VRIJE UNIVERSITEIT BRUSSEL

INTERUNIVERSITY PROGRAMME MOLECULAR BIOLOGY (IPMB)

GENERAL PRACTICAL COURSE

TRAINING MANUAL

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1.0 INTRODUCTION

1.1 BACKGROUND

This training manual introduces first year Interuniversity Programme Molecular Biology (IPMB) students to basic/routine laboratory practices and molecular techniques. The training is hands-on practical demonstration. Therefore, to master the concepts, students are advised to pay attention and participate actively in all practical sessions. It is important to carefully follow procedures in this manual at all time during the experiments. To understand the theory behind each experiment, **read the manual in advance of a practical class**.

IMPORTANT: Your punctuality for practical (time management), motivation, attendance and participation in all practical will earn you Marks. Late coming (for **practical** or **seminar)** and failure to submit your **written report** in time will lead to loss of Marks**!**

1.2 ALLOCATION OF COURSE MARKS

IPMB general practical course will be examined as follows:

1.2.1 General report

This section will contribute 40% of the total marks.

General practical report should be written individually excluding the major experiment that will be reported in journal article format.

Outline of general report:
Introduction: Write a short background of the experiment done (i.e. what is known of the topic in few lines) and objective (s) of the experiment.

Methodology **(Materials and Method)**: For this section, do not copy the whole protocol but to give a summarized description of the protocol and the principle behind.

Result section: Show only relevant pictures, graphs or tables with appropriate labels (legends) and in few words describe the result (as they appear in the picture, table or graph).

Discussion and conclusion: Here briefly explain your results while referring to other authors work (pointing at deviation from or agreement with the author you cited). Then give relevant conclusion of your observation basing on your opinion.

References or Bibliography: These are lists of citations made in the text.

Report writing tips report:

- Outline the entire report into section and then write a draft
- Note all results and any deviation in procedure other than in the manual. Always consult your note book and training manual
- Discuss within your group
- Seek help from other groups or with the course instructor
- Study and organise results obtained from each experiments immediately to avoid confusion during the writing.

1.2.2 Journal article format report

This section will contribute to 30% of the total marks

Each practical subgroup will have to write one report in the format of journal article. Choose one of the two experiments (*outlined below*) for this report.

Report any of these major experiments in article format:

1) Cloning Nanobody gene
2) Expression of Nanobodies

Your article should have a **Title, an Abstract, Introduction, (Materials and Methods), Results, Discussion, Conclusion, and References**

*****Consult a journal article provided to guide your writing*****

1.2.3 Seminar

This section will contribute to 20% of the total marks.

Seminar is a presentation organized at the end of the practical training. Students, working in pairs, choose a topic on a modern technology or a method in Molecular biology and submit it for vetting. Once passed and the two workmate prepares a 15 min power point presentation (with additional 5 min allocated for questions from the audience). The presentation should be structured according to the guide lines listed below:

Expectation:

- General introduction of the topic
- Show the principle
- Brief description of operation (only if applicable)
- Show application (s) in science
- Be able to answer questions from the audience

1.2.4 Practical course test

This section will contribute to 10% of the total marks.

These are spot tests done every week during the practical. Questions are set from the practical topics which have been taught.

1.3 LABORATORY SAFETY

Laboratories have some materials which may be hazardous (or potentially harmful to your health), and equipments that are expensive and delicate. This section, therefore, provides you with basic concepts that will help you navigate safely in a laboratory premises. Following these guiding principles will avoid occurrence of accident.

1.3.1 Strict laboratory rules

- No eating, drinking or smoking in the lab.
- Wear lab coat and gloves while in the lab.

- Wearing open-toe shoes and shorts are forbidden in the lab.
- Do not pipette with your mouth
- Read instructions carefully before proceeding with experiments.
- Ask the instructor if there is something that you do not understand.
- Be responsible for your own mess (when you made it dirty, please clean it unless it is hazardous material which requires special attention!!!).
- Do not do something that you think to be unsafe.
- In case of fire out break call the emergency phone number (s) available in the laboratory

1.3.2 Using lab equipments

- Get a demonstration of its use.
- Turn off equipment after use, clean and ensure safe storage.
- Do not change settings unnecessarily.
- Do not ignore equipment alarm or flash lights, respond to it immediately.
- *Centrifuge is very expensive*. To avoid mishaps with centrifuge during or after centrifugation; balance the tubes, fill tubes until 1-2 cm from the top to avoid spillage, close the lid of the machine, clean it up every time, do not use cracked tubes for centrifugation, stick to the preferred centrifugation speed and time.

1.3.3 Waste disposal

- *Biological material*: All pipette tips, tubes, agar plates, and anything that touches a solution of bacteria or DNA should be deposited into a special waste container or placed in orange *Biohazard* bags. These wastes will be autoclaved and disposed appropriately.
- *Chemicals*: Discard waste in appropriate containers. Waste should be disposed off in a labeled container, not in the trash or sink.
- *Glass*: Sharp objects and broken glass must be placed in boxes labeled for that purpose.
- *Plastic ware*: Disposable pipettes and micropipettes tips should be placed temporally in special containers in your work place. Once full, place them in special containers. **Ask the instructor**.

1.3.4. Good laboratory practice (GLP)

- Take notes, record experimental procedures and results in your notebook before leaving the laboratory otherwise you are bound to forget.
- Read protocol before starting the experiment.
- Label and store your samples appropriately.
- Before leaving the laboratory check that your work place is organize and clean.
- All solutions and everything stored in an incubator, refrigerator, etc. must be appropriately labeled with a name and date.
- Glass and plastic ware must be scrupulously cleaned with soap after use. All labels should be removed, and the glassware should be placed in the dirty dish bin.

2.0 PRACTICAL SESSIONS

2.1 PIPETTING TECHNIQUE

Measuring very small volumes is routine in molecular biology laboratory. Therefore, good results in experiments will depend on your ability to accurately measure small volumes of solution using micropipettes. The two most prevalent units of liquid measurement are the milliliter (ml) and the microliter (µl). Micropipettes are available in many different models and volume range. They can take volumes of up to 1, 10, 20, 50, 100, 200 and 1000 µl.

1ml = 0.001 liter or **1000 ml** = 1 liter; **1 µl** = 0.001ml or **1000µl** = 1 ml; **1 µl** = 0.000001 liter or 1 000 000µl = **1 liter**

2.1.1 Drawing and dispensing sample with micropipette

- Take the right micropipette for a desired volume (fig. A).
- Dial the amount into the window (fig. B).
- Fit the micropipette end with a right pipette tip
- Hold the pipette in one hand and the open tube with the other hand.
- Push down the plunger to the first stop (1) and hold it in this position. Do not pass the first stop!
- Dip the tip into the solution. Do not touch the bottom of the tube with the tip and do not touch the solution with the pipette (fig. C).
- Slowly release the plunger (2) taking fluid into the tip.
- Take the new tube and touch the wall with the micropipette tip (fig. D).
- Slowly push down the plunger first to the first stop (1) and then to the second stop (3). Hold the plunger in this position while removing the pipette out of the tube!
- Remove the tip from the pipette and place it in the appropriated waste container!

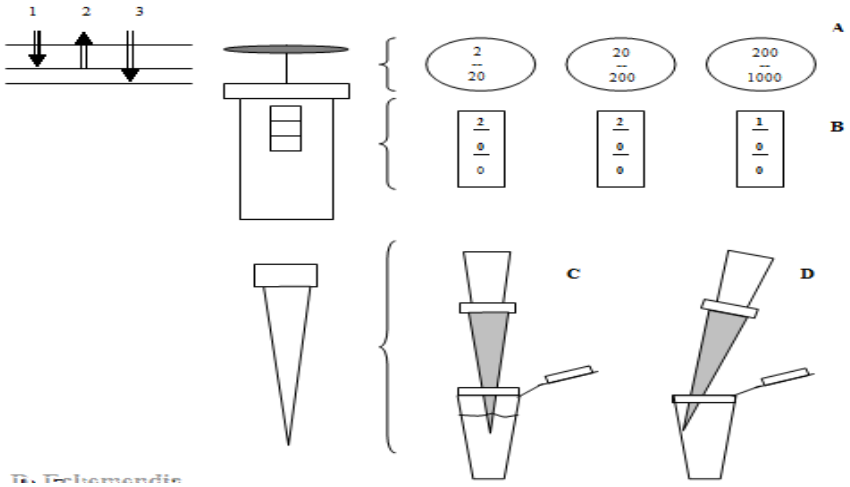


Figure 1: Using micropipette to draw and dispense solution.

Exercise

This laboratory activity will make you familiar with the pipetting techniques. You will find at your working place three different volume range micropipettes: P-20, P-200 and P-1000. The range of volume you can take with these pipettes is:

- P-20 (from 2 to 20 µl) – use yellow tips!
- P-200 (from 20 to 200 µl) - use yellow tips!
- P-1000 (from 200 to 1000 µl) - use blue tips!

a) Complete the following conversions (between ml and µl):

1- 1 µl = _____ml
2- ___ µl = 1.5 ml
3- 100 µl = ____ ml
4- ___ µl = 0.06 ml
5- 250 µl = ____ ml
6- ___ µl = 0.003 ml

b) Which pipette will you use to take the following volumes (P-20, P-200 or P-1000)?

1- 15 µl _____
2- 560 µl _____
3- 120 µl _____
4- 35 µl _____
5- 180 µl _____
6- 840 µl _____

c) How will you set the following pipettes to take the desired volume? Please label (with pencil) the window of each of the micropipettes in the picture (left to right) to correspond with the following volumes: 18 µl, 50 µl, 580 µl, 15 µl, 25 µl and 250 µl.

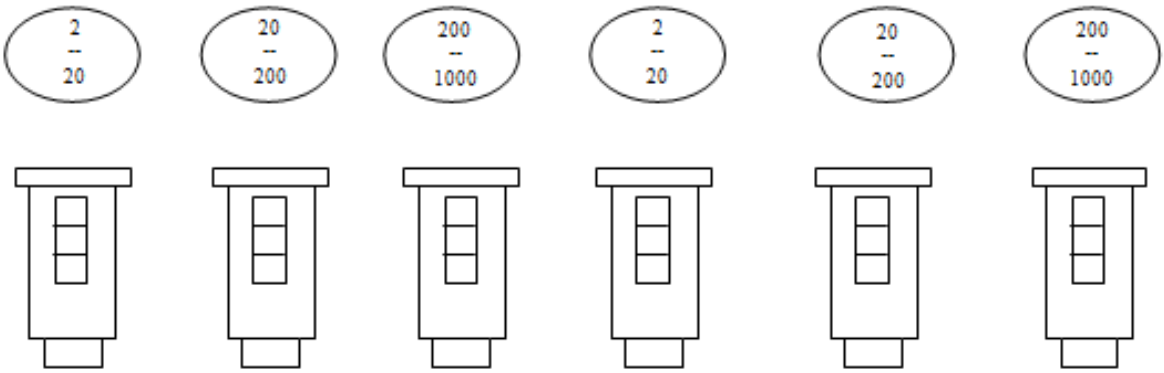


Figure 2. Windows of micropipettes of different sizes

c) Practicing with micropipette (please read the general remarks bellow): First check that you have the right pipette, then dial the desired volume and finally push the proper-size tip to the end of the pipette. Take the following volumes of water and dispose them into eppendorf tubes.

5.5 µl	335 µl	25 µl	650 µl	85.5 µl
155 µl	25.5 µl	40 µl	190 µl	250.5 µl

General Remarks

- When using a micropipette first apply the tip.
- Set pipette volume only within the range specified for the pipette!
- Always keep a micropipette in a vertical position when there is fluid inside the tip!
- Be careful and do not allow liquid to accidentally run into the piston!
- Control the speed at which the plunger rises after taking up or ejecting fluid. If you pull up too fast, the liquid will jump up and you will get air in the tip and will probably contaminate the pipette.
- Always change tips for each new reagent you need to pipette.
- Always take care not to drop micropipette!

Remember that micropipettes are critical for your work, treat them with care!

2.2. REFRESHER CALCULATIONS (MAKING SOLUTIONS)

2.2.1 Molar solution

A molar solution is one in which 1 L of solution contains the number of grams equal to its molecular weight. 1M (molar) = 1 mole of solute/L solution.

To obtain number of mole of a compound from a given weight (g), divide that weight by relative molecular weight (rMW) of the compound.

- Example: How to make up 500 ml of 1M NaCl

Molecular weight (MW) of NaCl = 58.45, it means that you need 58.45 g of NaCl in 1L to have 1M aqueous solution. Then, to have 1M solution in 500 ml you proceed:

MW (g) x final volume (L) x final concentration needed (M) = grams needed

= 58.45 g x 1M x 0.5 L = 29.29 g; add water to a final volume of 500 ml.

2.2.2 Percent solution

'Percent weight per volume': % (w/v) = weigh the solute (g) and then add solvent until 100 ml

- Example: 1% aqueous solution of NaCl in water is made by weighing 1 g of NaCl and adding water until 100 ml mark.

'Percent volume per volume': % (v/v) = measure the liquid solute (ml) and add solvent until 100ml.

- Example: A 40% aqueous solution of ethanol is made by measuring 40 ml of ethanol and adding water until 100 ml mark.

2.2.3 Preparation of working solutions from concentrated stock solutions

Many buffers or solutions require the same components but often in varying concentrations. To avoid having to make every buffer from scratch, it is useful to prepare several concentrated stock solutions and dilute them as needed. The following formula is useful for calculating amounts of stock solution needed:

$$\mathbf{C_i \times V_i = C_f \times V_f}$$

Where: C_i = initial concentration, or concentration in stock solution.
V_i = initial volume or amount of stock solution needed.
C_f = final concentration or concentration in desired solution.
V_f = final volume of desired solution.

- Example: Prepare a 100 ml 0.05M NaOH from 1.5M solution

Ci = 1.5M, Vi = ?, Cf = 0.05M, Vf = 100 ml
0.05 x 100 = 1.5 x **Vi**
Vi = 3.33 ml of 1.5M NaOH diluted to 100 ml with distilled water.

2.2.4 Concentrated solutions

Many enzymes and buffers are prepared starting from concentrated solutions, e.g. 5X or 10X etc..… (Five times or ten times the concentration of the working solution) and are diluted in such way that the final concentration of the buffer reaction is 1X.

- Example: Prepare 100 ml 1 X solution of PBS buffer from a 10 X stock solution.

The stock solution is 10 times concentrated, then to make 100 ml 1X concentrated solution, it is necessary to take 10 ml from the 10 X PBS solution and fill up to 100 ml with water.

Exercises:

How will you proceed to?

1) Prepare 400 ml of 2.2M solution of Tris (solid). MW of Tris is 121.
2) Prepare 1.5 L of a 75 mM solution of NaCl (solid). MW of NaCl is 58.5.
3) Prepare 500 ml of 50 mM NaCl if you have on hand a 2.5M stock.
4) Prepare 500 ml of 30% ethanol (liquid).
5) Prepare 1.5 L of 10% sucrose from a 60% stock sucrose solution.
6) Prepare 0.8% of agarose (solid) in 150 ml of 1X TBE buffer that is at 5X.
7) Prepare 2 L of 1X buffer from the 5X stock solution.
8) What is the molarity of a nitric acid solution made by diluting 25 ml of 15.8 M nitric acid solution to 1.5 L?
9) Make 500 ml of a 10X TE stock (Tris, EDTA). 1X TE is 10mM Tris and 1mM EDTA. MW of Tris is 121.1 and EDTA is 187.
10) Prepare 500 ml of the following stock solution: 0.6M Tris, 60mM SDS, 1% powdered milk and 1.3M NaCl. MW: Tris = 121.1, SDS = 257, powdered milk = 49, NaCl =58,44
11) You have a 150X stock of buffer and you need 2.5 ml of 1X. How do you do this?
12) How would you make 125 ml of a 0.750M NaOH solution from 2M NaOH solution?
13) How would you make 250 ml of 0.5M of sodium carbonate (solid) in water?
14) You have a 50X stock of buffer and you need 150 µl of 1X. How do you make this?
15) You have a DNA solution that is 21.3µg/µl and you want 75µl that is 50ng/µl. How do you do that?
16) Make 200 ml of a 15mM Tris, 50 mM EDTA and 0.03M NaCl from stock solutions that are 1M Tris, 0.5 M EDTA and 5000mM NaCl.
17) How do you make a 500 ml solution that is 1.5% (w/v) NaCl, 2mM Tris, 7% (w/v) powdered milk and 0.02% (v/v) antifoam?
18) Make 20 µl of 1X restriction enzyme buffer from a 10X stock.
19) How will you make 150 µl of 1X buffer from 50X stock?
20) What would be the percent concentration of a solution made when 40g of $CaCl_2$ is dissolved in 500 ml of water?

General Remarks:

- When making solutions, be sure all glassware is clean. Rinse them at least twice with distilled water.
- Weigh solids on appropriate weighing balance for the desired weight, use correct weighing boat and spatula.
- Dissolve the solid in a beaker with about one-half of a desired volume of a solvent.
- Transfer the solution to a volumetric flask or graduated cylinder.
- Rinse the beaker with small amount of distilled water at least 3 times and add washes to the solution.
- Measure the pH if is necessary.
- Fill the flask or cylinder to the desired level.
- Pour the solution into a storage vessel and mix well.
- When working with more concentrated solutions you should take the calculated volume of the more concentrated solution to a volumetric flask or graduated cylinder and fill up to a desired volume with distilled water.

'From now, turn to the appendix **for composition of buffers we need for our practical work'**

2.3 DNA TECHNIQUES

Deoxyribonucleic acid (DNA)

- *Genomic DNA***:** Chromosomal DNA
- *Extra-genomic DNA*: Plasmid, mitochondrial and chloroplast DNA

2.3.1 Cloning Nanobody® gene

Introduction

In the early 70's following elucidation of DNA structure (by Watson and Crick) and the discovery of restriction enzyme (Arber et al.), Paul Berg of Stanford University (USA) was able to recombine two DNA fragments obtained from different organisms, a technique which became popularly known as recombinant DNA technology.

To-date using the principle of recombinant DNA technology, we can **isolate** genomic DNA (or synthesize cDNA from mRNA) of an organism (donor), fragment the DNA isolate (by **restriction digestion**) and transfer the desired fragment (usually a **gene**) into another organism (recipient/host) by a process called **transfection** or **transformation** in case of bacteria) where it will eventually get incorporated into the genome of the host. Alternatively the gene is inserted into a vehicle (vector e.g. plasmid) by a process called **ligation** to produce a **recombinant DNA molecule** prior to transfer into host cell. Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries. When the host cell divides copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place. After a large number of cell divisions a colony or clone of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; **the gene carried by the recombinant molecule is said to be cloned**. In animals and plants, the end result of the whole process leads to creation of recipient with a piece of foreign DNA (a transgenic organism).

Reasons for creating a transgenic: (a) uses a recipient organism for amplification of a piece of foreign DNA (a process called cloning), (b) to cause high expression of foreign gene in a recipient organism (recombinant expression of heterologous protein).

Applications: in the fields of medicine, agriculture, environment, food manufacturing industry and in the generation of renewable energy sources.

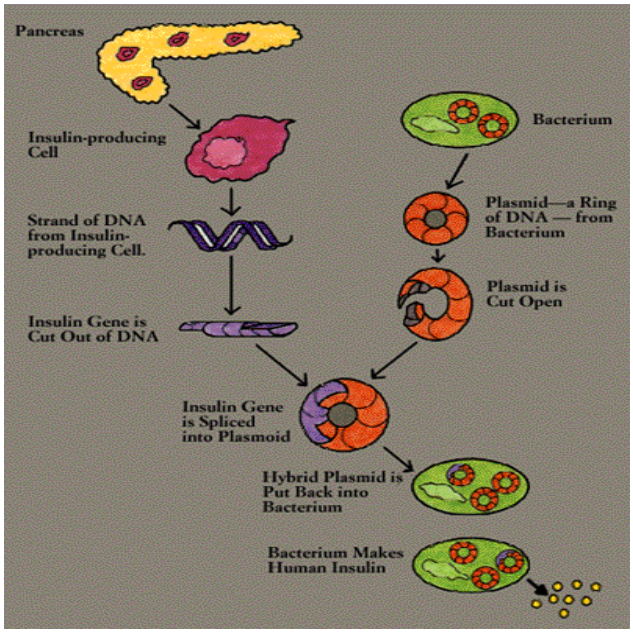


Figure 3: Isolation of insulin gene segment from pancreatic cell, splicing in expression plasmid, transformation and recombinant expression of insulin.

Outline of cloning experiment

In this experiment we are going to clone Nanobody gene into pHEN6c plasmid. First, we will begin by isolating pHEN6c plasmid. The plasmid and the Nanobody gene are then digested in parallel. The digests will be spliced (ligation) and introduced (transformation) into WK6 *E. coli*. The transformants (hosts that took up vector alone or vector with Nanobody gene) will grow on ampicillin-agar plate (selection). Transformed cells will grow on AMP-agar plate because pHEN6c plasmid contain ampicillin resistant gene (*selectable marker*) which expresses β-lactamase protein capable of destroying ampicillin. Then Colony PCR will be used to screen transformants carrying Nanobody gene. For colony PCR, a pair of primer annealing to either end of Nanobody gene will be used for amplification of the Nanobody gene insert. Amplified product will be analyzed on 1% agarose gel.

2.3.1.1 Plasmid Isolation (by alkaline denaturation)

a) Principle of alkaline denaturation

Ref: A Text book by T. A Brown, 2008 (Gene cloning and DNA Analysis: An Introduction 5th Ed) ➔*IPMB library*.

b) Materials

- Sterilized 2ml eppendorf tubes
- Sterilized micropipettes tips
- Sterilized bacterial culture tubes
- Ice in box
- Centrifuge
- Luria broth (LB) media (liquid)
- Cell re-suspension solution (P1)
- Cell Lysis solution (P2)
- Neutralization Solution (P3)
- Isopropanol (keep at -20^{0}C)
- 70% $_{(v/v)}$ Ethanol, (keep at -20^{0}C)
- Sterilized water
- Waste beaker/disinfectants
- *E. coli cell* WK 1168 containing PHEN6c

c) Recipes

P1	P2	P3
Obtain 10 mM EDTA & 50 mM Tris-HCl, add water to 100 ml. (pH 8.0) Autoclave 100 µg/ml RNase	Obtain 0.2 M NaOH & 1% $_{(w/v)}$ SDS, add dH_2O to 100 ml. (Make up fresh)	Obtain 2.55 M Potassium Acetate, add dH_2O to 100 ml pH 4.8 Autoclave

d) Protocol

i. Inoculate a single colony of *E. coli* 1168 or scrapping from glycerol stock into a 5ml of LB (supplemented with ampicillin) in a sterile 50ml tube.
ii. Incubate the tube at 37°C overnight while shaking at 250 rpm.
iii. To harvest overnight culture, put approximately 1.5 ml of the culture into an eppendorf tube. Centrifuge in the microcentrifuge at high speed for 1 min.
iv. Decant the supernatant into a waste beaker (containing disinfectant) and add the rest of the culture and centrifuge as in step 1.
v. Decant the supernatant and resuspend the pellet in 200 µl of solution P1 ("Cell Resuspension Solution"). Resuspend by pipetting up and down the pellet until there are no more solid pieces. You can also vortex. Be sure the pellet is totally resuspended before going on.
vi. Add 200 µl of freshly prepared solution P2 freshly prepared ("Cell Lysis Solution"). Close the tube and gently invert several times to mix the solutions. **Do not vortex!** Do not allow the process to proceed for more than 5 min.
vii. Add 200 µl of solution P3 ("Neutralization Solution") and gently invert several times the capped tube. Place on ice during 5 min.
viii. Centrifuge in a microcentrifuge at high speed for 15 min.
ix. Carefully remove the aqueous (upper) layer with a pipette and place it into new eppendorf tube. Be careful not to take any of the interface with the upper layer (it's better to have a low yield of clean DNA than a high yield of dirty DNA).
x. Add 0.8 volumes of isopropanol to precipitate the DNA. Incubate 15-30 minutes at RT.
xi. Centrifuge in a microcentrifuge at high speed for 15 min.
xii. Wash the pellet with 500 µl of 70 % ethanol and centrifuge in a microcentrifuge for 10 minutes at high speed.
xiii. Repeat step 10.
xiv. Pour off the last traces of ethanol. Invert the tubes on a paper towel for a couple of seconds to allow excess ethanol to run out of the tube and allow drying at 37°C for at least 30 minutes.
xv. Dissolve the pellet in 50 µl of sterile water and wait 15-30 minutes.
xvi. Determine the concentration of the purified plasmid DNA by Nanodrop™ (*also see DNA determination Method*).

The above procedure will serve whenever you do not have a kit. ***In our experiment we will use GenElute™Plasmid miniprep Kit (Sigma) which employs similar principle.***

2.3.1.2 Determination of plasmid DNA concentration

Introduction

Simple method for quantifying nucleic acid in solution is by reading the UV absorbance of the solution at 260 nm. An OD_{260} or A_{260} of 1 in a 1 cm path length corresponds to 50µg/ml for double-stranded DNA, 37µg/ml for single-stranded DNA and 40µg/ml for single-stranded RNA.

An absorbance ratio of 260 nm and 280 nm also gives an estimate of the purity of the solution. Pure DNA and RNA solutions have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. For DNA, Ratios of less than 1.8 indicate that the preparation is contaminated either with protein or with phenol. This method is not useful for small quantities of DNA or RNA (<1 µg/ml).

Principle:

The Beer-Lambert law (or Beer's law) is the linear relationship between absorbance and concentration of an absorbing species. The general Beer-Lambert law is usually written as: A=a(ʎ) * b * c, where A is the measured absorbance, a(ʎ) is a wavelength-dependent absorption coefficient, b is the path length, and c is the concentration of analyte.

a) Materials

- Sterilized eppendorf tubes
- Sterilized micropipettes tips
- Sterilized water
- Spectrophotometer
- Quartz Cuvettes

b) Protocol

1. Prepare 1 ml of a 1/100 dilution of the DNA sample in ddH_2O in an eppendorf tube.
2. Turn on the spectrophotometer and wait at least 15 min to allow it to warm up.
3. Set the wavelengths at 260 nm.
4. Set reference (zero) with distilled H_2O or the buffer in which your sample is dissolved.
5. Transfer diluted DNA sample into a **quartz cuvette** and read the absorbance.
6. Repeat the measurement at 280 nm.
7. Calculate A260/A280 ratio and DNA concentration: [DNA] in µg/µl = (50 X dilution factor X absorbance at 260 nm) divided by 1000.

***Total A_{260} units* = (A $_{260}$ absorbance) x (dilution factor)**

***Concentration (µg/ml)* = (Total A_{260} units) x (50 µg/ml)**

***Concentration (µg/µl)*= Concentration (µg/ml)/1000**

***Yield (µg)* = Concentration (µg/µl) x (total volume in µl)**

2.3.1.3 Restriction Endonuclease digestion of Nanobody gene and pHEN6c plasmid

Introduction

Gene cloning requires that DNA molecules be cut in a very precise and reproducible fashion using restriction endonuclease. Restriction endonuclease was discovered in 1950´s when it was shown that some strains of bacteria were immune to bacteriophage infection (**host-controlled restriction**). Restriction occurs because the bacterium produces an enzyme that degrades the phage DNA before it has time to replicate and direct synthesis of new phage particles. The bacterium own DNA is protected from the attack because it carries additional methyl groups that block the degradative enzyme action.

Vector must be cleaved to open up the circle so that new DNA can be inserted. Often it is also necessary to cleave the DNA molecule so that a single gene is obtained. But sometimes cleavage is done to obtain fragments small enough to be carried by the vector.

NB: if the DNA fragment produced by restriction is to be used for cloning, destroy (by short incubation at 70°C or phenol or addition of EDTA) the enzyme to prevent accidental digestion of other DNA molecules that will be added at a later stage.

a) Materials

- PCR fragment (Nanobody gene)
- 10x O-buffer (Fermentas)
- PCR clean up kit –GenElute
- Spectrophotometer/Nanodrop™
- Water bath (37°C)
- *Eco*91I (Fermentas, 10 units/µl)
- *Pst*I (Fermentas, 10 units/µl)

b) DNA digestion protocol

Digestion of Nanobody gene (PCR product)

1) **In a 1.5 ml tube (T1) add** 1.5 µg PCR fragment (cDNA) + 5µl buffer-O (Fermentas) + 1µl *Pst*I (Fermentas, 10 units/µl) + 1µl *Eco*91I (Fermentas, 10 units/µl), and top the mixture to 50 µl with distilled water.
2) Incubate at 37°C overnight.
3) Clean the digested cDNA (use PCR clean up kit -GenElute).
4) Elute in 50 µl distilled H_2O.
5) Measure concentration (Nanodrop™).

Digestion of vector (plasmid)

1) **In another 1.5 ml tube (T2) add**: Add 10 µg plasmid + 5 µl buffer-O (Fermentas) + 1µl *Eco91*I (Fermentas, 10 units/µl) + 1µl *Pst*I (Fermentas, 10 units /µl) and top with water to 50µl.
2) Digest at 37°C overnight.
3) Clean the digested vector (use PCR clean up kit-GenElute).
4) Elute PCR fragment with 50 µl distilled H_2O.
5) Measure its concentration and purity on Nanodrop™.

2.3.1.4 Ligation

This is linking DNA fragments. The process is catalyzed by DNA ligase. In a cell DNA ligase repairs single-stranded breaks in double-stranded DNA molecules which occur, for

example, during replication. The chemical reaction involved in ligation of two DNA molecules is formation of a phosphodiester bond. In genetic engineering, T4 DNA ligase (an enzyme purified from *E. coli* infected with T4 phage) is commonly used for ligation purpose.

a) Materials

- Eppendorfs
- Digested vector & PCR fragments
- Water bath
- dH_2O
- T4DNA ligase (5 units /µl)
- 10x ligation buffer

b) DNA ligation protocol

- In 1.5 ml eppendorf tube, add *50ng vector + 50ng PCR fragment* + 2µl ligation buffer + 1µl T4 DNA ligase (5units/µl). **Add ligase last!** Top reaction mixture to 20 µl with distilled H_2O
- Incubate the tube for 1-2 hr at **room temperature**.

2.3.1.5 Transformation

Introduction

Transformation is a term for artificial introduction of DNA into bacteria. Bacterium can be transformed in several ways (see Fig.5). The simplicity of bacterial structure and their fast growth make them suitable organisms for cloning, expression and study of gene of interest. After transformation, bacteria are spread on media where they can replicate, resulting in replication of the transformed foreign DNA fragment.

The first step in transformation is to make cells **competent** (ability to take up the DNA). In *E. coli* for example, chemical competence is made by incubation of the early log phase bacteria in ice with cold $CaCl_2$ solution (known as the pre-incubation step). Treatment with $CaCl_2$ is thought to cause precipitation of DNA onto the outside of the cells. After such treatment the cells are then incubated with plasmid at 42°C for 90 seconds. This is known as the "heat shock", followed by a brief incubation with non-selective growth media at 37°C to allow expression of antibiotic resistant gene prior to plating on solid media with antibiotics. Only those cells transformed with plasmids are expected to be able to growth.

Although the requirements for bacterial growth may vary, the most commonly used culture medium composition is LB. This medium contains: yeast extract (nucleic acids, cofactors, inorganic salts, and carbohydrates), tryptic digest casein (peptides and amino acids), sodium chloride, water and agar in the case of solid media.

Heat-shock transformation

i. <u>Generation of $CaCl_2$ competent *E. coli* cells</u>

a) Materials

<u>Reagents</u>

- LB medium
- Sterile ice cold 0.1 M $MgCl_2$
- Sterile ice cold 0.1 M $CaCl_2$
- Sterile ice cold 100 % glycerol

<u>Equipment</u>

- 50 ml blue caps
- Sterile 1.5 ml eppendorfs
- Shaking flask with baffles
- Cooled centrifuge for 50 ml tubes

- Fresh *E. coli* WK6 403 strain
- Laminar air flow
- Spectrophotometer

b) Protocol

1. Prepare 5 ml of LB (without antibiotics) in one sterile 50 ml tube. Inoculate the tube with a single colony of *E. coli* WK403 from a fresh plate or a scraping from a glycerol stock. Incubate the tube at 37°C, shaking vigorously at 230 rpm overnight.
2. The next morning inoculate 20 ml LB with 0.2 ml of the overnight culture
3. Grow to early log phase, OD_{600} nm in cuvette = 0.2 (-0.4 max.) (90-180 minutes)
4. After growth put the 50 ml tube containing the cell culture on ice for 5-10 minutes.
5. Pellet cells for 7 min at 3000 rpm in an Eppendorf centrifuge at 4°C.
6. Pour supernatant and gently re-suspend pellet in 10 ml sterile ice cold 0.1 M $MgCl_2$.
7. Centrifuge 7 min 3000 rpm in 4°C Eppendorf centrifuge
8. Pour supernatant and gently re-suspend pellet in 10 ml sterile ice cold 0.1 M $CaCl_2$.
9. Incubate at least 30 min on ice (better 1-2h).
10. Centrifuge 7 min 3000 rpm in 4°C eppendorf centrifuge
11. Remove supernatant and put 2 ml sterile ice cold 0.1 M $CaCl_2$ on the bacteria as well as 0.3 ml sterile ice cold 100 % glycerol. Incubate 30 min on ice.
12. Aliquot 100 µl cell suspension in three 1.5 ml eppendorf tubes labelled **T1**, **T2** and **T3** (keep tubes on ice!).

Heat-shock transformation

a) Materials

- Sterilized eppendorf tubes
- Sterilized micropipettes tips
- LB-Ampicillin-Glucose agar plates (*see appendix*)
- Water bath
- Ice
- Laminar flow
- Shaking incubator
- LB media (liquid) and 15g/l agar plate
- Sterilized water
- Centrifuge
- Glass spreader/bunsen flame/match stick
- Competent cells

b) Protocol

1. Get the three tubes of the 100 µl cell aliquots. **Tube 1**: add **~50ng** (note the volume) of purified intact plasmid DNA (for calculating transformation efficiency – overall it will help you to know if your transformation was successful). **Tube 2**: add **10 µl** of unpurified ligation (for PCR screening of colonies transformed with pHEN6 plasmid carrying nanobody DNA plus calculation of the nanobody DNA size). **Tube 3:** no DNA added (to check for contamination with cells possessing resistance to ampicillin). Gently mix by pipetting up and down.
2. Incubate tubes on ice for 30 minutes.
3. Place tubes in a warm bath at 42°C for exactly 90 seconds.
4. Put tubes back on ice for 2 minutes.
5. Add 1 ml of LB medium to each tube and place them in an incubator at 37°C for 45 minutes.
6. Plate cells on LB agar containing ampicillin. Using pipette dispense 100µL of each of transformation preparation on two LB-AMP agar plates. Do the same for control

(untransformed cells). With a sterile lazy spreader (one per plate to avoid cross-contamination) spread the liquid on agar until all is absorbed into the medium.

7. Once the plates are dry, incubate at 37°C upside down overnight.
8. From 16 to 20 hours later, count number of colonies on each of the two plates plated with cells transformed with the intact plasmid DNA. Obtain average count and use it for calculation of transformation efficiency. Spare plates transformed with ligation for colony PCR.

c) Calculation of transformation efficiency

Start with calculating the amount of intact plasmid DNA plated:

- If 0.5µg of DNA, for example, in 5µl was added to 100 µl cells then concentration of DNA in the solution = (0.5/105) µg/µl ≈ 0.0048 µg/µl. Later, 1500µl LB was added during phenotypic expression, new concentration =0.5/1605 µg/µl = $3.1x10^{-4}$ µg/µl
- After, 100µl of culture was added to each of the plates, therefore amount of intact plasmid DNA plated on each plate is calculated as:
  Per 100 µl = $3.1x10^{-4}x100 = 3.1x10^{-2}$µg (incase of dilution, take it into account)
- DNA plated per ml= $3.1x10^{-4}x1000 = 3.1x10^{-1}$µg

Transformation efficiency = $\frac{\text{Average number of colonies on LB-AMP plate}}{3.1x10^{-1}\mu g}$

2.3.1.6 Polymerase Chain Reaction (PCR)

Introduction

PCR is a procedure used for amplification of a segment of DNA from a few copies to thousands or millions of copies. The technique was made possible by the discovery of *Thermus aquaticus* (*Taq*) polymerase, a DNA polymerase that is used by the bacterium *Thermus aquaticus* that was discovered in hot springs. This DNA polymerase is stable at high temperatures, where other DNA polymerases become denatured.

PCR is valuable because the reaction is highly specific, easily automated, and capable of amplifying large amounts of sample. For these reasons, PCR has revolutionized molecular biology and is important in clinical medicine, diagnosis of genetic diseases, forensic science, and evolutionary biology. It is also used in DNA sequencing, screening for genetic disorders, site-specific mutation of DNA, and in gene cloning.

Basic steps in a PCR experiment

1) Template DNA is placed in a mixture containing the four DNA nucleotides, pair of primers flanking the target sequence, Taq DNA polymerase, buffer and water. **2)** The mixture is heated at 94°C to separate the hydrogen bonds holding the two strands of double-stranded DNA molecules together a process called **denaturation**. **3)** The mixture is cooled down to 50-60°C allowing the primers to **anneal** to specific target on the single stranded DNA molecules. **4)** The temperature is again raised to 74°C. This is the optimum working

temperature for ***Taq* DNA polymerase** present in the mixture. Taq DNA polymerase attaches to one end of each primer and synthesizes new strands of DNA complementary to the template DNA molecules at this step. Now we have four strands of DNA instead of the two we started with. **5)** The temperature is raised back to 94°C. The double-stranded DNA molecules, each of which consists of one strand of the original molecule and one new strand of DNA, denature into single strands. This begins a second cycle of denaturation-annealing-synthesis at the end of which there are eight DNA strands. By repeating the cycle 25 times the double stranded molecule that we began with is converted into over 50 million new double-stranded molecules, with each one of them being a copy of the starting molecule delineated by the annealing sites of the two primers.

PCR reaction is performed in a thermocycler machine, which is a programmable heating block that cycles between melting, annealing and polymerization temperatures. While a very powerful technique, PCR can also be very tricky. The polymerase reaction is very sensitive to the levels of divalent cations (especially Mg^{2+}) and nucleotides. The primers designed for the reaction must be very specific for the template to be amplified. Cross reactivity with non-target DNA sequences results in non-specific amplification of DNA. Also, the primers must not be capable of annealing with each other or form a hair pin loop, as this will result in undesirable amplification product. The reaction is also limited in the size of the DNAs to be amplified; the most efficient amplification is in the 300 - 1000 bp range. Also, *Taq* polymerase has been reported to make frequent mismatch mistakes when incorporating new bases into a strand. The total error rate of *Taq* is between 1×10^{-4} to 2×10^{-5} errors per base pair.

***Objective of this experiment*:** To screen colonies transformed with pHEN6c plasmid carrying the Nanobody gene. Positive colonies will have amplification product corresponding to certain size which will be inferred from the DNA ladder run alongside and by calculation.

a) Materials

- Laminar air flow
- Thermocycler
- PCR tubes
- Oligonucelotides,
- *Taq* DNA polymerase
- Primers, 10xPCR buffer
- dH_2O
- Ice

b) Protocol

1. Make PCR **master mix** for total of 6 tubes in a single 1.5 ml eppendorf. The **master mix** (below) is for 1 PCR tube; calculate for 6 PCR tubes.

10x PCR buffer	5 µl
dNTP (10 mM total)	1 µl
FP primer (20 µM)	1 µl
RP primer (20 µM)	1 µl
Taq DNA polymerase	0.25 µl
dH_2O	41.75 µl
Total	50 µl/PCR tube

2. Dispense 50µl/tube in 5 PCR tubes, discard extra.

3. Using sterile pipette tips randomly pick 1 colony from a positive plate, 3 from ligation plates. Dip in separate tubes with master mix. Tube 5 should serve as negative control (i.e. no colony added)
4. Leave tips in the tubes for at least 10 min, then swing and remove with care (Avoid contamination of the negative tube).
5. Close the tube and transfer them to a Thermocycler programmed as below:

Program:	**Pre-cycle**	95 °C	3 min
	28 cycle	94°C	30 sec
		57°C	30 sec
		72°C	45 sec
	Post-cycle	72 °C	10 min
		4°C	"until take out"

2.3.1.7 Analysis of DNA by gel electrophoresis

Gel electrophoresis

Gel electrophoresis is a technique developed in early 1970's. The technique is used to separate and characterize a mixture of charged molecules, especially **proteins** and **nucleic acids**. Standard electrophoretic methods are based on the principle that charged molecules will migrate through a liquid or semi-solid medium when subjected to an electric field. Each molecule migrates at a characteristic rate depending on its *charge*, *size*, and *shape* resulting into distinct bands in a gel.

In gel electrophoresis, a matrix consisting of either **polyacrylamide** (for proteins and small nucleic acids) or **agarose** (for larger nucleic acids) is prepared. *The gel serves two purposes*. (a) It serves to diffuse convective currents that would result in localized heating in the matrix, which would result in irregular migration patterns. (b) It creates a molecular sieve that enhances the separation based on molecular mass.

Application of gel electrophoresis: for determination of protein and DNA molecular weight, visual analysis of protein and DNA sample purity; verification of DNA and protein concentration, detection of proteolysis, identification of immunoprecipitated proteins, first stage of immunoblotting, detection of protein modification, separation and concentration of antigenic proteins for antibody production, and separation of radioactively labeled proteins.

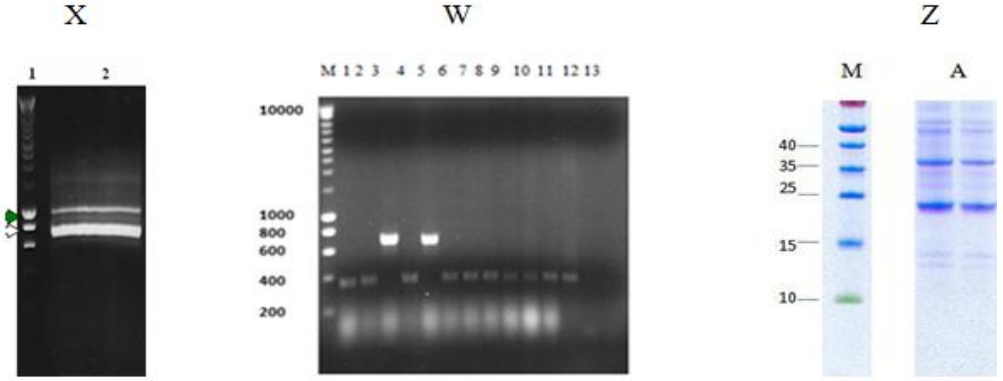


Figure 6: Resolution of DNA and protein by gel electrophoresis. Panel X and W: DNA fragments separated on 1% Agarose gel. Panel Z: Proteins expressed in *E. coli* and separated on 12% Sodium dodecyl polyacryamide gel (SDS PAGE).

***Objective of this experiment*:**

- To analyse PCR amplification of Nanobody gene fragment by electrophoresis on 1% agarose gel
- To determine the molecular weight of a DNA fragment on gel.

2.3.1.7.1 Analysis of PCR product by Agarose Gel electrophoresis

Separation of DNA molecules

The most common gel electrophoresis matrix for DNA molecules is agarose. Agarose is a long linear polysaccharide. When melted and allowed to solidify, the polysaccharide chains forms woven (mesh-like) structure with pores which DNA molecules would be forced to migrate through with the help of electric current. The rate of DNA migration is dependent on four main parameters: **(a)** the molecular size of the DNA, **(b)** the agarose concentration (Gels containing a high percentage of agarose have smaller pores than those containing a low percentage of agarose). A 0.5cm thick slab of 0.5% agarose, which has relatively large pores, would be used for molecules in the size range 1-3 kb, allowing, for example, molecules of 10 and 12 kb to separate clearly. At the other end of the scale, a very thin (0.3 mm) 40% polyacrylamide gel, with extremely small pores, would be used to separate much smaller DNA molecules, in the range1-300 bp, and could distinguish molecules differing in length by just a single nucleotide. **(c)** Conformation of the DNA (covalently closed circular DNA-*fastest*, linear DNA form-*moderate* and nicked circular-*slowest*), and **(d)** applied voltage.
Agarose gels are usually poured and run horizontally. Nucleic acids being negatively charged migrate towards the positive electrode (anode).

Table 1: Size estimation of DNA fragments in Agarose Multi-purpose gels

Agarose (%)	Range of separation of linear ds DNA fragment sizes (in kb)
0.4	2.5-30
0.8	1.0-15
1	0.5-10
1.5	0.25-5
2	0.1-2.5

Visualizing DNA molecules by staining a gel

The easiest way to observe the results of a gel electrophoresis experiment is to stain the gel with a compound that makes the DNA visible. Ethidium bromide (EtBr) is routinely used to stain DNA in agarose and poly acrylamide gels. EtBr binds to DNA molecules by intercalating between adjacent base pairs. Bands showing the positions of the different size classes of DNA fragment are clearly visible under ultraviolet (UV) irradiation after EtBr staining, so long as sufficient DNA is present. Unfortunately, **the process is very hazardous because EtBr is a mutagen and UV can cause serious burn.** Because of this other dyes are now being used.

a) Materials:

- Gloves
- Electrophoresis cuves
- Small plastic transparent gel holder (small)
- 2 black gel borders (put at each side of the transparent gel holder)
- 1% (w/v) agarose gel in 1xTBE kept at 60°C in oven.
- TBE 1x buffer from 10x stock
- EtBr
- 20 µl micropipettes + tips
- DNA loading buffer 6x (to increase the density of samples so that they sink to the bottom of slots/wells)
- Smart ladder kept at 4 °C
- Goggles

d) Protocol:

- Put on gloves.
- Pour molten 1% (w/v) agarose gel in 1xTBE (kept in the oven at 60°C) in a tray
- Add 30 µl ethidium bromide at 10 mg/ml to molten gel and mix by stirring with the pipette tip.
- Put a comb (with the teeth pointed downwards) on the gel holder.
- Remove all air bubbles using clean pipette tip.
- Let the gel solidify. It will get a milky appearance, when ready (~20 min).
- Put solid gel with tray in an electrophoresis buffer tank
- Pour 1xTBE electrophoresis buffer to completely cover the gel. Certainly air bubbles inside the slots must be avoided, because they cause cross- contamination of neighbouring slot!
- Mix 16 µl of each PCR product (and negative control) with 10 µl 3x loading buffer and load 15 µl from slot number two onwards (*this volume varies with size of the slots*)
- Load negative control into the last slot.
- Then load 5 µl smart ladder (marker) on the first slot. A black background can be put under the slots row to visualize the slots better.
- Put lid on the buffer tank, connect the positive (red) and negative (black) electrodes.
- Put the voltage at 125 V and set the time to 40 minutes. Push start.
- When finished, the current will automatically shut down.
- Shut down the power supply
- Take the gel carefully from the gel holder. Look at the gel by putting it on the UV light booth.

NB: U.V. light will damage your eyes if not protected by plastic cover lid or goggles.

2.3.1.7.2 Molecular weight determination of DNA after resolution by Gel electrophoresis

Gel electrophoresis separates DNA molecules of different length, with the smallest molecules travelling the greatest distance towards the cathode. If several DNA fragments are present then a series of bands is observed in the gel. How can the sizes of these fragments be determined? A much simpler though less precise method is used. It utilizes a standard restriction digest (ladder/molecular weight marker), comprising fragments of known size which is included in each electrophoresis gel that is run to estimate the size(s) DNA in a samples. As the sizes of the standards are known, the fragment sizes in the experimental sample can be estimated by comparing the positions of the bands in the two tracks. Although not precise this method has below 5% error, which is quite satisfactory for most purposes.

The most accurate method is to make use of the mathematical relationship that links migration rate to molecular mass. The molecular size of an unknown piece of DNA can be estimated by comparing its distance of migration in a gel with that of the standards. Plot the log of the molecular weight (in bp) of each band of standard (Y) against relative distance traveled from the well (X).

$$\text{Relative distance travelled} = \frac{\text{Distance of DNA migration from the slot}}{\text{Distance of migration of dye from the slot}}$$

Draw a line of best fit connecting the points. From this you should be able to determine the molecular size of the DNA fragment.

2.4 PROTEIN TECHNIQUES

Introduction

Central dogma: DNA➔mRNA➔Protein. One of the goals for cloning is to produce **recombinant protein** in the end. The protein obtained may be used for development recombinant vaccines, therapeutics, diagnostic tool etc. Therefore, we can identify a gene expressing protein of interest in plant or animal, insert the gene into cloning vector and introduce the gene into a bacterium or yeast where it is expressed as a recombinant protein. But sometimes we can purify protein direct from an organism without necessarily under going through cloning. Cloning is done mainly to obtain large quantity of protein which would otherwise be insufficient if we would purify from its natural source.

Scientists usually proceed to study the properties and characteristics of the protein isolate. Most of the experiments which follow protein isolation are purification, analysis of purity, determination of concentration, establishment of molecular weight, its primary sequence, its charge, its structure and other functional properties.

2.4.1 Recombinant protein expression in *E. coli* and extraction

2.4.1.1 Recombinant protein expression

Production of recombinant protein in *E.coli* requires special types of cloning vector called expression vector. This is because the vectors contain special signals which surround the gene to be expressed. These signals which are short sequences of nucleotides advertise the presence of the gene and provide instructions for the transcription and translational apparatus of the cell. The three most important signals for the *E. coli* genes are: **1)** the **promoter**, which marks the point at which transcription of the gene should start. In *E. coli* the promoter is recognized by the δ subunit of the transcribing enzyme RNA polymerase. **2**) The **terminator**, which marks the point at the end of the gene where transcription should stop. This is nucleotide sequence base pair with itself to form a **stem-loop** structure. **3)** The **ribosome binding site**, a short nucleotide sequence recognized by the ribosome as the point at which it should attach to the mRNA molecule. The initiation codon of the gene is always a few nucleotides downstream of this site.

Promoters are usually constructed in such that regulation of gene expressed by expression vector can be done. Regulation is done because of the following reasons **a)** recombinant protein may be harmful to the host (bacterium), then synthesis must be closely monitored to prevent accumulation of toxic levels **b)** continuously high level of transcription may affect the ability of the recombinant plasmid to replicate, leading to its eventual loss from the culture. The regulations of gene expression mostly occur by induction and repression. Expression can be regulated by use of regulatory chemical. In this experiment, we will use Isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce expression of Nanobody gene. Addition of IPTG into the growth medium switches on transcription of the gene inserted downstream of the *lac* promoter carried by the expression vector.

***Objective of this experiment*:**

- To express protein (Nanobody) in *E. coli* WK6 transformed with pHEN6c containing Nanobody gene (Nanobody gene).
- To extract the protein by osmotic shock.

a) Materials

- Terrific broth (TB) medium
- LB medium
- Ampicillin (100mg/ml)
- 50 ml Falcon tubes
- 1M IPTG
- Micropipettes and sterile tips
- Cells kept in glycerol stock

b) Protocol

Prepare starter culture:

1. Work under sterile conditions in a laminar air flow
2. Dispense 15 ml of LB in two 50 ml Falcon tube, one tube will be negative control
3. Add 15 µl of 1000xAmpicillin stock.
4. Inoculate a single colony obtained from LB-ampicillin glucose agar plate or scrap from glycerol stock with a sterile tip and dip in 15 ml LB with ampicillin in the Falcon tube
5. Grow culture overnight at 37 °C and 200 RPM

Expression of Nanobodies in WK6 *E. coli* cell periplasm

1. Work under sterile conditions in a laminar air flow
2. Add to the 330 ml TB media in each baffled shaker flask:
   - 330 µl of the 100mg/ml ampicillin stock solution
   - 1.5 ml of 20% glucose stock solution
   - 330 µl of 2 M $MgCl_2$ stock solution
3. Inoculate 1 ml of overnight starter culture into each of the the 330 ml TB media baffled shaker flask
4. Grow at 37 °C and 200 RPM until OD_{600} reaches 0.6 to 0.9 (± 2-4 hours).
5. Add 330 µl of 1M IPTG stock per 330 ml culture to start protein expression
6. Incubate further at 28 °C and shaking at 200 RPM overnight.

2.4.1.2 Extraction of the expressed protein (Nanobody)

a) Materials

- Sodium hypochlorite (disinfectant)
- 2M $MgCl_2$
- Tris EDTA Sucrose (TES)
- TES/4
- 500ml centrifuge bottle Bottle
- Beckman Coulter Avanti J-E centrifuge with rotor JA-10
- Spectrophotometer

b) Protocol

1. Measure turbidity (OD_{600} nm) after overnight expression (value should be between 20 and 30)
2. Harvest *E. coli* cells.
   - 330 ml culture from the shaker flask is added to 500 ml centrifuge bottle
   - Centrifuge for 8 min at 8,000 RPM and 14 °C in a 500 m rotor centrifuge

- Decant supernatant
- Reload the next 330 ml into the centrifuge bottle.
- Repeat centrifugation step
- Continue above steps until cultures have completely been harvested

3. Re-suspend the cell pellet:
    - Add 4 ml Tris EDTA Sucrose (TES) per pellet from 330 ml culture.
    - Pipette suspension up and down rigorously.
    - Ensure that suspension is free of cell clumps

4. Incubate mixture for 3 hours on ice while shaking at 200 RPM on a table top shaker

5. Later give osmotic shock which disrupts cell periplasma leading to release of its content which will contain the expressed Nb. For this perform the step below:
    - Add 8 ml TES/4 (i.e solution with 1 part TES: 3 parts distilled water water) per pellet from 330 ml culture
    - Pipette suspension up and down rigorously

6. Incubate further on ice shaking at 200 RPM for 2 hours in a table top shaker

7. Centrifuge for 30 min at 8,000 RPM in 500 ml a bottle centrifuge

8. Pipette supernatant, i.e. the periplasmic extract, into 50 ml Falcon tubes without disturbing pellet.

2.4.2 Protein purification (Affinity Chromatography)

Introduction:
Affinity chromatography encompasses diverse array of protein purification methods, all based on the interaction between a protein and its ligand. In all affinity purifications, the most effective strategy is to bind the protein of interest to a stationary phase and then to detach it with an eluent.

In this experiment we will use Immobilized metal affinity chromatography (IMAC) more specifically slurry of nickel beads for purification of Nanobody fused C-terminally to 6x histidine residues. Histidine which acts as electron donor will chelate nickel ion and whole protein is retained in the column. The bound protein is dislodged by adding imidazole solution which competes with histidine for binding to the nickel.

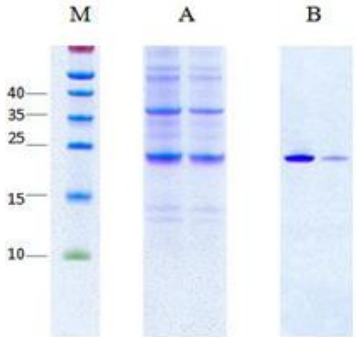


Figure 7. SDS-PAGE showing a protein sample loaded in duplicate before purification (panel A) and after purification (panel B). Purification achieved removal of unnecessary proteins from the sample.

Objective of this experiment:

- To purify the expressed 6x his-tagged Nanobody by affinity chromatography (IMAC).

a) Materials:

- His select solution (Nickle beads)
- PD-10 column
- Clamp
- Centrifuge
- 0.5 M imidazole in PBS
- Nanodrop™
- PBS

b) Protocol

1. Insert frit (the white filter) into an empty PD-10 column, and push it to plug the bottom of column
2. Re-suspend the Nickel slurry by swirling it vigorously.
3. Load 2-3 ml of the Nickel slurry solution into the column (the slurry volume depends on the amount of protein in the sample to be purified).
4. Wash the packed Nickel slurry off the storage buffer by passing stream of PBS (not less than 20 ml) into the column.
5. Load the column with periplasmic extract solution and allow it to drain by gravity. Collect 500 µl of the flow through for analysis.
6. Wash column off non specific binding proteins with atleast 20 ml of 1xPBS
6. Elute the protein by adding 500µl-1 ml of 0.5 M imidazole and seal the column outlet for 10 min. Open the outlet and collect the flowing eluate in eppendorf tube. Repeat this step twice, from the second elution onward there is no need for the 10 min incubation.
7. Measure the OD_{280} nm of the imidazole elutions on the Nanodrop™. If third elution fraction has an OD_{280}nm > 0.2, subsequent elution can be performed until OD_{280}nm < 0.2.
8. Keep fraction with highest OD_{280nm} at 4°C for SDS-PAGE and western blot analysis.

2.4.3 Analyzing protein by SDS PAGE & Western blot

2.4.3.1 SDS-PAGE

Objectives of this experiment:

- To analyse expression and purity of protein sample
- To determine the molecular weight of recombinant protein expressed.

Principle

SDS PAGE technique separates protein based primarily on their molecular weights. Proteins may be resolved under denaturing or non-denaturing conditions. Under denaturing conditions, the negatively charged detergent, sodium dodecyl sulfate (SDS), is added to the protein sample and incorporated in the gel. SDS binds to the hydrophobic portions of a protein, disrupting its folded structure allowing the proteins in the sample to exist stably in an extended conformation. As such, protein molecules will migrate irrespective of their native hydrodynamic properties. The SDS molecules that bind to a polypeptide also confer to each molecule a large net negative charge.

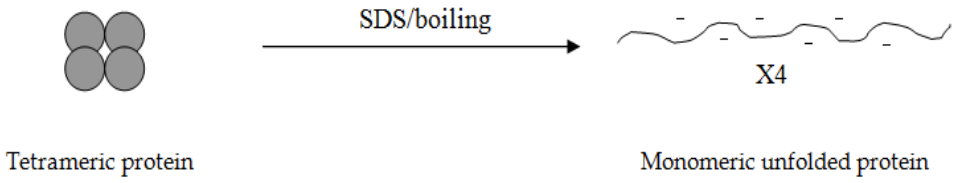


Figure 8. Protein denaturation by boiling and SDS treatment

The negatively charged molecules migrate through the gel matrix to the anode at a velocity that is roughly proportional to the logarithm of the molecular mass of each polypeptide. The speed at which the polypeptide travels is dependent on the pore size of the gel matrix and the molecular mass of the protein.

Gel electrophoresis of proteins almost exclusively utilizes polyacrylamide (*acrylamide and bisacrylamide*) as the solid matrix that functions as a sort of sieve through which the electric current transports them.

Acryl amide (%)	Range of polypeptides separation (in kDa)
6	60-200
8	40-100
10	20-70
12	20-60
15	10-40

Sometimes SDS-PAGE is performed in the presence of *reducing agents*, such as *β-mercaptoethanol*, which will reduce all intra- and inter-chain disulfide bonds in a protein. Thus, apparently single protein may exhibit a set of small fragments under reducing PAGE conditions. *When SDS-PAGE is used for investigating the subunit structure of a protein and determining the molecular mass of the polypeptides, it is essential that the electrophoresis is run in reducing conditions (see figure bellow).*

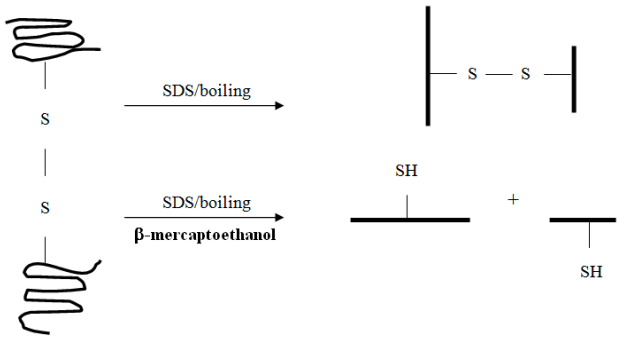


Figure 9. SDS-PAGE under non-reducing (SDS/boiling) and reducing (SDS/boiling/ β-mercaptoethanol) conditions. Under reducing condition (lower picture) the disulfide bond breaks and the protein separate into two distinct subunits.

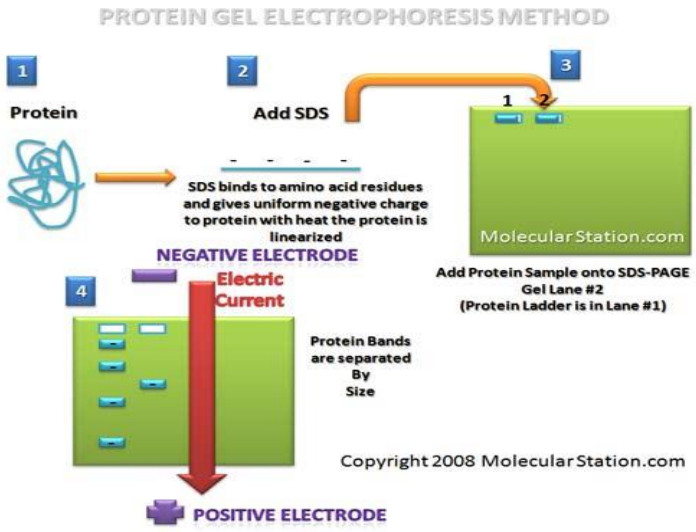


Figure 10. Illustration of SDS-PAGE

Applications of SDS-PAGE: establishing protein size, protein identification, determining sample purity, identifying inter-chain disulfide bonds (the existence of subunits), qualitative quantification of proteins, blotting applications among others.

2.4.3.1.1 Analysis of expression and purity of protein sample by SDS-PAGE

a) Materials

- Acrylamide
- Bisacrylamide
- Hydrochloric acid
- Ammonium Persulfate
- TEMED
- Dithiothreitol (DTT)
- Glycine
- Mercaptoethanol
- Molecular Weight Marker
- Eppendorf tubes
- Heat block
- Electrophoresis apparatus
- Power supply
- Gel dryer
- Rocking shaker

b) Working solutions (See preparation in appendix)

• 30% acrylamide stock solution, 100ml	• 10% ammonium persulfate, 5 ml
• TEMED	• SDS-PAGE electrophoresis buffer, 1L
• 10% SDS, 100 ml	• 5x Sample buffer, 10 ml
• Distilled water	• 1.5 M Tris-HCl pH 8.8& 1M tris pH 6.8
• 50% Glycerol, 100ml	• 1% Bromophenol blue, 10ml

c) Protocol

Pouring the Separating Gel:

1. Prepare all solutions and buffers (above).
2. Assemble the glass plates.
3. Prepare 12% gel (*refer to the table on page 30*).
4. Upon adding ammonium persulfate and TEMED, gently mix by swirling. Work rapidly at this point because polymerization is underway.
5. Using 1ml micropipette, dispense gel solution between the assembled glass plates until 0.5 cm below the level where the teeth of comb will reach. Avoid trapping air bubbles by pipetting along a spacer.

6. Layer 1-5 mm of water on top of the separating gel solution. The water will flattened the gel surface at the same time preventing it from undergoing oxidation.
7. Allow polymerization to occur (for 30-60 min)

Pouring Stacking gel:

1. Pour off water covering the Separating Gel.
2. Prepare 4 % stacking gel in a test tube (*again refer to the table on page 30*)
3. Add ammonium persulfate and TEMED, mix by gently swirling or inverting.
4. Pipette stacking gel solution onto separating gel until solution reaches top of front plate.
5. Carefully insert comb into gel sandwich until bottom of teeth reaches the top of front plate, avoid trapping air bubbles between the teeth.
6. Allow stacking gel to polymerize (~30min).
7. Remove the comb carefully.
8. Place the gel into electrophoresis chamber after attaching to the electrode assembly.
9. Add electrophoresis buffer to the inner and outer reservoir. Make sure that both the top and bottom of the gel are immersed.

Preparing and loading samples:

1. Mix 17 µl protein samples with 5 µl 5x Sample buffer in an eppendorf tube.
2. Heat at 100°C for 5 min on heating block.
3. Spin down protein solution at maximum speed for 1 minute in a microcentrifuge.
4. Introduce sample solution into well.
5. Include molecular weight standards in one outside well.

Running a Gel:

1. Attach electrode plugs to proper electrodes.
2. Turn on power supply to 150 V.
3. The dye front should migrate to 1-5 mm from the bottom of the Gel.
4. Turn off power supply
5. Remove electrode plugs
6. Remove gel plates from electrode assembly.
7. Pry apart the gel plates, the gel will stick to one of the plates

Staining gel with Coomassie Blue to visualize protein band

1. Wear gloves. Pick up a gel and transfer it into a small container containing about 20 ml of coomassie stain
2. Agitate for 5-15 minutes
3. Pour out the stain
4. Add about 50 ml coomassie destain and continue agitating for about 30 minutes. A piece of styroform can be added to absorb coomassie stain which difuses from the gel.
5. Visualize the protein bands.
6. Preserve the gel and estimate the molecular mass of your protein.

***** apply only in reducing conditions. *Handle with care, β-mercaptoethanol is very toxic for the liver!*

* Protect the solution from the light. *Acrylamide is a potent neurotoxic and is absorbed through the skin; always use gloves when handling the gel and solutions!*

Gel preparation: **(amounts for different % of one 0.75 mm gel)**

Running gel	**7%**	**10%**	**12%**	**15%**
30% Acryl/bis	2.3 ml	3.3 ml	***4.0 ml***	5 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	***2.5 ml***	2.5 ml
Water	5.1 ml	4.1 ml	***3.4 ml***	2.4 ml
10% SDS	100 µL	100 µL	***100 µL***	100 µL
10% APS	100 µL	100 µL	***100 µL***	100 µL
TEMED	5 µL	5 µL	***5 µL***	5 µL

Stacking gel	**4%**	**5%**
30% Acryl/bis	***0.650 ml***	1.25 ml
1.0 M Tris pH6.8	***0.650 ml***	1.9 ml
Water	***3.645 ml***	4.3 ml
10% SDS	***50 µL***	75 µl
10% APS	***25 µL***	20 µL
TEMED	***5 µL***	15µL

2.4.3.1.2 Protein molecular weight determination by SDS - PAGE

SDS polyacrylamide gel electrophoresis is frequently used to determine the molecular weight of a protein since migration is generally proportional to the mass of the protein. A standard curve is generated with proteins of known molecular weight, and the molecular weight of the protein of interest can be calculated.

Protocol

1. Following gel electrophoresis and staining, measure the distance of migration of the proteins as well as that of the tracking dye (bromophenol blue). Distance of migration is measured from the beginning of the separating gel to the leading edge of a protein band.

2. Calculate relative mobility (R_f) values

$$R_f = \frac{\text{Distance of protein migration from the origin}}{\text{Distance of tracking dye migration by from the origin}}$$

3. Plot the $\log_{10}$ of the known protein molecular weights as a function of their R_f

4. Draw a line connecting the points. From this you will generate equation that will help you determine the molecular weight of the protein.

Example:

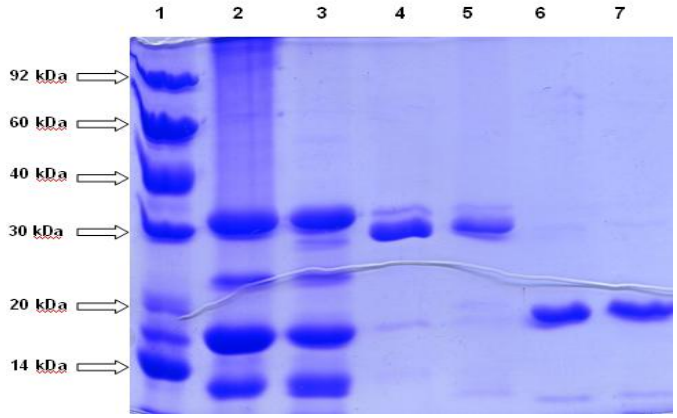


Figure 11. A 12% SDS-PAGE. Lane 1, Marker; lanes 2-7 protein sample of unknown molecular weight. Lane 4 contain purified sample and is the sample of interest.

Table 2: Values of various Relative migration front (R_f) and transformation of MW (log) of protein standards

Protein marker	1	2	3	4	5	6
Rf (X)	0.15	0.29	0.42	0.54	0.74	0.87
MW (kDa)	92	60	40	30	20	14
Log MW (Y)	1.96	1.78	1.6	1.48	1.3	1.15

R_f value of sample = 0.56.

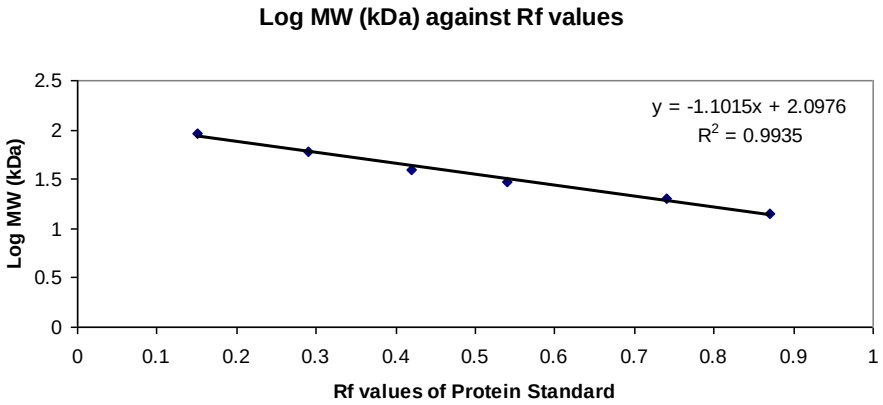


Figure 12. Graph of Log. MW (kDa) against relative mobility

Calculation

Y = -1.1015x + 2.0976

= -1.1015 (0.56) + 2.0976

= 1.48

Therefore, the molecular weight of the sample equals to the antilog of 1.48 = <u>30.3kDa</u>

2.4.3.2 WESTERN BLOT

Introduction

Western blotting is used here to confirm expression. The technique detects protein after immobilization on a matrix. It uses a monoclonal or polyclonal antibody which recognizes protein in a crude extract or a more purified form. Western blot is a very sensitive (sensitivity is up to 10 picogram with HRP/AP) technique used for identifying a single protein in a complex mixture following separation based on its molecular weight (SDS – PAGE), size and charge (non-denaturing gel electrophoresis) or isolelectric point (isoelectric focusing).

In western blotting, protein in a sample is separated by electrophoresis and transferred on blotting membrane (nylon or nitrocellulose). Once the proteins have been transferred from the polyacrylamide gel to the membrane, detection of specific proteins proceed by the use of antibodies. Prior to the addition of antibody, the membrane is coated with a blocking agent, typically a 3% solution of bovine serum albumin (BSA) in Tris-Buffered saline (TBS). Blocking prevents nonspecific binding of antibodies to the membrane. The first antibody (primary antibody) recognizes the protein of interest while the second antibody recognizes the F_c portion of the first antibody. The second antibody is coupled to an enzyme (horseradish peroxidise or alkaline phosphatase) which produces a coloured product which stains the membrane.

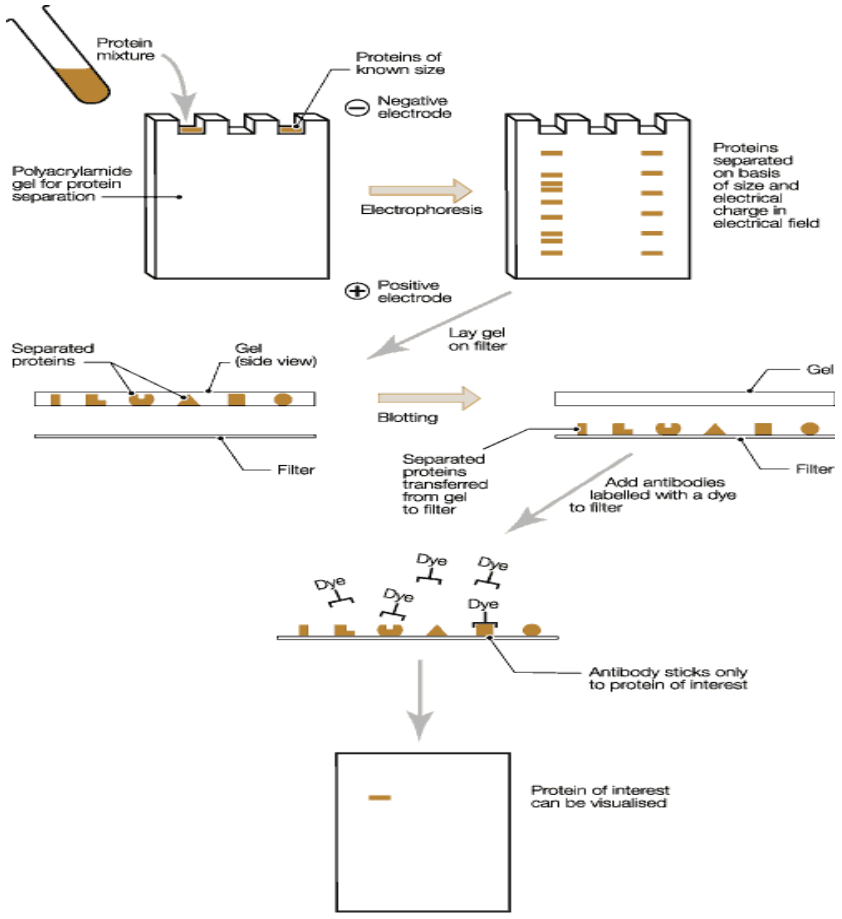


Figure 13. Illustration of western blot

*Applications of western blot***:** Epitope mapping, dot ELISA, ligand binding, antibody purification, renaturing proteins for functional assay, cutting out protein band for antibody production, improved autoradiography, structural domain analysis, protein identification: amino acid analysis and protein sequencing.

Objective of this experiment:

- To confirm expression in WK6 *E. coli*. The Nanobody expressed contain 6x histidine tail attached to the C-terminal end. His-tagged Nanobody will be identified by probing with anti-his antibody.

2.4.3.2.1 Confirming protein expression by Western blot

a) Materials

- Western blot cassette
- Transfer Buffer tank
- Frozen cooling unit
- Rocker
- Power supply (200 V, 0.6 A)
- Nitrocellulose membrane (0.2 or 0.45 µm pore size) store in dark cool place
- Glycine
- Tris base
- Magnetic stir plate
- Magnetic bar
- Whatman 3MM paper
- Shallow tray
- Gloves
- Forceps and scissors

b) Working solutions (See preparation in appendix)

- Transfer buffer, 1L
- Phosphate buffered saline (1xPBS), 1L
- Horseradish peroxidase developing reagent
- 2.5% (w/v) milk in PBS
- 0.05 % (v/v) Tween 20 in 1xPBS (PBS-T)
- 5% nonfat dry milk in PBS
- Alkaline phosphatase developing reagent
- Mouse anti-his IgG
- Anti mouse IgG HRP/AP
- BCIP solution, AP buffer, NBT solution

Protocol for western blotting

Run SDS-PAGE:

1. Prepare 12% gel, load protein sample and run as described in SDS-PAGE

Transfer protein to nitrocellulose membrane:

1. Wear gloves. Cut nitrocellulose membrane (6 cm x 8 cm) and wet in transfer buffer (30 ml – 40 ml) for 15-20 minutes.
2. Rinse buffer chamber with distilled water
3. Insert Trans-Blot electrode insert and small stir bar into buffer chamber, and add transfer buffer until half full (about 40ml)
4. Insert frozen cooling unit
5. In a shallow tray, open the transfer cassette and place a wetted sheet of Whatman paper 3MM paper on a well-soaked (in transfer buffer) filter pad on the black panel of the transfer cassette.

6. Carefully place the gel on the wet filter paper on black side of the assembly and arrange well ears and gel so that air bubbles are removed.
7. Wet the gel and carefully lay a wetted sheet of nitrocellulose on top, beginning from one side so air bubbles are removed to the edge of the gel.
8. Place a wetted sheet of 3MM Whatman paper over the nitrocellulose and roll a small test tube or pipette over the sandwich to remove any air bubbles.
9. Cover with the second well-soaked fibre pad, close the transfer cassette and slide it into the electrode insert in the buffer tank, keeping the black side of the cassette on the same side as the black panel of the electrode assembly.
10. Fill the buffer tank with transfer buffer.
11. Place entire Trans-blot apparatus on a magnetic stir plate and begin stirring.
12. Attach the electrodes
13. Set the power supply to 100 V and transfer for 1 h.

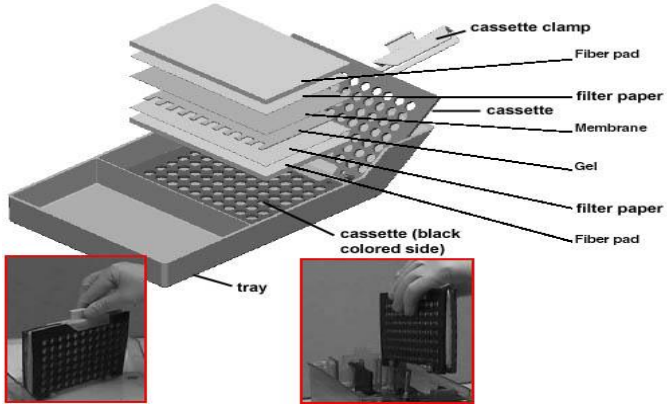


Figure 14: Assembling polyacrylamide gel and nitrocellulose membrane in a cassette prior to protein transfer (blotting)

Immunodetection:

Protocol of immunodetection with horse raddish peroxidise (HRP)/ alkaline phosphatase (AP) conjugate

1. Disconnect transfer apparatus, remove transfer cassette, and peel 3MM paper from nitrocellulose.
2. Using forceps or wearing gloves, remove nitrocellulose membrane from transfer apparatus into a small container
3. Block unbound sites on the membrane with 20 ml 5% (w/v) milk in PBS.
4. Rock the filter gently for 1.5 h.
5. Pour off blocking solution and rinse briefly (3x) with 0.05 % (v/v) Tween 20 in 1xPBS (PBS-T).
6. Add first antibody (mouse anti-his IgG antibody) diluted (1/1000) 15 ml 2.5% (w/v) milk in PBS
7. Rock gently for at least 1h.
8. Pour off first antibody solution from membrane wash (3x) with PBS-T.
9. Pour off PBS-T. Add second antibody (anti-mouse IgG HRP/AP) diluted to (1/1000) in 15 ml 2.5% milk.
10. Rock the membrane gently for at least an hour.

11. Pour off second antibody solution from membrane.
12. Rinse the membrane with PBS-T (3x)
13. ****<u>**If anti-mouse IgG HRP conjugate was used,**</u>
14. Prepare a developing reagent for HRP (*see in the appendix*) and add on the membrane
15. Rock nitrocellulose gently while monitoring development (which should occur in 5-30 mins).
16. Stop development by washing with excess distilled water.
17. Dry the membrane with adsorbent paper and store the membrane protected from light and atmosphere. Wrapping membrane with aluminium foil in note book is usually suitable.
18. Photograph or scan within a week, since the signal may fade with time or the nitrocellulose may begin to turn yellow.

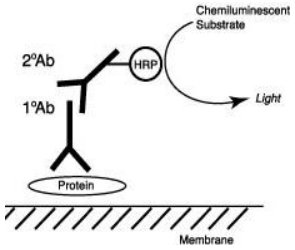


Figure 15. Indirect recogntion blotted protein by secondary antibody (2° Ab) which specifically recognises primary antibody (1°Ab).

13. ***** <u>**If anti-mouse IgG AP conjugate is used,**</u>
14. Prepare a developing reagents: AP buffer, BCIP & NBT solutions (*see in the appendix*)
15. Wash membrane for 5 mins in AP blot buffer
16. Mix developing reagent and use within 1 h: 66 µl NBT solution+10 ml AP buffer (mix well) and add 33 µl BCIP solution.
17. Pour off the AP buffer and add 10 ml developing reagent to nitrocellulose.
18. Incubate at room temperature or at 37°C to speed reaction.
19. Reaction is mostly complete within 30 mins and can be stopped by rinsing with 20mM EDTA in PBS.

<u>If using AP kit</u>: Mix 1ml 10x NBT+1ml 10x BCIP+ 8ml dH_2O in a 15ml Falcon tube, add to washed membrane and keep in the dark for at least 30 seconds and wash it immediately when colour has develop

2.4.4 Determination of protein concentration

Introduction
Although there are other methods, protein concentration can be easily determined by BCA method (Smith *et al*., 1985). This method is easy to perform and highly sensitive (up to 1 µg of protein can be detected).

Principle:
The proteins react with Cu^{2+}, which produces Cu^{+} in complex with BCA. The greenish color of the BCA is then converted into the purple color of the Cu^{+}-BCA complex. The absorbance

can be read at or near 595/562 nm. The BCA analysis gives a linear relationship ([protein] vs. OD).

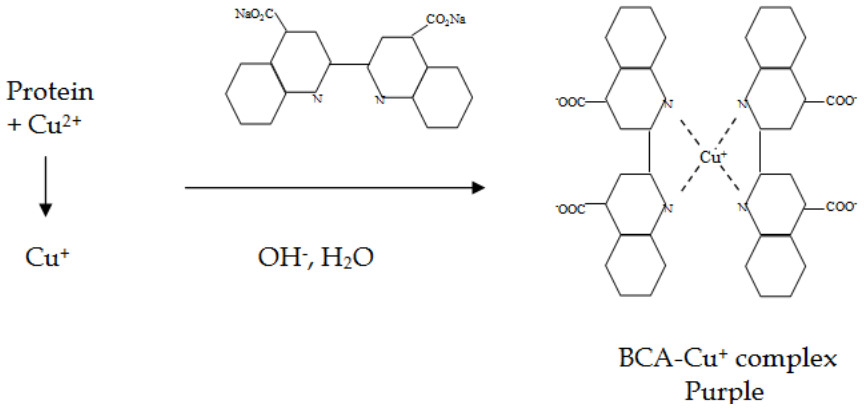


Figure 16. BCA reaction scheme

This assay can be performed in either test tubes or microtiter plates. A drawback of the BCA assay is that it is incompatible with agents such as EDTA, DTT, Mn^{2+}, mercaptoethanol, thio-urea and ammonium sulphate. When present, these interfering agents have to be removed by dialysis prior to the protein analysis.
A standard curve, from a series of protein solutions of known concentrations should be made in order to estimate the protein concentration of in protein sample solution of unknown concentration.

Objective of this experiment: To determine the concentration of purified protein (Nanobody) by Bicinchoninic Acid (BCA) Assay.

a) Materials

- Eppendorf tubes
- Micropipettes tips
- Distilled water
- Bovine Serum Albumin (BSA)
- Incubator at 37°C
- Pierce Protein Assay Kit
- 96-wells flat -bottom plates
- Microtiter plate reader (in CMIM lab)
- /Spectrophotometer

b) Recipes

Reagent A, 1 L	**Reagent B, 50 ml**
10 g BCA (1%) 20 g Sodium carbonate ($Na_2CO_3.H_2O$) - 2% 1.6 g Sodium tartrate ($Na_2C_4H_4O_6.2H_2O$) - 0.16% 4 g Sodium hydroxide (NaOH) - 0.4% 9.5 g $NaHCO_3$ (0.95%) Add distilled water to 1L. If needed add NaOH or solid Na HCO_3 to adjust pH to 11.25	2 g $CuSO_4.5H_2O$ (4%) Add distilled water to 50ml

Stability*: Reagents A* and *B* is stable for at least 12 months at *RT*. Standard Working Reagent: 50 volume reagent A and 1 volume reagent B stable for a week

c) Protocol

1. Prepare a set of protein standards of known protein concentration. From 2 mg/ml of BSA stock, prepare 320 µl solution containing: 40µg/ml, 36 µg/ml, 32 µg/ml, 28 µg/ml, 24 µg/ml, 20 µg/ml, 16 µg/ml, 12 µg/ml, 8 µg/ml and 4 µg/ml in 1.5 ml eppendorf tubes. Make dilutions in PBS buffer because it is desired to use the same buffer as the one containing your samples.

2. Prepare 2-fold serial dilutions of sample in 9 eppendorfs. Starting from a 640 µl sample get 320 µl and transfer to first eppendorf with 320 µl PBS mix up and down, take fresh pipette tip and transfer 320 µl to the next eppendorf tube with same volume of PBS. Continue until the last until the last tube where you mix and discard 320 µl as indicated in the diagram of microplate (*see the plate lay out below for your guidance*)
3. Fill the wells of a flat-bottom microtiter plate by pipetting 150 µl of each standard and sample in duplicate into the wells. Do not forget to include a blank lacking protein (PBS only).

	1	2	3	4	5	6	7	8	9	10	11	12
A	40	36	32	28	24	20	16	12	8	4	PBS	BSA µg/ml
B	40	36	32	28	24	20	16	12	8	4	PBS	
C	Conc sample	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	PBS	2-fold sample dilution
D	Conc sample	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	PBS	
E												
F												
G												
H												

4. Prepare the working reagent (WR) by mixing reagents A, B, and C in the proportion of 25:24:1. Add 150 µl WR in all the working wells (BSA and sample wells). Total volume of WR required should be determined in advance.
5. Place the plate at 37°C for 2h.
6. Read the **OD_{595}nm** in a microtiter plate reader and copy the data in your notebook.
7. With microsoft excel programme, plot the protein standard concentration (X-axis) against absorbance at 595 nm (Y-axis). Derive the best linear fit (y = mx + b) where y is the absorbance at 595 nm, m is the slope of the line, b is the y-intercept, and x is the concentration of bovine serum albumin in µg/ml.
8. Calculate the concentration of unknown samples using equation of straight line derived.

Note: Values obtained for samples should lie within the range obtained with the BSA standards and when a second plate has to be made, the standards have to be repeated in the same way. Always dilute sample to ensure they will lie within the range obtained with the BSA

Example:

BSA(mg/ml)	2	1.8	1.6	1.4	1.2	1	0.8	0.6	0.4	0.2	0.1
Mean A_{595} - Blank	0.54	0.5	0.46	0.42	0.39	0.31	0.25	0.2	0.13	0.08	0.04

Sample dilution	Conc.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Mean A_{595} - Blank	2.18	1.59	0.97	0.52	0.31	0.18	0.11	0.08	0.02	0.01

Absorbance at 1/8 dilution (0.52 nm) was used to calculate protein concentration in the sample because it is the first to fall within the range of the standard.

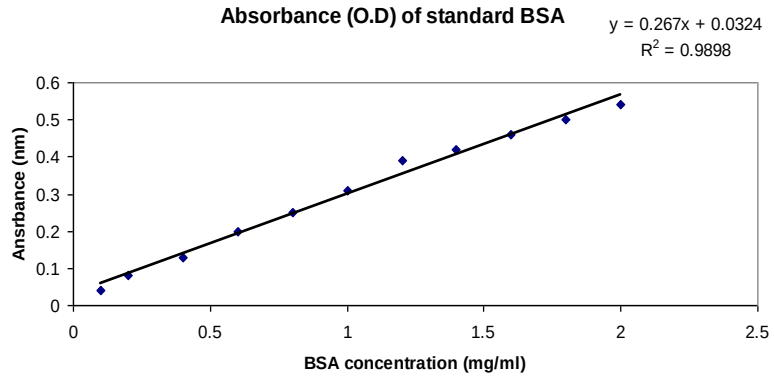


Figure 17. BCA Assay Standard Curve

Therefore, calculation to obtain protein concentration in the sample will proceed as below:
y = 0.267x + 0.0324
0.52 = 0.267x + 0.0324
0.4876 = 0.267x
x = 1.826
Concentration at 1/8 is 1.826 mg/ml, then concentration of undiluted protein equals to
8 (i.e. dilution factor) x 1.826 = 14.608mg/ml

2.5 ENZYME LINK IMMUNOSORBENT ASSAY (ELISA)

Introduction

ELISA is a useful and powerful diagnostic method. This method combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily assayed enzyme.

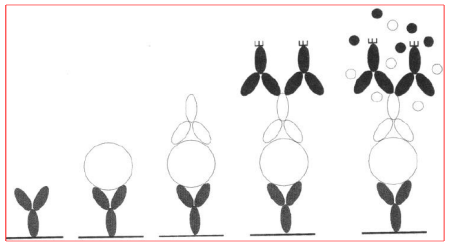
The ELISA can be used to detect the presence of antigens provided we have its antibody and vice versa. Depending on the kind of ELISA you perform, you can, for example, measure the antigen affinity by competitive assay using antibody and a competing substance or determine the strength and/or amount of antibody response in a sample.

In this experiment we are going to test sera provided for presence of *Trypanosoma congolense* antigen by Nanobody (Nb) sandwich ELISA

Stages of classical indirect sandwich ELISA:

I-Ab + Ag + AB + Anti-AB*E + S → Read
W W W W

Stage (i): Passive adsorption of Ab.
Stage (ii): Wash.
Stage (iii): Addition of antigen.
Stage (iv): Wash.
Stage (v) Addition of antibody from different species vs antigen.
Stage (vi): Wash.
Stage (vii): Addition of enzyme-labeled antispecies (directed against AB)



Ag = Antigen, **Ab** = Antibody directed against antigen, **AB** = Antibody from another animal species as compared to Ab, **Anti-Ab** = Species-specific antiserum, e.g., if Ab was raised in a mouse, anti-Ab is anti-mouse serum, ***E** = Enzyme attached to a particular antibody (Anti-Ab*E = anti-species antibody linked to enzyme, e.g., anti-mouse), **I** = Solid-phase to which reagent is attached passively, **S** = Substrate addition and color development, **+** = Addition of reagents and incubation, **Read** = Measurement of color using spectrophotometer, **W** = Separation of bound and free reagents by washing.

Material for our indirect sandwich ELISA using Nanobody other then Antibody:

Ag = trypanosome antigen, **Ab**= histidine-tagged Nanobody (Nb), **AB**= biotinylated Nb, **Anti-Ab*E** conjugate = Streptavidin horse-radish peroxidase (HRP) conjugate because streptavidin binds to biotin with high affinity and **S**=Tetra-methylbenzidine (TMB) substrate.

Protocol

1. Dilute Nb4741 to 0.02µg/mL in 1xPBS and add 100 µL into six wells (from C2-C7) on Nunc plate. To wells C8 and C9 add PBS only. Incubate overnight at 4°C.
2. In the morning wash off unbound Nb (x3) with PBS-T. Blocked with (250-300) µL 5% milk (w/v) in 1xPBS for 2hrs at room temperature.
3. After coating wash (x3) like in step (2).
4. Add 100 µl sera. Add positive control serum to wells C2 and C3; test serum to C4 and C5; and negative control serum to C6 and C7. Fill C8 and C9 with 5 % milk.
5. Incubate plate for an hour at room temperature and wash (x3).
6. Add 100 µL of biotin labelled Nb4741 (at 0.02µg/ml in 5% milk) in all the wells.
7. Incubate plate at room temperature for 1 hr.
8. Wash (x4).
9. Then add to all wells 100 µL of streptavidin-HRP conjugate diluted to 1/1000 in 5 % (w/v) milk.
10. Incubate for 1 h at room temperature.
11. Washed (x4).
12. Add 100 µL 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate.
13. Stop reaction by adding 50 µL 1MH_2SO_4 after 15-30 min of color development.
14. Using spectrophotometer, read absorbance at 450nm.

Question: Calculate the cut-off OD and interpret the test result.

APPENDIX

1.1 Culture media and reagents/buffers

Medium	Recipes	Reference or source
LB (1L)	10 g Tryptone/ Peptone (Duchefa Biochemie), 5g Yeast (Duchefa Biochemie), 10g NaCl (Fisher scientific), filled with distilled water to 900ml, adjust pH to 7.0 with 10M NaOH, adjust volume to 1L with dH_2O autoclaved and stored at room temperature	Sambrook and Russell (2001)
TB (5L)	11.5g KH_2PO_4 (Merck), 82g K_2HPO_4 (Merck), 60g Tryptone/Peptone (Duchefa Biochemie), 120g Yeast (Duchefa Biochemie), 20ml 100%Glycerol (Duchefa Biochemie), filled with distilled water to 5L , autoclaved and stored at room temperature	Sambrook and Russell (2001)
Ampicillin glucose LB agar plate	In 1000ml scot duran bottle, add 15g Bacto/Micro- agar, 25g LB broth high salt (10 g tryptone, 10 g NaCl & 5 g yeast) and top with dH_2O until 900ml then autoclave. Allow the medium to cool to 55°C. Add 100ml autoclaved glucose 20% & 1ml of a 100mg/ml ampicillin solution. Mix by rolling the closed bottle back & forth in a laminar flow. Pour ±20ml into sterile plates. Allow the medium to solidify (±1h) and mark plates with black and red. Store the dishes at 4°C in an inverted position. Before use, incubate the plate at 37°C in the inverted position for 1 h to remove condensation within the plate.	CMIM protocol

Reagents/biological buffers	Recipes	Reference or source
20% (w/v) D-Glucose solution	20g D-Glucose (Duchefa Biochemie) in 100ml distilled water, autoclave and store at room temperature.	Sambrook and Russell (2001)
2M $MgCl_2$ (50ml)	20.33 g $MgCl_2$ (Merck) in 50ml water and autoclaved	Sambrook and Russell (2001)
1M $CaCl_2$ (1L)	Dissolve 219.08g $CaCl_2$-$6H_2O$ in 800ml H_2O. Adjust volume to 1L with water and sterilize by autoclaving. Store at RT	RAS LAB FAQs
1M isopropyl-ß-D-thiogalactoside (IPTG) solution	11.92g IPTG in 50 ml distilled water, filter with 0.2µm Gyrodisc CA-PC 30mm (Orange Scientific), aliquot and store at -20°C.	Sambrook and Russell (2001)
0.5 M Na_2EDTA (pH 8.0) (1L)	Dissolve 186.12g Na2EDTA-$2H_2O$ in 800 ml H_2O; Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with (~20g NaOH	RAS LAB FAQs

	pellets) divide into aliquot and store at RT	
100mM EDTA	1.86g EDTA (Duchefa Biochemie) in 50ml water and autoclave	Sambrook and Russell (2001)
1M Tris-HCl (pH 8.0)	121.1g Tris (sigma), mix and solubilised 900ml distilled water, adjust to correct pH with 37% HCl solution, fill with distilled water to 1L, autoclave and store at room temperature.	Sambrook and Russell (2001)
TES (1L)	171.15g sucrose (Duchefa Biochemie), 5ml 100 mM EDTA (Duchefa Biochemie), 200ml 1M TrisHCl (pH 8.0), Keep in brown bottle at 4°C	Sambrook and Russell (2001)
TES/4 (1L)	750mls distilled water and 250mls TES (3:1), store in brown bottle at 4 °C	Nanobody expression protocol (CMIM Laboratory)
10x Phosphate buffered saline (PBS) (1L)	2g KCl, (Merck), 80g NaCl (Fisher Scientific) 2.4g KH_2PO_4 (Merck), 26.8 g Na_2HPO_4 (Merck), mix and solubilised 900ml distilled water, fill with distilled water to 1L, autoclave, filter with 0.2µm Gyrodisc CA-PC 30mm (Orange Scientific) and store at 4 °C/RT.	Sambrook and Russell (2001)
0.5M imidazole in PBS (Prepared fresh)	3.5 g imidazole (sigma), mix and solubilised in 90ml PBS adjust pH to 7.5 with 37% HCl (Merck), bring to 100ml with PBS and with 0.2µm Gyrodisc CA-PC 30mm (Orange Scientific) and store at 4 °C.	Sambrook and Russell (2001)
Antibiotics	**Preparation**	**Reference or source**
50 ml Ampicillin (100mg/ml)	5g Ampicillin (Duchefa Biochemie) in 70% Ethanol (35ml ethanol+15ml dH_2O), filter with 0.22µm filter & store at -20 °C	Sambrook and Russell (2001)

1.2 Buffers/reagents for DNA, protein electrophoresis and western blot

DNA	**Recipes**	**Reference or source**
10x TBE (1L)	108g Tris , 55g Boric acid in 900 ml H_2O and 40ml 0.5 M Na_2EDTA (pH 0.8) adjust volume to 1L, store at RT	Sambrook and Russell (2001)
1% Agarose gel	5g Agarose (Invitrogen) in 500mls 1xTBE , boil until it dissolves, keep at 50°C	Sambrook and Russell (2001)
Ethiduim bromide (10mg/ml)	1g ethiduim bromide and dissolve in 100mls of water and store at room temperature	Sambrook and Russell (2001)
6x DNA loading buffer (100ml)	0.25%bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400; Pharmacia) in dH_2O store at RT	Sambrook and Russell (2001)

Protein	Recipes	Reference or source
1x Transfer buffer (1 L)	5.8 g Tris Base, 1.9 g Glycine, 0.37 g SDS in 200ml methanol adds dH_2O to make 1 L, Store at 4 °C.	Bollag *et al.* (1996)
20xMES buffer (1L)	195.2g [2-(N-Morpholino) ethanesulfonicacid] monohydrate (MES) (Duchefa Biochemie), 121.2g Tris (sigma), 20g Sodium dodecy sulphate (SDS) (BDH), 6g EDTA (Duchefa Biochemie) and bring to 1L with distilled water.	Sambrook and Russell (2001)
2M Tris-HCl (pH 8.8), 100 ml	24.2 g Tris base, add 50 ml dH_2O, add conc HCl slowly until pH 8.8 (allow cooling to RT pH ↑) top with dH_2O to 100ml	Bollag *et al.* (1996)
1M Tris-HCl (pH 6.8), 100 ml	12.1 g Tris base, add 50 ml dH_2O, add conc HCl slowly until pH 6.8 (allow cooling to RT pH ↑) top with dH_2O to 100ml	Bollag *et al.* (1996)
10% SDS (w/v), 100 ml	10 g SDS (may heat to dissolve), add dH_2O to 100 ml and store at RT	Bollag *et al.* (1996)
50% glycerol (v/v), 100 ml	Pour 50ml glycerol 100%, add 50 ml dH_2O	Bollag *et al.* (1996)
1% bromophenol blue (w/v), 10 ml	Weigh 100 mg bromphenol blue, bring to 10 ml with dH_2O stir until dissolve and filter to remove aggregated dye	Bollag *et al.* (1996)
Acrylamide stock solution, 100 ml 30% (w/v) acryamide, 0.8% (w/v) bis-acrylamide	29.2 g acrylamide, 0.8 g bis-acrylamide, add distilled H_2O until 100 ml, stir to dissolve. Work under hood and cover beaker with parafilm untilpowder dissolves. Store at 4°C.	Bollag *et al.* (1996)
10% Ammonium persulfate, 5ml	0.5 g ammonium persulfate, 5ml H_2O, store at 4°C.	Bollag *et al.* (1996)
Electrophoresis buffer (1L)	3 g Tris, 14.4 g glycine, 1 g SDS, add dH_2O until 1L keep at RT.	Bollag *et al.* (1996)
5x Sample buffer, 10 ml	0.6 ml 1M Tris – HCl (pH 6.8), 5 ml 50% glycerol, 2 ml 10% SDS, 0.5 ml 2-mercaptoethanol, 1ml 1% bromophenol blue, 0.9 ml H_2O stable for months in -20°C	Bollag *et al.* (1996)
Coomassie blue (1L)	50% methanol (LP), 10% Acetic acid (Merck), 1.25g coomassie brilliant blue R250 (MP Biomedical Inc.) and bring to 1L with water.	Bollag *et al.* (1996)
Coomassie destain (1L)	400ml methanol (LP), 100ml Acetic acid (Merck) and bring to 1L with water.	Bollag *et al.* (1996)
SDS-gel loading buffer (Dye 8x) (1ml)	400µl NuPAGE® reducing agent (invitrogen™), 500µl NuPAGE® dye 4x (invitrogen™) and 100µl 100% glycerol	invitrogen

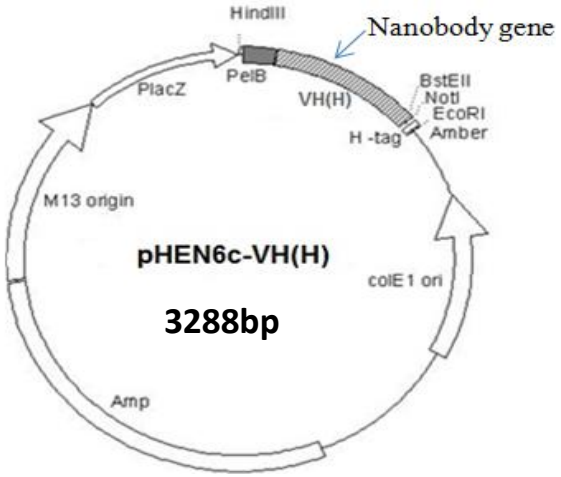
	(Duchefa Biochemie)	
Horseradish peroxidase (HRP) substrate (prepared fresh)	45 mg 4-chloro-1-naphthol (Sigma), 45 ml PBS, 100 µl Hydrogen peroxide (Merck), 15 ml Methanol (LP).	Bollag *et al.* (1996)
0.05% PBS-T (1L)	0.5ml Tween®20 (Sigma) in 1L PBS	Bollag *et al.* (1996)
Alkaline Phosphatase Buffer	0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 0.05 M $MgCl_2.6H_2O$ prepared, filtered and stored at 4°C for > one year.	Bollag *et al.* (1996)
BCIP Solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide	Bollag *et al.* (1996)
NBT Solution	50 mg/ml *p*-nitro blue tetrazolium chloride in 70% dimethylformamide	Bollag *et al.* (1996)

1.3 Bacterial strain

Bacterial strain:	**Genotype**	**Reference or source**
• *E. coli* WK6	Δ(*1ac-proA B*) *galE strAIF'lacI*^{q} *lacZ* ΔM 15 *proA*$^+$*B*$^+$	Stanssens *et al.,* (1989)

1.4 Plasmid Map

Plasmid:	**Genotype**	**Reference or source**
• pHEN6c	AmpR	Conrath *et al.,* (2001)

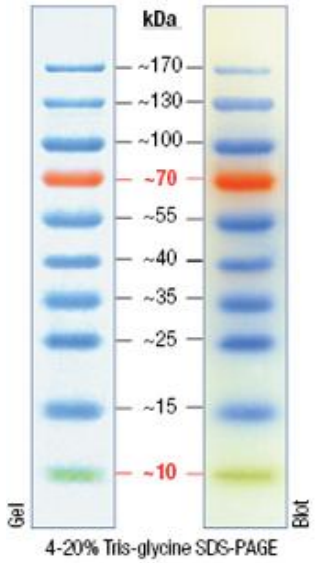


1.5 Enzymes:

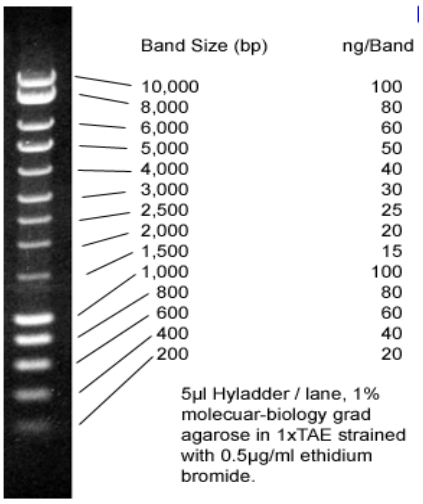
a) Restriction enzymes:	**Reference or source**
• *Eco91I* (10u/µl)	Fermentas
• *NotI* (10u/ µl)	Fermentas

• *PstI* (10u/µl)		Fermentas
• *XhoI* (10u/ µl)		Fermentas
b) Polymerase and DNA ligase		**Reference or source**
• *T4DNA ligase* (5u/µl)		Fermentas
• *Taq polymerase* (5u/µl)		Roche
Primers:	**Sequence (5'→3')**	**Reference or source**
• *Foward primer (FP)*	TTCCCAGTCACGAC	Arbabi Ghahroudi et al., (1997)
• *Reverse (RP)*	CACACAGGAAACAGCTATGAC	Arbabi Ghahroudi et al., (1997)

1.6 PageRuler: Pre-stained (2 colours) protein Ladder (Euromedex.com)



1.7 DNA Ladder: Hyladder™ 10kb (www.denville.ca)



1.8 Calculations and conversion units

Calculations

$$\text{molarity of a solution} = \frac{\text{g of solute}}{[\text{molecular mass of solute (g/mol)}] \times (\text{L of solution})}$$

molecular mass of a ds DNA fragment = (number of bp) × (660 Da/bp)

moles of ends of a ds DNA fragment = 2 × (g of DNA)/[molecular mass of DNA (Da)]
or = 2 × (g of DNA)/[(number of bp) × (660 Da/bp)]

moles of ends generated by a restriction endonuclease
circular DNA: 2 × (moles of DNA) × (number of sites)
linear DNA: 2 × (moles of DNA) × (number of sites) + 2 (original ends)

Units of Radioactivity

1 μCi = 2.2×10^6 disintegrations per minute = 3.7×10^4 becquerels
1 becquerel = 1 disintegration per second

Half-life of Common Radioisotopes

Radioisotope	Half-life
Carbon-14 (^{14}C)	5,730 years
Iodine-125 (^{125}I)	60 days
Phosphorus-32 (^{32}P)	14.3 days
Sulfur-35 (^{35}S)	87.4 days
Tritium (^{3}H)	12.4 years

Metric Prefixes

M = mega = 10^6	n = nano = 10^{-9}
k = kilo = 10^3	p = pico = 10^{-12}
m = milli = 10^{-3}	f = femto = 10^{-15}
μ = micro = 10^{-6}	a = atto = 10^{-18}

Abbreviations

ds	double-stranded (as in ds DNA)
ss	single-stranded (as in ss DNA)
bp	basepair
kb	kilobase: 1,000 bases or basepairs, as appropriate
Da	Dalton, the unit of molecular mass
mol	mole
M	molarity, moles of solute per liter of solution

Conversion Factors

base ↔ mass

1 kb ds DNA (Na^+) = 6.6×10^5 Da
1 kb ss DNA (Na^+) = 3.3×10^5 Da
1 kb ss RNA (Na^+) = 3.4×10^5 Da
1 MDa ds DNA (Na^+) = 1.52 kb
average mass of dNMP = 330 Da
average mass of dNMP bp = 660 Da

base ↔ protein

1 kb coding capacity = 333 amino acids = 40,000 Da protein
10,000 Da protein = ~270 bp DNA
50,000 Da protein = ~1.35 kb DNA
100,000 Da protein = ~2.7 kb DNA
average mass of amino acid = 120 Da

mass of nucleic acid ↔ moles of nucleic acid

1 µg/ml of nucleic acid = 3.0 µM phosphate
1 µg of a 1-kb DNA fragment = 1.5 pmol; 3.0 pmol ends
1 pmol of a 1-kb DNA = 0.66 µg
1 µg pBR322 = 0.36 pmol DNA
1 pmol pBR322 = 2.8 µg
35 nmol of a 24-mer = 277 µg

absorbance of nucleic acid ↔ concentration of nucleic acid

Nucleic acid	Concentration (µg/ml) for 1 A_{260} unit
ds DNA	50
ss DNA	33
ss RNA	40

mass of protein ↔ moles of protein

Molecular weight (Da)	1 µg	1 nmol
10,000	100 pmol or 6×10^{13} molecules	10 µg
50,000	20 pmol or 1.2×10^{13} molecules	50 µg
100,000	10 pmol or 6×10^{12} molecules	100 µg
150,000	6.7 pmol or 4×10^{12} molecules	150 µg

concentration of protein ↔ absorbance of protein

Protein	A_{280} units for 1 mg/ml
IgG	1.35
IgM	1.2
IgA	1.3
protein A	0.17
avidin	1.5
streptavidin	3.4
bovine serum albumin	0.7

Protein Molar Conversions

100pmols of 100,000 MW protein = 10µg
100pmols of 50,000 MW protein = 5µg
100pmols of 10,000 MW protein = 1µg

Protein/DNA Conversions

1kb of DNA = 333 amino acids of coding capacity = 3.7×10^4 MW
10,000 MW protein = 270bp DNA
30,000 MW protein = 810bp DNA
50,000 MW protein = 1.35kb DNA
100,000 MW protein = 2.7kb DNA

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