3) Gently mix and briefly spin the tube in a microcentrifuge to collect to the bottom of the tube.

4) Program the thermocycler with the following conditions. Load the tubes and run the reactions.

Initial denaturation	95°C	3 minutes	1 cycle
Denaturation	95°C	30 seconds	
Annealing	55°C	30 seconds	35 cycles
Extension	72°C	1 min/kb	
Final extension	72°C	10 mins	1 cycle
Hold	10°C	-	-

5) Once run, store the reactions on ice or at -20°C awaiting further analysis

6) Use 5-10 µl from the PCR reaction mixture via agaorse gel electrophoresis to analyse the success of PCR reaction

Notes:
All polymerases are different and could be of conventional *Taq* type, or alternatively high-fidelity or hot-start. Also, many polymerases are pre-supplied as a 2x mastermix with a agarose gel-loading dye added to the reaction. Because of the many varieties of enzyme it is essential to read the product information leaflet to find out how to design primers, set up the reaction correctly and to use the right amplification conditions.

References:
Joshi, M. & Deshpande, J.D. (2011) POLYMERASE CHAIN REACTION: METHODS, PRINCIPLES AND APPLICATION International Journal of Biomedical Research 2(1). Pp. 81-97. http://ijbr.ssjournals.com/index.php/journal/article/view/24

Sambrook, J. & Russell, D.W. (2001). Chapter 8. *In Vitro* Amplification of DNA by the Polymerase Chain Reaction. In: *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp. 8.1-8.24.

Purification of PCR products

Purification of PCR products (using PCR purification kit)

Outline: The removal of unused primers, polymerase, dNTPs and buffer salts from the desirable PCR product is usually the first step after carrying out a PCR reaction. These impurities interfere with many downstream applications of PCR such as ligation reactions. Originally, purification was carried out by phenol:chloroform extraction, but this has now been largely superseded by commercial kits that allow quick purification on a single column. These columns are quicker and more efficient – they also have a 90 to 100 bp size cut-off on the column meaning DNA fragments smaller than this are lost. Because of the commercialised nature of this process the protocol outlined in QIAquick PCR Purification Kit is shown here.

Materials, reagents and equipment

1. PCR products (unpurified)
2. QIAquick PCR Purification Kit
3. 1.5 ml microcentrifuge tubes
4. Microcentrifuge

QIAquick PCR Purification Kit Protocol

Using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of #7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.

- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene. For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel. Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Notes: The protocol shown here is copied from the kit.

References: http://sevierlab.vet.cornell.edu/resources/EN-QIAquick-Spin-Handbook.pdf

Purification of PCR products (using Gel extraction method)

Outline: This protocol outlines the method used for recovering DNA from an agarose gel. Initially, a mixture of DNA species is separated out by agarose gel electrophoresis; an isolated DNA fragment (band) of a precise size is then cut from the gel and purified for downstream cloning. You will follow the protocol outlined in the commercial QIAquick® Gel Extraction Kit.

Materials, reagents and equipment

1. Agarose gel that has been run.
2. QIAquick® Gel Extraction Kit
3. 1.5 ml microcentrifuge tubes
4. Microcentrifuge
5. UV Transilluminator ***WARNING***

Protocol:

1. Run the DNA on an agarose gel, such that the gel has been run long enough for the band(s) of interest to be individually cut from the gel.

2. Cut the bands out on a UV transilluminator using the low energy (higher wavelength) setting (365 nm not 302 nm) to minimise damage to the DNA. Note: UV light is dangerous - only use the transilluminator if you have been trained and always wear gloves, a lab coat and a UV-safe face mask keeping all skin covered.

3. Follow the protocol that accompanies the kit.

QIAquick® Gel Extraction Kit - Spin Protocol

The QIAquick Gel Extraction Kit (cat. nos. 28704 and 28706) can be stored at room temperature (15–25°C) for up to 12 month.

Further information

- *QIAquick Spin Handbook*: www.qiagen.com/HB-1196
- Safety Data Sheets: www.qiagen.com/safety
- Technical assistance: support.qiagen.com

Materials and Equipment to be supplied by User:

- Heat block or water bath capable of 60°C
- Microcentrifuge capable of at least 13,000 x g
- Vortex Mixer
- Nuclease-free 1.5 mL microcentrifuge tubes
- 100% ethanol

Notes before starting

- This protocol is for the purification of up to 10 µg DNA (70 bp to 10 kb).
- The yellow color of Buffer QG indicates a pH ≤7.5. DNA adsorption to the membrane is only efficient at pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at 17,900 *x g* (13,000 rpm) in a conventional table-top microcentrifuge.

1. Perform agarose gel/midori green electrophoresis to fractionate DNA fragments. Any type or grade of agarose may be used. However, it is strongly recommended that fresh TAE buffer or TBE buffer be used as running buffer. Do not reuse running buffer as its pH will increase and reduce yields.
2. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
3. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6

volumes Buffer QG.

4. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.
5. Add 1 gel volume isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.
7. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 µl Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
8. To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

   Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt- ended ligation), let the column stand 2–5 min after addition of Buffer PE.

   Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
9. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
10. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.
11. If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Protein Analysis

Goal

In this experiment we want to confirm the expression of protein NatMx-Myc9 tag in yeast. The cells will be grown as usual and the expression level will be analysed using SDS PAGE followed by western blotting using anti-myc antibody.

Growth of cells

- Grow 10 ml culture in YPD broth at 30°C overnight with shaking or rolling of universal tubes.

Yeast Protein Extraction using Trichloroacetic acid (TCA) method (for Western blotting)

Procedure

Steps 1 – 6 (keep samples on ice)

1. Exponentially growing cells (4 0 OD_{600}) cells were collected by centrifugation at 3,500 rpm x 2 min
2. Re-suspend the pellet with 1 ml of ice cold water and transfer to 1.5 ml microfuge tube.
3. Spin down the cells at 13,000 rpm x 10 seconds and discard the supernatant. (The sample can be stored at -80oC at this step).
4. Re-suspend the cells with 1 ml of ice cold water.
5. Add 150 µl freshly prepared buffer D (1.85 M NaOH, 7.5% β-mercaptoethanol) and incubate on ice for 15 minutes.
6. Add 150 µl of 55% TCA and incubate on ice for 10 minutes (mix by inverting tube).
7. Spin down at 13,200 rpm for 10 minutes (room temperature) and remove as much supernatant as possible.
8. Re-suspend in 150 µl 2x protein loading buffer and mix with pipette 6 – 8 times.
9. Add 50 µl of 1 M Tris (pH 8.8) to adjust the pH. Vortex samples.
10. Incubate at 95oC for 5 minutes. (Can be sonicated at 7% for 10 seconds). (May freeze samples).
11. Spin down the cell debris at 13,200 rpm for 10 minutes.
12. Use 10 µl for SDS – PAGE separation.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a technique used to separate proteins either from a complicated mixture of proteins (i.e. whole cell lysate) or to check the purity of an enriched protein. Proteins are coated in the detergent SDS, giving a uniform negative charge, which causes them to migrate to the anode when a current is applied. The speed of migration is dependent solely on the molecular mass. SDS-PAGE gels are nearly always run vertically and use a discontinuous buffer system to aid stacking of the proteins in the gel.

Material and Equipment required for SDS PAGE:

1. Gel tank with lid and power cables, electrode assembly.
2. Buffer dam if one gel is being run.
3. Gel casting frame
4. Glass plates (one thick and one thin per gel) usually 1.0 mm
5. Comb (10 or 15 well) of same thickness as plates.
6. 30% Acrylamide (37.5:1 ratio acrylamide to bis-acrylamide)
7. 10% SDS ($^{w}/_{v}$)
8. 1M Tris pH 6.8
9. 1.5 M Tris pH 8.8
10. TEMED
11. 10% Ammonium persulfate solution ($^{w}/_{v}$) in water (freshly made)
12. 2X OR 5X SDS-PAGE Sample-loading buffer
13. 1x Tris-SDS-Glycine Buffer (Running Buffer)
14. Protein loading markers
15. Staining container
16. Staining solution
17. Destaining solution

18. Camera to take picture of gel
19. Orbital shaker

Protocol: (For casting two 10% gels)

1. Clean the glass plates and assemble them together in the casting frame.
2. Prepare the **Resolving gel** (10%) in a universal tube by combining the following and pour into the frame as demonstrated:

Composition of Resolving Gel (Lower Gel)	
30% Acrylamide (37.5:1 ratio)	3.8 ml
Water	3.4 ml
Tris HCl 1.5M (pH=8.8)	2.6 ml
SDS 10% (w/v)	0.1 ml
Ammonium persulfate (APS) 10% (w/v)	0.1 ml
TEMED	0.01 ml
Note: Add the TEMED and APS last (the crosslinking reagents), mix by swirling to minimise air bubbles and pour into the frame leaving approximately 15 mm space for the stacking gel and comb. Add 1 ml isopropanol as top layer to removing bubbles.	

3. Add water or water saturated isopropanol to the gel to ensure a flat join between stacking and resolving gels. This excludes air and aids polymerisation of the gel.
4. Wait 20 to 30 minutes until set.
5. Pour off the water or isopropanol.
6. Dry with clean filter paper or air dry.
7. Prepare the **stacking gel** (5%) by adding the following:

Composition of Stacking Gel (Upper Gel)	
Acrylamide 30% (37.5:1 ratio bis- to mono-acrylamide)	0.67 ml
Water	2.98 ml
Tris HCl 1.0 M (pH=6.8)	1.25 ml
SDS 10% (w/v)	0.05 ml
Ammonium persulfate (APS) 10% (w/v)	0.05 ml
TEMED	0.005 ml
Note: Add the TEMED and APS last, pour the mixture in between plates as demonstrated and push the comb into the stacking gel.	

8. Wait 20 to 30 minutes for the gel to set.
9. Remove the polymerised gel/plates from the casting frame and place it into the electrode assembly in the gel tank (with the comb still attached). If only one gel is needed, use the buffer bam in the other side of the electrode assembly.
10. Fill the tank up with 1x running buffer so that the inner chamber is full and the outer chamber is nearly to the top of the gel.
11. Remove the combs from the gel and wash out each well with 1x running buffer from the tank using a P200 pipette to remove debris and unpolymerised acrylamide.
12. Take the protein samples to be run (i.e. 12 µl of a purification fraction) and add 3 µl of 5x sample loading buffer and mix by pipetting. Alternatively, if using culture resuspend the pellet in 1x sample loading buffer (use 1 ml per 2.5 OD_{600} units of culture).
13. Boil for 5 minutes to denature the proteins. Spin 1 minute at 14k in a microcentrifuge to remove debris/insoluble proteins.
14. Load 5 µl protein marker in one of the wells (NB. Pre-stained markers are quite useful for visualising the progress of the electrophoresis and also for verifying protein transfer if immunoblotting.
15. Load 15 µl of protein sample per well. Add 15 µl of 1x SDS-PAGE loading buffer to the empty wells as well so that they do not run empty.

16. Place the lid on the tank and connect up the cables to the power supply.
17. Run at 200V for 45 minutes, or until the gel has run sufficiently to visualise your proteins based on the migration of the pre-stained markers.

For the optimal resolution of different size proteins, the concentration of acrylamide in the resolving gel can be altered. This is done by varying the amount of 30% acrylamide and water that is added to the gel during preparation.

Acrylamide %	M.W. separation Range
7%	50 kDa - 500 kDa
10%	20 kDa - 300 kDa
12%	10 kDa - 200 kDa
15%	3 kDa - 100 kDa

Coomassie Staining of SDS-PAGE Gels

Coomassie-staining of SDS-PAGE gels allows visualization of the proteins directly in the gel. The whole gel is firstly fixed and stained with the staining solution, before de-staining solution is applied, which washes stain from the gel, leaving just blue protein bands. (Coomassie Brilliant Blue R reacts non-specifically with proteins). The detection limit is approximately 0.1 µg per band; however, if using a purified protein, then loading 0.5-1 µg gives a nice band.

Protocol

1. Take a run gel, separate the plates using a gel knife and carefully using a vertical cutting motion cut off and remove the staking gel.
2. Place the resolving gel in the staining container and cover it with staining solution (enough so that the gel can move about freely).
3. Place on the orbital shaker with moderate agitation (50 rpm) for 1 to 2 hours.
4. Pour the stain back into the bottle (it is re-usable).

De-Staining of SDS-PAGE Gels

Remove the gel from the Coomassie staining solution and gently agitate the stained gel in de-staining solution until the background becomes clear (1–2 h to overnight).

Protocol

1. Add copious amounts of destain solution and place back on the orbital shaker.
2. Change the destain solution every 30 minutes until bands are easily visualised and the background is low.
3. Take a picture of the gel (using gel documentation system).

Buffer for SDS PAGE	Concentration	Required volume
Tris-SDS-Glycine Buffer (for SDS-PAGE) Tris base, 25 mM (pH 8.3) Glycine, 250 mM, SDS, 0.1%	A 5X stock can be made by dissolving 15.1 gm of Tris base and 94 gm of Glycine in 900 ml of H_2O. Then, 50 ml of a 10% (w/v) stock solution of electrophoresis – grade SDS is added, and volume is adjusted to 1000 ml with H_2O.	1 Lit
SDS-PAGE sample buffers (2X) 0.09 M Tris·Cl, pH 6.8 20% glycerol 2% SDS 0.02% bromophenol blue 0.1 M DTT	2X	10 ml
Coomassie staining solutions 0.05% (w/v) Coomassie Brilliant Blue R-250*; 40% (v/v) ethanol; 10% (v/v) glacial acetic acid. For 1 litre, dissolve 500 mg Coomassie Brilliant Blue R-250 in 400 ml	1X	1 Lit

100% ethanol. Add 100 ml glacial acetic acid and water to 1 litre. Filter before use.		
De-staining solution 25% (v/v) ethanol; 5% (v/v) glacial acetic acid; 70% water	1X	2 Lit

References

de Boer, H. A. and A. S. Hui (1990). "Sequences within ribosome binding site affecting messenger RNA translatability and method to direct ribosomes to single messenger RNA species." Methods Enzymol **185**: 103-114.

Dubendorff, J. W. and F. W. Studier (1991). "Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor." J Mol Biol **219**(1): 45-59.

Looman, A. C., J. Bodlaender, et al. (1987). "Influence of the codon following the AUG initiation codon on the expression of a modified lacZ gene in Escherichia coli." EMBO J **6**(8): 2489-2492.

Studier, F. W., A. H. Rosenberg, et al. (1990). "Use of T7 RNA polymerase to direct expression of cloned genes." Methods Enzymol **185**: 60-89.

QIAexpress Ni-NTA Fast Start Hnadbook for purification and detection of recombinant 6xHis tagged proteins available at (2005) available at www.qiagen.com

Protein Purification and Detection Tools (2nd Ed) available at www.merckbio.eu

1. Protein quantitation

There are many different ways of measuring protein concentration. The Bio-Rad protein assay is one of the most popular and is based on the Bradford dye-binding method. A standard curve first needs to be carried out – most standard curves use BSA as the reference protein. A measurement using your concentrated protein can then be made and compared to the standard curve.

For the standard curve:

1. Label 11 tubes as described in the table.

Tube Number	Amt of BSA	0.5 µg/ml BSA (µl)	Water (µl)
1	25 µg	50	0
2	22.5 µg	45	5
3	20 µg	40	10
4	17.5µg	35	15
5	15 µg	30	20
6	12.5 µg	25	25
7	10 µg	20	30
8	7.5 µg	15	35
9	5 µg	10	40
10	2.5 µg	5	45
11	0 µg (BLANK)	0	50

2. You have been supplied with a 0.5 µg/ml BSA solution. Add BSA and water to each of the tubes as described in the table.
3. Add 750 µl water to bring the final volume to 800 µl.
4. Add 200 µl Bio-Rad protein assay reagent.
5. Vortex briefly.
6. Incubate 5 minutes at room temperature.
7. Measure A_{595} vs the 0 mg blank.
8. Plot out the absorbance on a graph.

Western immunoblotting

Western immunoblotting is a detection technique that allows quantification of protein samples. In its most common form, protein species are resolved on a SDS-PAGE gel, transferred onto a membrane, which is then subsequently probed quantitatively using antibodies. The transfer of protein takes place in a plastic clamp, which is held in a wet-transfer tank. The clamp contains the gel and membrane between two pieces of filter paper that in turn are between two pieces of sponge. The proteins migrate from the negative terminal to the positive terminal.

Equipment:

1. Wet transfer tank with lid and power cables.
2. Wet transfer cassette with sponges and Perspex clamp.
3. Blotting paper
4. Membrane (Nitrocellulose 0.45 µm)
5. PBS-Tween
6. 0.05% Tween 20 in PBS.
7. Dried Skimmed Milk Powder
8. 1x Wet transfer buffer
9. Primary antibody
10. Secondary antibody
11. ECL detection reagents
12. Gel visualiser

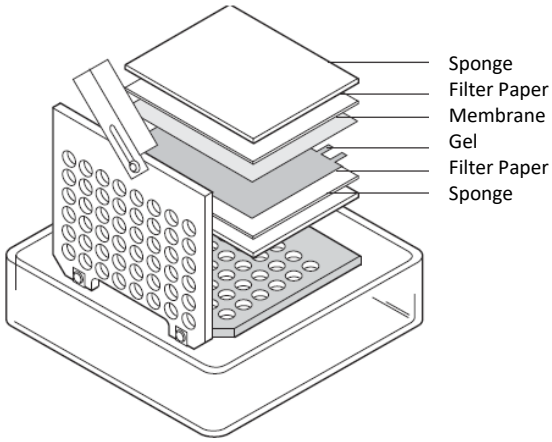
Protocol:

The initial step of a western blot requires the separation of proteins by SDS-PAGE. It is imperative to load the same quantity of material per lane; therefore, some normalisation is usually required. This can take the place of a protein quantitation assay or loading the same proportion of cells per lane (based on culture OD_{600} measurements). Alternatively, an identical SDS-PAGE gel can be run, coomassie-stained and quantitated to show equal loading or to provide an idea of the differences in loading quantities for retrospective normalization.

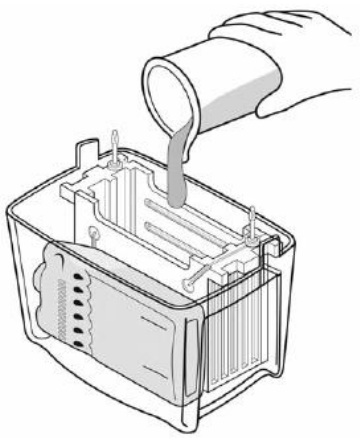
1. Run a SDS-PAGE gel to separate the proteins. Load equal quantities per lane and include positive and negative controls if possible. This will not be coomassie stained.
2. While the gel is running:
    a. Take two pads of blotting paper (pre-cut to gel size) and place them in a clean bowl containing 1x Wet-transfer Buffer to soak for 15 minutes. At the same time soak two of the sponge pads.
    b. Cut some 0.45 µm membrane (GE Healthcare) to the correct size (use the pre-cut blotting paper as a guide). Do not touch the membrane directly and use gloves. Dip in distilled water and then place in 1x Wet-transfer buffer for 15 minutes.
    c. Clean the blotting tank.

3. Disconnect the power supply and remove the SDS-PAGE gel from the tank. Separate the plates. Remove the stacking gel with the gel knife.

4. Using a large bowl filled with blotting buffer, open the plastic clamp. Onto the black (negative) side add a soaked sponge and then soaked filter paper, gel, nylon membrane, soaked filter paper and finally the soaked sponge. Try to minimise the bubbles.



5. Close the clamp and secure it shut with the sliding lock. This can be difficult as the bundle of sponges, paper, gel and membrane is very thick.
6. Place the shut clamp into a half-full blotting tank in the black and red electrodes/clamp holder. Up to two gels may be blotted at the same time. Ensure the black (negative) side of the clamp faces the black electrode in the tank and the clear side of the clamp faces the red electrode.
7. Add the pre-cooled Bio-Ice cooling unit to ensure the apparatus does not overheat as it transfers.
8. Fill up the tank with buffer to the top.



9. Place the lid on the tank and plug the cables into the power supply..
   Transfer for:
   - 100V, constant 350 mAmps for 1 hr (hydrophilic proteins)
   - 100V, constant 350 mAmps for 1.5-2 hrs (large hydrophilic proteins (>150 kDa) and hydrophobic proteins)
   - 30V, constant 90 mAmps overnight

Note: The optimal transfer time varies from protein to protein. Larger and hydrophobic proteins take longer to transfer than smaller hydrophilic proteins. If you transfer to long, your protein will transfer off the membrane into the filter paper.

10. Upon completion of the transfer, turn off the power supply, disassemble the blotting sandwich and remove the membrane. A successful transfer can be verified by the presence of pre-stained markers on the membrane.
11. Remember which side of the gel the faced the gel and so has the protein on it (usually by cutting off two of the corners).

12. If you are transferring your sample for the first time, you will want to stain the transfer gel(s) to see if the transfer was efficient.
13. Stain the gel if needed.

Immuno Detection:

1. Rinse the membrane in PBS.
2. Prepare Blocking solution (5% Marvel in PBS-Tween) and add 20 -25 ml to the membrane in a square Petri dish or box. Block the membrane by incubation at room temperature with agitation, (can be blocked overnight at 4°C).
3. Wash in T-PBS (1x15 min, 2x5 min)
4. Replace the wash buffer buffer with fresh 25 ml blocking buffer and add 5µl of primary antibody (1:5000 diln.) mix briefly. Leave to shake for 1 hour at room temperature.
5. Tip off primary antibody. Wash in T-PBS (1x15 min, 2x5 min)
6. Add 5 µl of secondary antibody with fresh 25 ml blocking buffer (1:500o dilution) and mix briefly. Leave to shake 20 minutes to 1 hour at room temperature with agitation.
7. Drain off secondary antibody
8. Wash in T-PBS (1x15 min, 2x5 min)
9. Quick rinse in PBS to remove Tween-20. (Wash once for 5 minutes with 1x PBS (no tween))
10. Just prior to finishing the washes prepare the working solution by mixing an equal volume of the stable peroxide solution and the luminal/enhancer solution (Millipore High Sensitivity ECL). Use 0.125 ml per cm^2 of membrane. The working solution should be stable for 8 hours at room temp if kept in the dark. For our 8.6 x 6.8 cm gels, 1 ml is sufficient.
11. Drain the excess wash buffer from the washed membrane and place it, protein side up, on a sheet of cling film or other clean surface. Pipette the mixed detection reagent on to the membrane.
12. Incubate for 5 minutes at room temperature, covered by a box to minimise light. The reagents should cover the entire surface of the membrane, held by surface tension to the surface of the membrane.
13. Drain off excess detection reagent by holding the membrane with forceps and touching the edge against a tissue. Place the blots protein side down onto a fresh piece of cling film, wrap up the blots and gently smooth out any air bubbles using an absorbent tissue. This forms an envelope (or detection pocket) - avoid using pressure on the membrane. Ensure there is no free detection reagent in the membrane.

Take the membrane to a chemilluminescence detector (e.g. G-Box in our lab) to visualise where the light is being produced on the membrane, and hence where the protein of interest is most abundant.

Appendices

USE OF AUTOMATIC PIPETTES

In lab classes we will be using pipettes which are adjustable to dispense different volumes. Pipetman® is a continuously adjustable pipette, eight models are available covering a range from 0.1 µl to 10 µl. We are using the P200 (20-200 µl delivery) and P1000 (200-1000 µl).

The pipettes have an adjustment (referred to as a 'digital volumeter' by the manufacturer) which is used to set the volume. The volume is continuously adjustable within the volume range for the pipette. The maximum volume for the pipette is shown on the push-button and corresponds to the model (see table).

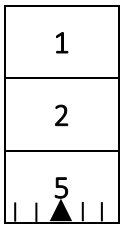
Model	Volume Range (µl)
P200	20-200
P1000	200-1000

The pipettes are equipped with a metallic tip-ejector and use disposable tips made of polypropylene. You are protected from contamination because you are not required to handle the tips after use; carry-over between transferred liquids is eliminated by using a new tip for each transfer.

SETTING THE VOLUME

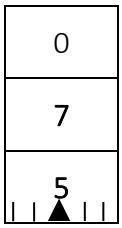
The volumeter consists of three number dials (which are used to set the volume of liquid to be transferred) (see diagram). They are read from top (most significant digit) to bottom (least significant digit). A marker is used to set exact or intermediate volumes using the scale on the bottom dial. According to the model, the dials are coloured either black or red (only black in the diagram).

Model	Colour of volumeter numbers	
	Black	Red
P200	microlitres	
P1000	microlitres	millilitres



P200

125 µl



P1000

0.75 ml

The volume is set by turning the black knurled adjustment ring or the adjustment knob of the push-button. The adjustment knob makes it easier and quicker to set volumes, especially when wearing gloves. The adjustment ring may be used to slowly reach the required setting. To obtain maximum accuracy when changing the volumeter setting, follow the recommendations below:

- When **decreasing** the volume setting, slowly reach the required setting, making sure not to overshoot the mark.

- When **increasing** the volume setting, pass the required volume by 1/3 of a turn and then slowly decrease the volume to reach the required setting, making sure not to overshoot the mark.

OPERATION

Firstly, fit a tip to the tip-holder of the pipette. You must fit the tip that is appropriate for the Pipetman model that you are using. The colour of the tip should match the crown of the push button.
Push the tip onto the tip-holder using a slight twisting motion to ensure a firm and airtight seal.

Always fit a tip to your Pipetman before aspirating any liquid.

Aspirating

- Press the push-button to the first positive stop.
- Holding the pipette vertically, immerse the tip into the liquid.
- Release the push-button slowly and smoothly to aspirate the liquid.
- Wait one second and withdraw the tip from the liquid. Wipe any droplets away from the outside of the tip using a medical wipe.

Avoid touching the orifice of the tip.

Dispensing

- Place the end of the tip against the inside wall of the vessel at an angle of 10 to 40 degrees.
- Press the push-button smoothly to the first stop. Wait one second. Press the push-button to the second stop to expel any remaining liquid.
- Keeping the push-button pressed to the end, remove the pipette by drawing the tip along the inside surface of the vessel.
- Release the push-button.
- Eject the tip by pressing the tip-ejector button. It is only necessary to change the tip if different liquid is being transferred.

SUMMARY OF OPERATION

1. Attach a disposable tip on the shaft of the pipette by pushing the end of the pipette onto a tip in the pipette tip boxes provided. (Use blue tips for the P1000 and yellow tips for the P200). Press on firmly with a slight twisting motion to ensure a positive, airtight seal.
2. Depress the push-button to the first positive stop.
3. While holding the pipette vertically immerse the tip 3 or 4 mm into the sample liquid.
4. Release the push button **slowly** to draw up the sample.
5. Wait 1 or 2 seconds, and then withdraw the tip from the sample. Wipe any fluid from the outside of the tip, taking care not to touch the orifice of the tip.
6. To dispense the sample, place the tip end against the inside wall of the vessel and depress the push-button **slowly** to the first stop. Wait a couple of seconds and then depress the push-button completely to expel any residual liquid.
7. With the push-button fully depressed, carefully withdraw the Pipetman with tip sliding along the wall of the vessel.
8. Release the push-button.
9. Remove the used tip by depressing the tip ejector button.
10. Never

    - ™ Allow liquid to enter the barrel of the pipette.
    - ™ Place the pipette horizontally or vertically when the tip contains liquid.
    - ™ Contaminate the handle or any external surface of the pipette.
    - ™ Turn the volume adjustment to a position higher than the maximum; the gearing will seize up.

GILSON PIPETMAN

P1000 Pipettes up to 1 ml (1000 microlitres)

Use between range of 1000 – 200 µl only

Max. No. (1000 µl)

0.1→ 0.9 (100 to 900 µl)

0.01 → 0.09 (10 to 90 µl)

0.001 → 0.009 (1 to 9 µl)

DO NOT attempt to adjust above 1000 µl

If for example you want to pipette 1.5 ml, adjust to 0.75 and use 2 squirts

P200 Pipettes up to 0.2 ml (200 microlitres)

Use between range of 200 – 20 µl only

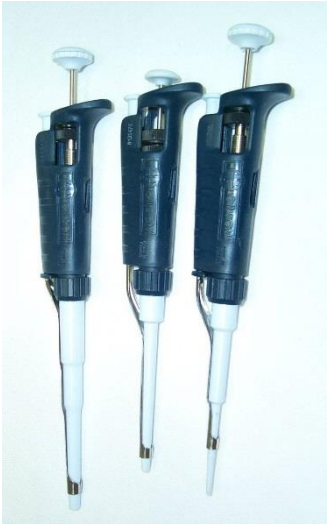
Max. No. (200 µl) [0.2 ml]

0.01→ 0.09 (10 to 99 µl)

0.001→ 0.009 (1 to 9 µl)

0.0001 → 0.0009 (0.1 to 0.9 µl)

DO NOT attempt to adjust above 200 µl



P20 Pipettes up to 0.02 ml (20 microlitres)

Use between range of 20 – 1 µl only

Max. No. (20 µl) [0.02 ml]

0.001→ 0.009 (1 to 9 µl)

0.0001→ 0.0009 (0.1 to 0.9 µl)

0.00001→ 0.00009 (0.01 to 0.09 µl)

DO NOT attempt to adjust above 20 µl

REMEMBER! EACH PIPETMAN COSTS OVER £100!
FOLLOW THESE GUIDELINES CAREFULLY.

Plasmids used in this study (plasmids maps shown at the end)

	Plasmid	Used for
1	pBH750	Responsible for the expression of Cas9 gene and gRNA-Met15
2	pBH756	Met15 repair vector for sub-cloning NatMx
3	pQIS-NatMx-Myc9 OR NatMx donor plasmid	Plasmid carrying NatMx-Myc9 integration cassette (will be used for Western blotting experiment) Donor plasmid for NatMx resistance gene (for Streptothricin antibiotic Nourseothricin) (also called NTC or clonNAT). Used as yeast selection marker (responsible for inactivation of NTC by mono-acetylation of ß-amino group of the ß-lysine by Nouresothricin N-acetyltransferase the product of the *sat1* or *nat1* genes)
4	New plasmid **pMSc-MBB (NatMx-Myc9)**	To be constructed by yourself (by sub cloning NatMx / clonNAT in pBH756 using restriction enzymes).

Primers to be used for diagnostic PCR to check integration of NatMx

Primer name	Sequence 5' – 3'
Met15OutF	5'-ATTTGCGTCATCTTCTAACACCG-3'
Met15OutR	5'-TATTATGGCCTCTAGCAGCAACG-3'
NatF	5'-GACCGTCGAGGACATCGAGGTCG-3'
NatR	5'-TCGTCGGGGAACACCTTGGTCAG-3'

Sequence of gRNA (MET15-targetting sequence and gRNA scaffold)

Met15 targeting sequence	5'-GATACTGTTCAACTACACGC-3'
gRNA Scaffold	5'-GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGG-3'

Bacterial / Yeast Strains for this study

Strain	Genotype	Supplied by
NEB® 5-α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
SC K699	*MATa ade2-1 trp1-1 can1-100, leu2-3, 112 his3-11,15 ura3 Gal^{+}*	Gift from Dr Bin Hu, Department of MBB, University of Sheffield

Media for Bacterial Growth

LB broth — 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl.
pH adjust to 7.0 and sterilise by autoclaving.

LB agar — LB broth with 1.5% agar added prior to autoclaving.

Antibiotics stock solutions

Kanamycin	50 mg/ml stock (in water), use at 50 μg/ml.
Ampicillin	200 mg/ml stock (in water), use at 200 μg/ml.
Chloramphenicol	25 mg/ml stock (in EtOH), use at 25 μg/ml.
Nourseothricin	50 mg/ml stock (in water), use at 50 μg/ml.

Media for Yeast Growth

YPG-Media

This media is used to select yeast with healthy mitochondria.

Composition for YPG agar plates:

1% Bacto yeast extract,
2% Bacto peptone,
2% Glycerol and
2% Bacto agar
(Add the agar in the bottle after dissolving and making up the volume for the other nutrients)
Usually you should patch (spread cells on a small area onto the plate) in YPG and leave them overnight at 30°C.

YAPD-Media

This is a complete growth media.

a. Composition for YAPD broth:

1% Bacto yeast extract,
2% Bacto peptone,
2% D-glucose and
0.004% adeneine

b. Composition for YAPD agar plates:

1% Bacto yeast extract,
2% Bacto peptone,
2% D-glucose and
0.004% adeneine
2% Bacto agar
(Add the agar in the bottle after dissolving and making up the volume for the other nutrients)

Buffers for preparing Competent *E. coli*

TFB1: 100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM CaCl2, 15% glycerol, Adjust to pH 5.8;* sterile-filter	**TFB2**: 10 mM MOPS, 10 mM RbCl, 75 mM CaCl2, 15% glycerol, Adjust to pH 6.8 with KOH, sterile filter

Buffer for SDS PAGE and Western Blotting

Buffer required for SDS PAGE	Concentration	Required volume

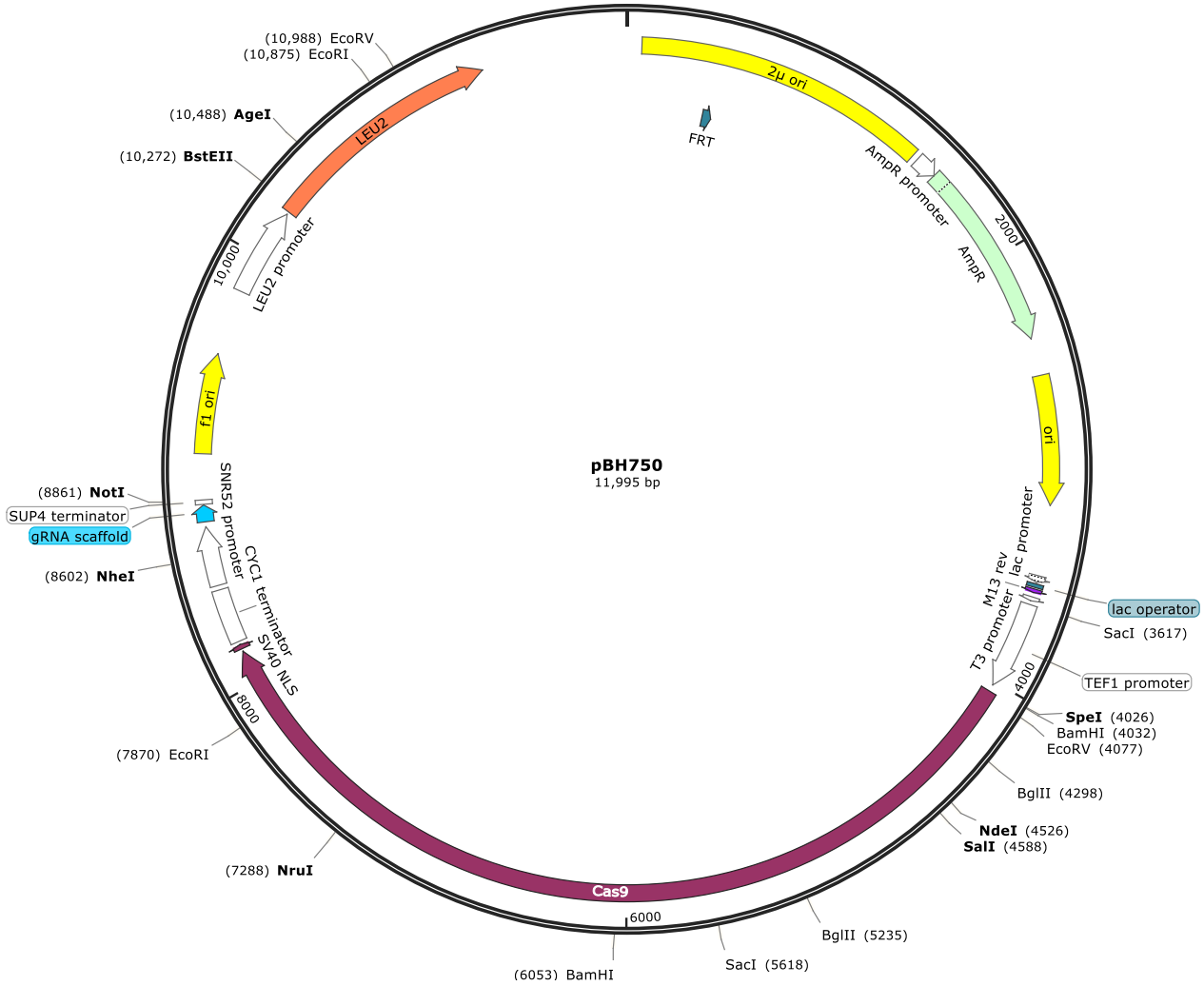
Tris-SDS-Glycine Buffer (for SDS-PAGE) Tris base, 25 mM (pH 8.3) Glycine, 250 mM, SDS, 0.1%	A 5X stock can be made by dissolving 15.1 gm of Tris base and 94 gm of Glycine in 900 ml of H2O. Then, 50 ml of a 10% (w/v) stock solution of electrophoresis – grade SDS is added, and volume is adjusted to 1000 ml with H2O.	1 Lit
SDS-PAGE sample buffers (2X) 0.09 M Tris·Cl, pH 6.8 20% glycerol 2% SDS 0.02% bromophenol blue 0.1 M DTT	2X or 5X	10 ml
Coomassie staining solutions 0.05% (w/v) Coomassie Brilliant Blue R-250*; 40% (v/v) ethanol; 10% (v/v) glacial acetic acid. For 1 litre, dissolve 500 mg Coomassie Brilliant Blue R-250 in 400 ml 100% ethanol. Add 100 ml glacial acetic acid and water to 1 litre. Filter before use.	1X	1 Lit
De-staining solution 25% (v/v) ethanol; 5% (v/v) glacial acetic acid; 70% water	1X	2 Lit

Buffer for Western Blotting

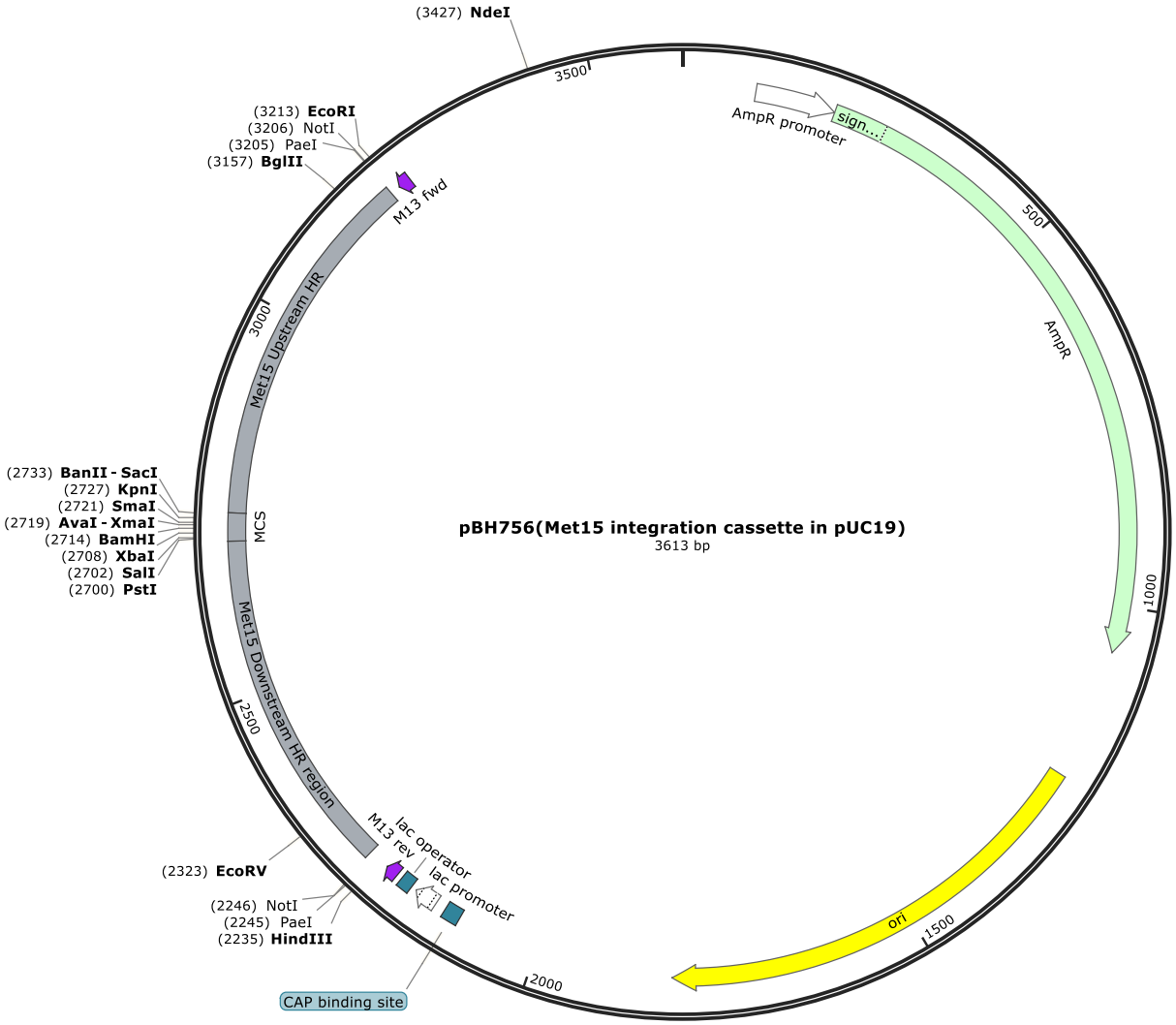
Buffers required (for Western Blotting)		
	Buffer	**Composition**
1	**PBS:**	(pH 7.5) 11.5 g di-sodium hydrogen orthophosphate anhydrous (80 mM), 2.96 g sodium di-hydrogen orthophosphate (20 mM), 5.84 g NaCl (100 mM). Dilute to 1 liter dH2O. Check pH.
2	**T-PBS:**	0.1% of Tween-20 in PBS (it could be tried between 0.005-1%)
3	**Blocking Buffer:**	1% BSA or 5% Bloto/ Marvel in T-PBS
4	**Towbin Transfer Buffer** for SDS-proteins using nitrocellulose (with methanol) or ZetaProbe membrane (without methanol);	25 mM Tris, 192 mM Glycine (20% methanol), pH 8.3 Dissolve 3.03 g Tris and 14.4 g glycine in dH2O (add 200 ml methanol); adjust volume to 1 liter with dH2O. **Do not add acid or base to adjust the pH**.
Water and chemicals used for buffer preparation should be of high purity.		

Reagents for bacterial cell lysis
Bug Buster Protein Extraction Reagent
Benzonase / DNAase (Novagen)
DTT (Optional)
PMSF in Isopropanol or DMSO (Optional)
Antibiotic stock solution e.g. Ampicillin

Plasmid maps



Plasmid maps



Plasmid maps