
بسم الله الرحمن الرحيم


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# Practical Note

# Biotechnology & Genetic engineering

# (BCH 462)

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# LAB safety for the course BCH 462

## 1. Sterile technique for Bacterial cultures.

Observe good laboratory practice. All strains of *Escherichia coli* are potentially pathogenic. Before opening any culture vessel think carefully. What needs to be done before and after manipulation? What you are going to do with the culture vessel? What you are going to do with any equipment that has come into contact with its contents?

Safe sterile technique is largely a matter of common sense. It has two majoraims:

1. Preventing contaminant organisms from getting *into* your cultures. Contamination *into* cultures can occur from the air when cultures are opened for manipulation, so open them for a minimum time and use smooth movements to reduce sudden air currents. It may also occur from unsterile equipment, as when a pipette-tip touches your filthy, bacteria-laden hands, so be careful with any used pipette, dispose them immediately.
2. Preventing any organisms or accidental contaminants from getting out. Escape fromcultures can occur from dripping pipettes, from putting used spreaders and inoculating hoops on the bench without sterilising them first, by aerosols formed by blowing bubbles through cultures with pipettes, or by rapid air movement (particularly with dry fungal spores).

The media and glassware you have been provided will be autoclaved in steam at 121°C for 20 min. However, the mouths of culture tubes, inoculating hoops and spreaders will need to be flamed using with a Bunsen burner. Used pipettes and discarded tubes must be disposed of into disinfectant. Use proper technique whatever the organisms being used are: you never know when a culture may have been contaminated, and you owe a duty of care to yourself and others not to filthy your lab space with potentially pathogenic bacteria.

**Some Helpful hints to remember when following the methods below.**

1. Always wash your hands and spray with 70% ethanol or wear gloves.

2. Always keep the caps on the polystyrene tubes loose so that air can circulate. Only cap tightly when the cells are no longer growing and are being stored in the refrigerator until the transformation efficiency has been calculated.

3. When scraping the frozen cell sample, hold the eppendorf tube at the top rather than at the bottom, so that the sample does not fully thaw. (one may want to keep the frozen samples on dry ice.)

4. When doing these experiments always have a negative control. A negative control is used to show that the media is not contaminated and what is growing in the media are the bacteria cells and not contaminants.

5. Note that both x-gal and IPTG are light sensitive and therefore should only be taken out of the light proof box for as brief a time as possible.

6. Ampicillin can cause allergic reactions on contact with skin to those who are sensitive to penicillin. Do not touch the agar.

Experiment 1

## 2. How to make Competent Bacterial cells.

### 2.1 **Introduction**:

Bacteria are able to take up DNA from their environment (exogenous DNA) in three ways; conjugation, transformation, and transduction. Only transformation is the direct uptake of DNA, since conjugation requires cell-cell contact via a sex pilus and transduction requires a bacteriophage intermediary to transfer DNA from one cell to another. For a bacterial cell to take up DNA from its surroundings, it must be in a special physiological state called competence. Experiments by Frederich Griffith in 1929 using competent *Streptococcus* (now *Enterococcus*) *pneumoniae*were instrumental in showing that DNA was the transforming principle – the genetic material. Natural competence is highly regulated in bacteria, and the factors leading to competence vary among genera. For some genera, only a portion of the population is competent at any time; for others, the entire population gains competence. A series of competence proteins is produced, which have some homology but differ in the Gram negative and the Gram positive bacteria. Once the DNA has been brought into the cell's cytoplasm, it may be degraded by cellular nucleases, or, if it is very similar to the cells own DNA, enzymes that normally repair DNA may recombine it with the chromosome. Natural transformation is very efficient for linear molecules such as fragments of chromosomal DNA but not for circular plasmid DNAs. Whereas, artificial competence is not encoded in the cell's genes. Instead it is a laboratory procedure in which cells are passively made permeable to DNA, using conditions that do not normally occur in nature. These procedures are comparatively easy and simple, and can be used to genetically engineer bacteria. Chilling cells in the presence of divalent cations such as $CaCl_2$ or $MgCl_2$ prepares the cell walls to become permeable to plasmid DNA. Cells that are undergoing very rapid growth are made competent more easily than cells in other stages of growth. Thus cells are brought into log phase before the procedure is begun. The log phase cells are all living, healthy, and actively metabolizing. Because this procedure can be very harsh on cells, the log-phase cells are more able to withstand this treatment.

2.2 **Theory**:

Since DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell's membrane. In order to make bacteria take in the plasmid, this is done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium.

There are two main methods for transformation of competent bacterial cells, the calcium chloride and the electroporation method. We will be using the calcium chloride method.
The protocol, explained here has developed approx. 30 years ago, is used to prepare batches of competent bacteria that yield 5 $x10^6$ to 2 x $10^7$ transformed colonies/μg of supercoiled plasmid DNA.

Materials and Methods:

1. Any Bacterial *E. Coli* strain such as XL 1 or DH5α

2.3 **Chemicals**:

1. Calcium ch;oride
2. Magnesium chloride.
3. Nacl
4. LB medium

2.4 **Equipments**:

1. Autoclave
2. Refregiated centrifuge
3. Water Bath
4. Ice container
5. Sorval GSA rotor
6. Spectrophotometer
7. Shaker and incubator

Glass ware:

1. Sterile glass universal tube

2. Petri dishes
3. 1L and 500ml Conical flask sterilised by outoclaving.

2. Luria Broth (LB) media:

Bactotryptone 10 g

Bacto yeast extract 5 g

NaCl 5 g

This was made up to 1L with distilled water and autoclaved immediately.

3. LB plates:

15g Bacto agar was added to a litre of LB media before autoclaving. Plates were then stored at $4^{o}$C.

4. 100mM $MgCl_2$
5. 100mM $CaCl_{2}..2H_2O$
6. 15% glycerol

2.5 **Objective**;

To prepare competent bacterial cells for transformation.

2.6 **Method**:

1. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 hours at 37°C. Transfer the colony into 100 ml SOB medium (LB-may be used) in a 1-liter flask. Incubate the culture for 3 hours at 37°C with vigorous agitation, monitoring the growth of the culture.
2. Incubate your 100 ml SOB medium (LB-may be used) in a 1-liter flask without any bacteria as a negative control.

3. Transfer the bacterial cells to sterile, disposable, ice-cold 50-ml polypropylene tubes. Cool the culturesto 0°C by storing the tubes on ice for 10 minutes.

4. Recover the cells by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
5. Decant the medium from the cell pellets. Stand the tubes in an inverted position on a pad of paper towels for 1 minute to allow the last traces of media to drain away
6. Resuspend each pellet by swirling or gentle vortexing in 30 ml of ice-cold .1M $MgCl_2$-.1M$CaCl_2$ solution.
7. Recover the cells by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
8. Decant the medium from the cell pellets. Stand the tubes in an inverted position on a pad of papertowels for 1 minute to allow the last traces of media to drain away.
9. Resuspend the pellet by swirling or gentle vortexing in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50ml of original culture.

When preparing competent cells, thaw a 10-ml aliquot of the $CaCl_2$ stock solution and dilute it to 100 ml with 90 ml of pure $H_2O$. Sterilize the solution by filtration through a prerinsed Nalgene filter (0.45-µmpore size), and then chill it to 0°C.

At this point, either use the cells directly for transformation or the pellet was gently resuspended in 2.5 mL of ice-cold 50 mM calcium chloride containing 10% sterile glycerol, aliquoted (200 mL each) in 1.5-mL centrifuge tubes, and stored at –70°C for further use.

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## 2.7 **Result and Discussion** ;.

2.8 **Questions**:

1. What precautions you would use for performing this experiment?
2. How do you know that you have successfully made competent cells?
3. Why is it always important to have a negative control?

2.9 **References**:

1. Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids.*J. Mol. Biol.* 166:557-580.
2. Sambrook,J., Fritsch, E.F. andManiatis, T.(2001) Molecular Cloning. A laboratory Manual. 3rd Edition. Cold Spring Harbor Laboratory Press

Experiment 2

3. **Transformation** :

**3.1 Introduction**:

This is a very basic technique that is used on a daily basis in a molecular biological laboratory. In 1971-1973 recombinant DNA technology was developed as gene cloning to be its major application. Molecular cloning is an important tool to understand the structure, function and regulation of individual genes and their products. The essence of cell-based DNA cloning involves four steps:

i. *Construction of recombinant DNA molecules* by *in vitro* covalent attachment (ligation) of the desired DNA fragment to a replicon. This step is facilitated by cutting the target DNA and replicon molecules with specific restriction endonucleases before joining the different DNA fragments using the enzyme DNA ligase.

ii. *Transformation.* The recombinant DNA molecules are transferred into host cells in which the chosen replicon can undergo DNA replication independently of the host cell chromosome(s).

iii. *Selective propagation of cell clones* involves two stages. Initially the transformed cells are plated out by spreading on an agar surface in order to encourage the growth of *well-separated* cell colonies. These are cell clones (populations of identical cells all descended from a single cell). Subsequently, *individual* colonies can be picked from a plate and the cells can be further expanded in liquid culture.

iv. *Isolation of recombinant DNA clones* by harvesting expanded cell cultures and selectively isolating the recombinant DNA.

**3.2 Theory**:

Introduction of recombinant plasmid into cells is achieved by the transformationof competent cells. Competent cells are prepared by treating the cell with adivalent cation like calcium chloride. Once the cells are made competent, theplasmid DNA is mixed with the cells. The competent cells are then subjectedto heat shock, which allows the DNA to

enter the cells. The cells are then platedonto a medium containing antibiotics to allow identification of recombinants. The recombinant plasmid can be amplified as well.

**3.3 Materials and Methods**:

1. Competent cells as prepared in our first experiment.
2. Appropriate antibiotics.

Chemicals:

1. Bacto agar
2. NaCl
3. Bactotryptone
4. Bacto yeast extract

**3.4 Equipments**:

Polysterine tubes

Sterile eppendorf tubes

Water bath

Disposable pipettes

Falcon tubes.

**3.5 Solutions**:

**DMSO**

Purchase a high grade of DMSO (dimethylsulfoxide, HPLC grade or better). Divide the contents of a fresh bottle into 1-ml aliquots in sterile tubes. Close the tubes tightly and store at -20°C. Use each aliquot only once and then discard

**Media Containing Agar or Agarose**

Prepare liquid media according to the recipes given. Just before autoclaving, add one of the following:

Bacto Agar (for plates)

15 g/liter

Bacto Agar (for top agar)

7 g/literagarose (for plates)

15 g/literagarose (for top agarose) 7 g/liter

Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm 2) on liquid cycle. When the medium is removed from the autoclave, swirl it gently to distribute the melted agar or agarose evenly throughout the solution. *Be careful!* The fluid may be superheated and may boil over when swirled. Allow the medium to cool to 50-60°C before adding thermolabile substances (e.g., antibiotics). To avoid producing air bubbles, mix the medium by swirling. Plates can then be poured directly from the flask; allow approx. 30-35 ml of medium per 90-mm plate. To remove bubbles from medium in the plate, flame the surface of the medium with a Bunsen burner before the agar or agarose hardens. Set up a colour code (e.g., two red stripes for LB-ampicillin plates; one black stripe for LB plates, etc.) and mark the edges of the plates with the appropriate colour markers.

When the medium has hardened completely, invert the plates and store them at 4°C until needed. The plates should be removed from storage 1-2 hours before they are used. If the plates are fresh, they will "sweat" when incubated at 37°C. When this condensation drops on the agar/agarose surface, it allows bacterial colonies or bacteriophage plaques to spread and increases the chances of cross-contamination. This problem can be avoided by wiping off the condensation from the lids of the plates and then incubating the plates for several hours at 37°C in an inverted position before they are used. Alternatively, remove the liquid by shaking the lid with a single, quick motion. To minimize the possibility of contamination, hold the open plate in an inverted position while removing the liquid from the lid.

**SOB**

deionized $H_2O$, to 950 ml

tryptone, 20 g

yeast extract, 5 g

NaCl, 0.5 g

For solid medium, please see Media Containing Agar or Agarose.

Shake until the solutes have dissolved. Add 10 ml of a 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of deionized H2O.) Adjust the pH of the medium to 7.0 with 5 N NaOH (approx. 0.2 ml). Adjust the volume of the solution to 1 liter with deionized $H_2O$. Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm 2) on liquid cycle. Just before use, add 5 ml of a sterile solution of 2 M $MgCl_2$. (This solution is made by dissolving 19 g of $MgCl_2$ in 90 ml of deionized H2O. Adjust the volume of the solution to 100 ml with deionized H2O and sterilize by autoclaving for 20 minutes at 15 psi [1.05 kg/cm2] on liquid cycle.)

**SOC**

deionized H2O, to 950 ml

tryptone, 20 g

yeast extract, 5 g

NaCl, 0.5 g

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SOC medium is identical to SOB medium, except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60°C or less. Add 20 ml of a sterile 1 M solution of glucose. (This solution is made by dissolving 18 g of glucose in 90 ml of deionized $H_2O$. After the sugar has dissolved, adjust the volume of the solution to 100 ml with deionized $H_2O$ and sterilize by passing it through a 0.22-μm filter.)

**Amphicillin stock solution:**

Ampicillin sodium salt was dissolved in AnalaR water to a concentration of 100mg/ml, and filtered through a 0.2μm filter. It was used in media at a final concentration of 100μg/ml. This solution was stored at $-20^{o}$ C.

**3.6 Objective:**

- To gain a hand on experience in recombinant DNA technology.
- To be able to transform the bacterial cells and calculate the transformation efficiency.

**3.7 Procedure:**

1. Frozen competent cells prepared from the first experiment will be used in this experiment.
2. Thaw the cells by holding the tube in the palm of the hand. Just as the cells thaw, transfer the tube to an ice bath. Store the cells on ice for 10 minutes.
3. To transform the $CaCl_2$-treated cells directly, transfer 200 µl of each suspension of competent cells to asterile, chilled 17 x 100-mm polypropylene tube using a chilled micropipette tip. Add DNA (no more than50 ng in a volume of 10 µl or less) to each tube. Include all of the appropriate positive and negative controls
4. Mix the contents of the tubes by swirling gently. Store the tubes on ice for 30 minutes.
5. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in therack for exactly 90 seconds. Do not shake the tubes.
6. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1-2 minutes.
7. Add 800 µl of SOC medium to each tube. Incubate the cultures for 45 minutes in a water bath set at37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by theplasmid.
8. Transfer the appropriate volume (up to 200 µl per 90-mm plate) of transformed competent cells ontoagar SOB medium containing 20 mM MgSO4 and the appropriate antibiotic.
9. Store the plates at room temperature until the liquid has been absorbed.
10. Invert the plates and incubate at 37°C. Transformed colonies should appear in 12-16 hours
11. Count cells. If the cells are spread relatively evenly over the plate, one can count the cells by dividing the plate into quarters, counting one quarter of the plate and then multiplying by four.
12. Calculate the efficiency (no. of colonies / ng DNA)

**3.8 Result and Discussion**:

Count white colonies as recombinant transformants and test for insert. Calculate the transformation efficiency in terms of the number of colony-forming units (CFU) per microgram of transforming DNA as follows:

$$\text{CFU/}\mu\text{g} = \frac{\text{CFU on Plate}}{\text{ng plasmid DNA used in transformation}} \times 101 \times \text{dilution factor}$$

**3.9 Questions**:

1. How do you know that you know that your transformation experiment is a success?
2. Why your transformed *E.coli* can now grow in the presence of antibiotics?

**3.10References**:

1. Cohen S.N., Chang A.C.Y., and Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA.*Proc. Natl. Acad. Sci.* 69: 2110-2114.
2. Sambrook,J., Fritsch, E.F. andManiatis, T.(2001) Molecular Cloning. A laboratory Manual. 3rd Edition. Cold Spring Harbor Laboratory Press

**CAUTIONS**

**DMSO**

DMSO (Dimethylsulfoxide) may be harmful by inhalation or skin absorption. Wear appropriate gloves and safety glasses. Use in a chemical fume hood. DMSO is also combustible. Store in a tightly closed container. Keep away from heat, sparks, and open flame

Experiment 3

4. **Screening of Recombinants by Qualitative estimation of β-galactosidase activity** produced by *Lac Z* gene induction by IPTG.

## 4.1 Introduction

Recombinant bacterial cells can be considered a protein-making factory. Under the right conditions, the majority of each cell's protein output can be the protein you're most interested in. The production of protein of interest can be turned on or off depending on the existence of an inducer.

The *lac* operon in *E. coli* is one of the simplest and best examples of such gene regulation. It consists of three genes—*lacZ, lacY,* and *lacA.* When the cell grows on rich medium or glucose minimal medium, transcription is blocked by *lac* repressor (product of the neighboring*lacI*gene) which binds to a single site (operator) upstream of *lacZ*and prevents RNA polymerase from binding to the promoter. When the cell grows on medium that contains lactose or certain related compounds, *lac* repressor no longer binds the operator, and RNA polymerase synthesizes a single mRNA which encodes *lacZ*, *lacY*, and *lacA*, i.e lactose (or related compound) induces the synthesis of the enzymes involved in its breakdown, particularly beta-galactosidase.

The bacterial cells we are using carry a plasmid that expresses β-galactosidase when Iso Propyl D- ThioGalactopyranoside (IPTG) is added to the growth media. b-galactosidase activity can be evaluated either quantitatively by using is *o*-nitrophenyl- β-D-Galactoside (ONPG) as a chromogenic substrate to estimate the enzyme activity in cell extract or either qualitatively by measuring the intensity of blue color produced by x-gal (5-bromo-4-chloro-3-indolyl-beta-galactoside ) inside the cell.

**4.2** Theory**:**

α-complementation occurs when two inactive fragments of *E. coli* -galactosidase associate to form a functional enzyme. Many plasmid vectors carry a short segment of DNA containing the coding information forthe first 146 amino acids of -galactosidase. Vectors of this type are used in host cells that express thecarboxy-terminal portion of the enzyme. Although neither the host nor

the plasmid-encoded fragments of β-galactosidase are themselves active, they can associate to form an enzymatically active protein. Lac+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of
the chromogenic substrate X-gal. However, insertion of a fragment of foreign DNA into the polycloning site ofthe plasmid almost invariably results in production of an amino-terminal fragment that is no longer capable of complementation. Bacteria carrying recombinant plasmids therefore form white colonies. The development ofthis simple blue-white colour test has greatly simplified the identification of recombinants constructed in plasmid vectors.

**4.3 Objective**:

1. To understand the functioning and regulation of the *lac* operon by α-complementation.
2. To identify the recombinant constructed in plasmid vectors.

Materials and Method:

Chemicals:

IPTG solution 20% w/v

X- Gal solution 2% w/v

**4.4 Media**:

1. Rich broth agar plates containing the appropriate antibiotic. Add amphicillin as 100µg/ml of LB.
2. Rich broth top agar ( as described before)
3. LB solid medium + IPTG + X-gal + amp
4. Components for Top Agar
   Amount of Reagent
   **Size of Plate Molten Top Agar X-gal IPTG**
   90 m 3 ml 40 µl7 µl
   150 mm 7 ml 100 µl20 µl

**Vectors and bacterialstrain**

1. *E. coli* strain, transformed with nonrecombinant plasmid (e.g., pUC, used as a negative control)
2. *E. coli* strain, transformed with recombinant plasmids

**4.5 Equipment**:

1. Shaking incubator
2. -70 freezer

**4.6 Procedure**:

1. Inoculate LB liquid medium (3 ml in sterile tube) with *E, coli* transformed with wild type *lac* operon containing plasmid.
2. Inoculate LB liquid medium (3 ml in sterile tube) with *E, coli* transformed with recombinant plasmid.
3. Streak glucose-containing LB plate with a loopful of *E coli* in order to obtain isolated colonies.
4. Streak IPTG+X-gal-containing plate with a loopful of *E coli* in order to obtain isolated colonies.
5. Incubate these two plates at 37 $^{o}$Cover night.
6. Selection of recombinants is based on the colour of the colony.
7. For a pUC vector, since the site used for insertion of foreign DNA is located within the lacZ gene, insertion of foreign DNA is monitored by the loss of 13-galactosidase activity upon transformation. Cells with the intact lacZ gene produce functional 13-galactosidase, which converts the colorless substrates X-gal to blue chromophor in presence of an inducer IPTG and therefore produce blue colonies. Transformed cells with recombinant plasmid do not demonstrate 13-galactosidase activity, and therefore, cannot act on X-gal resulting in the production of white colonies.

8. Colonies that carry wild-type plasmids contain active -galactosidase. These colonies are pale blue in the centre and dense blue at their periphery.
9. Colonies that carry recombinant plasmids do not contain active -galactosidase. These colonies are creamy-white or eggshell blue, sometimes with a faint blue spot in the center.
10. Select white colonies as clones.

**4.7 Storage of Clones**

1. Transfer the white colonies one by one onto a fresh LB agar plate containing ampicillin, IPTG, and X-Gal.
2. Incubate the plate at 37°C overnight. This is the master plate of the clones.
3. Inoculate the clones from the master plate to 1 mL of LB containing 100 µg/ml of ampicillin individually.
4. Incubate the tubes at 37°C overnight with constant shaking.
5. Add 150 mL of 100% glycerol to the wells of the microtiter plate.
6. Add 850 mL of the overnight grown culture to glycerol in a microtiter plate. Use 1 well per clone. Mix well.
7. Freeze the plate, cover, and store at –70°C.

**4.8 Result and Discussion**:

The induction of lac operon by IPTG in the presence of X-gal produce β-galactosidase which hydrolyzes X-gal to give blue color.

Absence of the inducer (IPTG) or the functional *lac* operon produces white colonies due to the absence of the degradation product of X-gal.

Fill in the following table.

| Bacterial strains with | Plate | Number of colonies formed |
|---|---|---|
| + Wild type plasmid | Plasmid+amp | |
| + Wild type plasmid | Plasmid + amp + IPTG+X-Gal | |
| + Wild type plasmid | Plasmid+amp (without IPTG+XGal) | |
| +Recombinant Plasmid | Plasmid + amp + IPTG+X-Gal | |

## 4.9 Questions

1. What do you expect if a wild-type *E. coli* strain containing *lac* operon was grown in minimal medium containing glucose and lactose as carbon sources and the metabolites were tested after 1 h, 4h, 10 h.

2. Describe the *lac* operon promoter/operator at these time periods including the relevant repressors and activators regulating this promoter.

## 4.10References:

2.9.1.1.1.1.1 Sambrook,J., Fritsch, E.F. andManiatis, T.(2001) Molecular Cloning. A laboratory Manual. 3rd Edition. Cold Spring Harbor Laboratory Press.

## 5. Quantitative estimation of □-galactosidase activity produced by *Lac Z* gene induction by IPTG

### 5.1 Introduction

Recombinant bacterial cells can be considered a protein-making factory. Under the right conditions, the majority of each cell's protein output can be the protein you're most interested in. The production of protein of interest can be turned on or off depending on the existence of an inducer.

The *lac* operon in *E. coli* is one of the simplest and best examples of such gene regulation. It consists of three genes—*lacZ, lacY,* and *lacA.* When the cell grows on rich medium or glucose minimal medium, transcription is blocked by *lac* repressor (product of the neighboring*lacI*gene) which binds to a single site (operator) upstream of *lacZ*and prevents RNA polymerase from binding to the promoter. When the cell grows on medium that contains lactose or certain related compounds, *lac* repressor no longer binds the operator, and RNA polymerase synthesizes a single mRNA which encodes *lacZ, lacY,* and *lacA,* i.e lactose (or related compound) induces the synthesis of the enzymes involved in its breakdown, particularly beta-galactosidase.

The bacterial cells we are using carry a plasmid that expresses beta-galactosidase when Iso Propyl D- ThioGalactopyranoside (IPTG) is added to the growth media. This expression method was originally described in 1986 (Studier and Moffatt *J. Mol. Biol.* Volume 189 (1): 113-130).

### 5.2 Theory

β-Galactosidase is encoded by the *lacZ* gene of the *lac* operon in *E. coli*. It is a large protein (120 kDa, >1000 amino acids) that forms a tetramer. The enzyme's function in the cell is to cleave β-D-Galactosides (like lactose). It can easily be measured with chromogenic substrates (colorless substrates which when hydrolyzed yield colored products). An example is *o*-nitrophenyl- β-D-Galactoside (ONPG). This compound is colorless, but in the presence of β-Galactosidase it is converted to galactose and *o*-nitrophenol. The latter compound is yellow and can be measured by its absorption at 420 nm. If the ONPG concentration is high enough, the amount of *o*-nitrophenol

produced is proportional both to the amount of enzyme present and to the time the enzyme reacts with the ONPG.

In 1972, Jeffrey Miller published a protocol for determining the amount of β-Gal with ONPG. Because of this, ONPG/β-Gal assays are referred to as "Miller" assays, and a standardized amount of β-Gal activity is a "Miller Unit".

Two specialized components are used. First is an expression plasmid in which the gene of interest (in this case the *LacZ* gene that encodes beta-galactosidase) is downstream of a special bacterial promoter (LacZ promoter). This promoter is activated by an RNA polymerase (from the T7 bacteriophage) cloned in *E. coli.* In the absence of inducer, there is no T7 RNA polymerase made and so no *LacZ* is expressed from the plasmid. In the presence of the inducer (galactose or its analogue IPTG), the lacZ promoter is de-repressed, leading to expression of the T7 RNA polymerase, which transcribes lots of *LacZ*, and lots of beta-galactosidase. High levels of expression can be expected since the T7 RNA polymerase promoter is strong and each cell maintains many copies of the expression plasmid.

### 5.3 Objectives of the experiment

In this experiment we will assess the over-production of beta-galactosidase in two samples (no IPTG & plus IPTG) through following the beta-galactosidase activity in these cells.

The goals are:

- To understand the principle of gene induction by measuring the inducible expression of the *lac* operon by IPTG.
- To understand the functioning and regulation of the *lac* operon by assaying β-Galactosidase from cultures grown under a variety of conditions.
- To understand enzyme induction.
- To understand catabolite repression.

## 5.4 Materials and Methods

### 5.4.1 Materials Required:

#### 5.4.1.1 *Chemicals* (80 µl per sample).

- 100 mM dibasic sodium phosphate ($Na_2HPO_4$)
- 20 mMKCl
- 2 mM $MgSO_4$
- 0.8 mg/ml CTAB (hexadecyltrimethylammonium bromide)
- 0.4 mg/ml sodium deoxycholate
- 5.4 µl/ml beta-mercaptoethanol

#### 5.4.1.2 *Substrate solution* (600 µl per sample).

- 60 mM $Na_2HPO_4$ $7H_2O$ (16.1 g /l)
- 40 mM $NaH_2PO_4$ $H_2O$ (5.5 g/l)
- *o*-nitrophenyl-β-D-Galactoside (ONPG) (1 g/l)
- 50 mMβ-mercaptoethanol(2.7 ml)

#### 5.4.1.3 *Stop solution* (700 µl per sample).

- 1 M Sodium Carbonate ($Na_2CO_3$)

The high pH of the stop solution denatures the β-Gal and approximately doubles the yellow color of the reaction.

#### *5.4.1.4 Z buffer*

16.1 g $Na_2HPO_4$ $7H_2O$ (60 mM final)

- g $NaH_2PO_4$ $H_2O$ (40 mM final)

0.75 g KC1 (10 mM final)

0.246 g $MgSO_4$ $7H_2O$ (1 mM final)

- ml 2-mercaptoethanol (50 mM final)

Adjust to pH 7.0 and bring to 1 liter with $H_2O$. Do not autoclave.

### 5.4.2 Equipment

Autoclave

Centrifuge

Automatic pipettes 1ml and 10µl

Vortex

Spectrophotometer

### 5.4.3 Glassware

Sterile Eppindorff tubes and tips

Glass spreader

Petri dishes

Plastic cuvettes

### 5.4.4 Procedure:

Before starting, determine how many cells you have in each of your samples. Transfer 0.5 ml culture solution to plastic cuvettes and measure the OD600 of each, blanking the spectrophotometer with water or M9, then convert the optical density to a concentration using the approximation that 1 OD600 is ~$1x10^9$ cells/ml.

| **Sample** | **OD600** | **cells/ml** |
|---|---|---|
| no IPTG | | |
| plus IPTG | | |

**Beta-galactosidase assay**

- Perform assays of each sample in triplicate to gain some confidence in the values you measure.
- Make 1 ml of a 1:10 dilution of each cell sample, using Zbuffer-BME as the diluent, then use 100 □ l of this dilution to make another 1:10, for a final concentration of 1:100.

1. Add 400 µl of Zbuffer-BME to 13 eppendorf tubes labeled 0-12.
2. Add 100 µl of the appropriate cell dilution to each tube (1:100 of no IPTG in tubes 1-3 etc). See chart below for guidance. Add 100 □ l of Zbuffer-BME to tube 0, to serve as your blank.
3. Next, lyse the cells by add 20 µl of 0.1% SDS to each eppendorf.
4. To better lyse the cells, add 30 µl of chloroform ($CHCl_3$) to each tube. Do this in the hood since chloroform is volatile and toxic. You will need to hold the pipet tip close to the eppendorf as you move between the chloroform stock bottle and your eppendorfs since chloroform has a low surface tension and will drip from you pipetmen. Be sure to dispose of your pipet tips in the chloroform waste container located on the right side of the hood.
5. To really lyse the cells, vortex the tubes for 10 seconds each.
6. Start the reactions by adding 100 µl of ONPG to each tube at 10 second intervals, including your blank.
7. Stop the reactions by adding 250 µl of $Na_2CO_3$ to each tube at 10 second intervals once sufficient yellow color has developed. "Sufficient" means yellow enough to give a reliable reading in the spectrophotometer, best between 0.3 and 1.0. Be sure to note the time you are stopping the reactions. Also be sure to remember that adding the $Na_2CO_3$ makes the reactions more yellow.
8. When all your samples have been stopped, add 250 µl of $Na_2CO_3$ to the blank and spin all the tubes in the microfuge for 1 minute at 13,000 RPM to pellet any cell debris.
9. Move 0.5 ml of each reaction to plastic cuvettes and read the absorbance at 420nm. These values reflect the amount of yellow color in each tube.
10. Read the absorbance of each at 550 nm. These values reflect the amount of cell debris and differences in the plastic cuvettes themselves.
11. Calculate the beta-galactosidase activity in each sample according to the following formula:

- Beta-gal Units = Abs at 420 minus (1.75 times Abs at 550nm) all divided by the product of time in minutes, volume of cells (from original culture) in ml and OD600 (of the original culture), then all times 1000.

Miller Unit = $$1000 * \frac{(Abs_{420} - (1.75 * Abs_{550}))}{(t * v * Abs_{600})}$$

where:

11. $Abs_{420}$ is the absorbance of the yellow o-nitrophenol,
12. $Abs_{550}$ is the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420nm,
13. $t$ = reaction time in minutes,
14. $v$ = volume of culture assayed in milliliters,
15. $Abs_{600}$† reflects cell density.

†Note that this value is different for each spectrophotometer used and should be calibrated by plating dilutions of known $Abs_{600}$ cultures to determine the colony-forming units per $Abs_{600}$.

12. Dispose of your samples properly. Place all cuvettes and tubes in the chloroform waste container in the hood.

**5.5 Results:**

| Sample | Tube | □ l used | Dil. | Start | Stop | $A_{420\ nm}$ | $A_{550\ nm}$ | Units |
|---|---|---|---|---|---|---|---|---|
| **Blank** | **0** | | | | | | | |
| **No IPTG** | **1** | | | | | | | |
| | **2** | | | | | | | |
| | **3** | | | | | | | |
| **+ IPTG** | **4** | | | | | | | |
| | **5** | | | | | | | |
| | **6** | | | | | | | |

### 5.6 Discussion:

The*LacZ* gene that encodes □-galactosidase is downstream of LacZ promoter. This promoter is activated by an RNA polymerase which induced by lactose or its analogue. In the absence of inducer, there is no T7 RNA polymerase made and so no *LacZ* is expressed. The addition of the inducer (IPTG) de-represses the lacZ promoter leading to the expression of the T7 RNA polymerase, which transcribes lots of *LacZ*, and lots of □-galactosidase. The β-Galactosidase converts ONPG to galactose and *o*-nitrophenol. The latter compound is yellow and the color intensity reflects the activity of β-Galactosidase induced by IPTG.

## 5.7 Questions:

a. What you expect if a wild-type *E. coli* strain containing *lac* operon was grown in minimal medium containing glucose and lactose as carbon sources and the metabolites were tested after 1 h, 4h, 10 h. Describe the *lac* operon promoter/operator at these time periods including the relevant repressors and activators regulating this promoter.

## 5.8 References:

1. Jeffrey H. Miller. *A short course in bacterial genetics.* Plainview, N.Y.: Cold Spring Harbor Laboratory Press, 1992.isbn:0879693495.

2. Zhang X and Bremer H. *Control of the Escherichia coli rrnB P1 promoter strength by ppGpp.* J BiolChem 1995 May 12; 270(19) 11181-9.

3. Jeffrey H. Miller. *Experiments in molecular genetics.* [Cold Spring Harbor, N.Y.] Cold Spring Harbor Laboratory, 1972.isbn:0879691069.

## 6. Small scale His-Tag fusion protein purification under denaturative conditions

### 6.1 Introduction

High levels of expression of recombinant proteins in a bacterial system can lead to the formation of insoluble aggregates, usually known as inclusion bodies (IB). 6M Guanidine-HCl (GuHCl), 8M Urea or other strong denaturants can be used to completely solubilized IB. Since under denaturating conditions the His tag is completely exposed, it will facilitate the binding to Ni columns. For most biochemical studies, proteins have to be renatured and refolded, and this can be done usually after elution or sometimes in the column itself before elution.

### 6.2 Aliquot of Cell Pellet after Induction

***The idea is to aliquot cells after induction, and keep at -80ºC enough cell pellet samples for optimization of small scale purification procedure and further scale-up. Once you set up the best purification conditions at low scale, you can scale-up the procedure.***

Example:

1) Grow 1L culture
2) Induce (IPTG, salt induction, etc. etc.)
3) Spin cell culture 10min 8000rpm 4ºC, discharge supernatant
4) Resuspend cell pellet at 4ºC very gently with 100ml cold PBS buffer. Aliquot as following:

    a) 10 tubes (1.5ml plastic tubes) with 1ml suspension (it means 10ml original culture per tube);

    b) 4 tubes (15ml plastic tubes) with 10ml suspension (it means 100ml original culture per tube)

    c) 1 tube (50ml plastic tube) with 50ml suspension (it means 500ml original culture).
5) Spin 10min 8000rpm 4ºC, discharge supernatant
6) Keep cell pellet at -80ºC

### 6.3 Equilibration of Ni-NTA agarose

Place 50ul beads (100ul suspension) of Ni-NTA agarose beads in 1.5ml plastic tube.

Wash with 2 x 1.5ml $H_2O$ and 2x 1.5ml equilibration buffer (washing: mix, spin 3min 3500rpm, discharge supernatant).

**Buffers**

Lysis buffer: 50mM $Na_2HPO_4$ pH 8.0, 0.3M NaCl, 1mM PMSF (or protease inhibitor cocktail for bacterial cells #P-8849 from Sigma) and strong denaturant as 6M Guanidine-HCl (GuHCl) or 6 to 8M Urea

*Optional additives to the lysis buffer*

*a) 1mM PMSF or protease inhibitor cocktail 1:200 (cocktail for bacterial cells #P-8849 from Sigma)*

*b) Dnase 100U/ml or 25-50ug/ml (SIGMA DN-25). Incubate 10min 4°C in the presence of 10mMMgCl₂*

*c) ßME up to 20mMas areducing agentif the protein has Cysteines.*

Equilibration buffer: 6 to 8M Urea, 50mM $Na_2HPO_4$ pH 8.0, 0.5M NaCl

Washing buffer: 6 to 8M Urea, 50mM $Na_2HPO_4$ pH 8.0, 0.5M NaCl

Elution buffer: 6 to 8M Urea, 20mM Tris pH 7.5, 100mM NaCl, and appropriate imidazole concentrations.

*Limitations*

*Do not exposed Ni matrices to reducing agents as DTT or DTE ( you can use ßME up to 20mM); chelating agents as EDTA and EGTA; $NH_4^+$ buffers and amino acids as Arg, Glu, Gly or His.*

### 6.4 Procedure I: Protein Extraction from Bacterial Pellet in the presence of a strong denaturant

1. Resuspend pellet of 10ml bacterial culture (or 100ml bacterial culture for very low expression level) in 1ml lysis buffer
2. Prepare lysis buffer containing urea 6 to 8M or Guanidine-HCl 6M (try 8M of Urea first, and if protein is soluble, titer down in the next experiments till minimal urea concentration is required for protein solubilization)
3. Sonicate on ice 3 x 20 seconds (depends of the sonicator)
4. Spin 15min max speed 4°C
5. Transfer supernatant into clean tube: crude extract (keep 40µl for PAGE-SDS)
6. Equilibrate 50µl Nil beads with equilibration buffer (see Equilibration of Ni-NTA agarose)
7. Add the crude extract to the beads and incubate 4°C / 1h (swirl)
8. Spin 3min 3500rpm. Dicharge unbound material (keep 40µl for PAGE-SDS)
9. Wash 3x1ml with wash buffer with appropriate urea concentration. Washing: mix, spin 3min 3500rpm, discharge supernatant (keep 40µl for PAGE-SDS)
10. Wash 2x1ml with wash buffer + 10mM imidazole (keep 40µl for PAGE-SDS)
11. Elute with 3x100µl elution buffer + 250mM imidazole (keep 40µl for PAGE-SDS) (elution: mix, keep 3min at 4°C, spin 3min 3500rpm, collect supernatant)
12. Run on PAGE-SDS gel 5µl of crude extract and unbound material, and 13µl of the wash and elution fractions.

### 6.5 Procedure II: Protein Extraction from Inclusion Bodies (IB) in the presence of a strong denaturant

1 Resuspend pellet of 10ml bacterial culture (or 100ml bacterial culture for very low expression level) in 1ml lysis buffer **without** denaturants as Urea or Guanidine-HCl

2 Sonicate on ice 3 x 20 seconds (depends of the sonicator)

4 Spin 15min max speed 4°C. Separate supernatant before solubilization (keep 40µl for PAGE-SDS) from pellet (IB).

5 Resuspend IB in 0.5ml lysis buffer containing urea 6 to 8M or Guanidine-HCl 6M (keep 30min at 30°C) **or** wash IB before solubilization as suggested in ""Contaminant Removal from Inclusion Bodies Before Solubilization"" (this step although more laborious, can help to reduce the background of protein contaminants in the final purification step). After IB solubilization spin 15min max speed 4°C. Separate supernatant after solubilization (keep 40µl for PAGE-SDS) from insolubilized pellet (suspend pellet in 0.5ml lysis buffer and keep 40µl for PAGE-SDS). Transfer supernatant into clean tube

6 Equilibrate 50µl Nil beads with equilibration buffer (see Equilibration of Ni-NTA agarose)

7 Add the last supernatant extract to the beads and incubate 4°C / 1h (swirl)

8 Spin 3min 3500rpm. Dicharge unbound material (keep 40µl for PAGE-SDS)

9 Wash 3x1ml with wash buffer with appropriate urea concentration. Washing: mix, spin 3min 3500rpm, discharge supernatant (keep 40µl for PAGE-SDS)

10 Wash 2x1ml with wash buffer + 10mM imidazole (keep 40µl for PAGE-SDS)

11 Elute with 3x100µl elution buffer + 250mM imidazole (keep 40µl for PAGE-SDS) (elution: mix, keep 3min at 4°C, spin 3min 3500rpm, collect supernatant)

12 Run on PAGE-SDS gel 5µl of supernatant extract before and after solubilization, insolubilized pellet, and unbound material, and 13µl of the wash and elution fractions.

## 7. Immuno-blotting (Western Blotting)

### 7.1 Introduction

Immunoblotting (often referred to as western blotting) is used to identify specific antigensrecognized by polyclonal or monoclonal antibodies.

### 7.2 Theory

Protein samples are solubilized,usually with sodium dodecyl sulfate (SDS) and in selected cases with reducing agentssuch as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME); some antibody epitopes aredestroyed if reducing conditions are used. Following solubilization, the material isseparated by SDS-PAGE). Theantigens are then electrophoretically transferred in a tank to a nitrocellulose, polyvinylidenedifluoride (PVDF), or nylon membrane. The process can bemonitored by a reversible staining procedure with Ponceau S. After staining, protein bandson the membrane can be photographed and/or the positions of the detected proteins canbe marked with indelible ink (e.g., Paper-Mate pen). The membrane is then completelydestained by soaking in water for an additional 10 min.At this point the transferred proteins are bound to the surface of the membrane, providingaccess for reaction with immunodetection reagents. All remaining binding sites areblocked by immersing the membrane in a solution containing either a protein or detergentblocking agent. After being probed with primary antibody, the membrane is washed andthe antibody-antigen complexes are identified using horseradish peroxidase (HRPO) oralkaline phosphatase (AP) enzymes coupled to the secondary anti-immunoglobulin-G(anti-IgG) antibody (e.g., goat anti-rabbit IgG). The enzymes are attached to the secondary antibody.Chromogenic or luminescent substrates are then used tovisualize the activity.

### 7.3 Immunoprobing with directly conjugated secondary antibody

After electrophoretic transfer to the membrane, the immobilized proteins areprobed with specific antibodies to identify and quantitate any antigens present. Themembrane is first immersed in blocking buffer to fill all protein-binding sites with anonreactive protein or detergent. Next, the membrane is placed in a solution containingan antibody directed against the antigen (primary antibody). The blot is washed and thenexposed to an enzyme-antibody conjugate directed against the primary antibody (secondaryantibody; e.g., goat anti-rabbit IgG). Antigens are identified by

chromogenic orluminescent visualization of the antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane. Tween 20 is acommon alternative to protein blocking agents for use with nitrocellulose or PVDF filters.

### *7.4* Objective

- To understand how large proteins (antigens) can be analyzed using antibodies raized against these proteins by Immunoblotting technique
- To understand the steps in the development of Western and antigen-antibody interaction and detection.

### *7.5* Materials and Method

***Materials***

Membrane with transferred proteins (nitrocellulose, polyvinylidenedifluoride [PVDF], or nylon membrane.

Blocking buffer appropriate for membrane and detection protocolPrimary antibody specific for protein of interest.

TTBS (nitrocellulose or PVDF) *or* TBS (neutral or positively charged nylon; seerecipes for both solutions)

Secondary antibody conjugate: horseradish peroxidase (HRPO)- or alkaline phosphatase.

Heat-sealable plastic bags

Powder-free gloves

Plastic box

Additional reagents and equipment for chromogenic or luminescent visualization

***Materials***

Membrane with transferred proteins and probed with antibody-enzyme complex.

TBS.

Chromogenic visualization solution.

Additional reagents and equipment for gel photograph

1. If final membrane wash (see Basic Protocol, step 7, or see Alternate Protocol, step 9)was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

2. Place membrane into chromogenic visualization solution. Bands should appear in 10to 30 min.

4. Terminate reaction by washing membrane in distilled water. Air dry and photograph for a permanent record.

## 7.6 REAGENTS AND SOLUTIONS

***Alkaline phosphate substrate buffer***

100 mMTrisHCl, pH 9.5

100 mMNaCl

5 mM MgCl2

***Blocking buffer***

*For nitrocellulose and PVDF:*

0.1% (v/v) Tween 20 in TBS (TTBS; see recipe).

*For neutral and positively charged nylon:*

Tris-buffered saline (TBS; see recipe)containing 10% (w/v) nonfat dry milk.

***Chromogenic visualization solutions***

*BCIP/NBT visualization solution:*

Mix 33 µl NBT stock [100 mg NBT in 2 ml 70% (v/v) dimethylformamide (DMF), stored <1 year at 4°C] and 5 ml alkaline phosphatesubstrate buffer (see recipe). Add 17 µl BCIP stock (100 mg BCIP in 2 ml 100%DMF, stored <1 year at 4°C) and mix. Stable 1 hr at room temperature.

*4CN visualization solution:*

Mix 20 ml ice-cold methanol with 60 mg 4-chloro-1-naphthol (4CN). Separately mix 60 µl of 30% (w/v) $H_2O_2$ with 100 ml TBS (seerecipe) at room temperature. Rapidly mix the two solutions and use immediately.

*DAB/NiCl2 visualization solution:*

5 ml 100 mMTris· Cl, pH 7.5

100 µl DAB stock (40 mg/ml in $H_2O$, stored in 100-µl aliquots at −20°C)

25 µl $NiCl_2$ stock (80 mg/ml in $H_2O$, stored in 100-µl aliquots at −20°C)

15 µl 3% (w/v) $H_2O_2$

Mix just before use

***Tris-buffered saline (TBS)***

100 mMTrisHCl, pH 7.5

0.9% (w/v) NaCl

Store up to several months at 4°C

***Tween 20/TBS (TTBS)***

0.1% (v/v) Tween 20 in Tris-buffered saline.

Store up to several months at 4°C

**7.7 Method:**

1. Place membrane in heat-sealable plastic bag with 5 ml blocking buffer and seal bag.

Incubate 30 min to 1 hr at room temperature with agitation on an orbital shaker orrocking platform.

2. Dilute primary antibody in blocking buffer.*1/100 to 1/1000 is convenient for apolyclonal antibody*.

3. Open bag and pour out blocking buffer. Replace with 5 ml diluted primary antibody solution and incubate 30 min to 1 hr at room temperature with constant agitation.

4. Remove membrane from plastic bag with gloved hand. Place in plastic box and wash4 times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10to 15 min each time.

5. Dilute secondary antibody HRPO- or AP-anti-Ig conjugate in blocking buffer. *1/200to 1/2000 (i.e., 20 _l/ml to 2 _l/ml)* is convenient dilution.

6. Place membrane in fresh heat-sealable plastic bag, add diluted HRPO- or AP-anti-Igconjugate, and incubate 30 min to 1 hr at room temperature with constant agitation.

7. Remove membrane from bag and wash as in step 4. Develop according to appropriatevisualization protocol (see Support Protocol 1 or see Support Protocol 2).

**7.8 Visualization with chromogenic substrates**

After incubation with primary and secondary antibody conjugates bound antigens are typically visualized with chromogenic substrates.

The substrates 4CN, DAB/NiCl2, and TMB are commonly used with horseradishperoxidase (HRPO)–based immunodetection procedures, whereas BCIP/NBT is recommendedfor alkaline phosphatase (AP)–based procedures.

Afterincubation with primary and secondary antibodies, the membrane is placed in theappropriate substrate solution. Protein bands usually appear within a few minutes.

## 7.9 Results

## 7.10Discussion

## 7.11References

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## 8. Single Strand Conformation Polymorphism (SSCP) Analysis by Nondenaturing PAGE

### 8.1 Principle

Single-strand conformation polymorphism (SSCP) technique is a simple and efficient means to detect any small alteration in PCR-amplified product. It is based on the assumption that subtle nucleic acid change affects the migration of single-stranded DNA fragment and, therefore, results in visible mobility shifts across a nondenaturing polyacrylamide gel (Orita et al., 1989). Polyacrylamide gel is used for analysis of DNA with specialized buffer systems and without urea. In nondenaturing PAGE the components used to synthesize matrix are acrylamide monomers, N, N-methylene bisacrylamide (Bis), ammonium persulphate (APS) and N,N,N',N'-tetramethylenediamine (TEMED). Ammonium persulphate (APS) when dissolved in water generates free radicals, which activate acrylamide monomers inducing them to react with other acrylamide molecules forming long chains. These chains cross-linked with Bis.TEMED act as catalyst for gel formation because of its ability to exist in free radical form. The acrylamide and bisacrylamide is used in 49:1 ratio using autoclaved HPLC water added to make 100 ml volume. This 49:1 acrylamide-bisacrylamide solution is dissolved completely using magnetic stirrer and kept it in refrigerator till used. SSCP is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair) which results in a different secondary structure and a measurable difference in mobility through a gel.

### 8.2 BACKGROUND

- The mobility of double-stranded DNA in gel electrophoresis is dependent on strand size and length but is relatively independent of the particular nucleotide sequence. The mobility of single strands, however, is noticeably affected by very small changes in sequence, possibly one changed nucleotide out of several hundred. Small changes are noticeable because of the relatively unstable nature of single-stranded DNA; in the absence of a complementary strand, the single strand may experience intrastrand base pairing, resulting in loops and folds that give the single strand a unique 3D structure, regardless of its length. A single nucleotide change could dramatically affect the strand's

mobility through a gel by altering the intrastrand base pairing and its resulting 3D conformation (Melcher, 2000).

- Single-strand conformation polymorphism analysis takes advantage of this quality of single-stranded DNA. First announced in 1989 as a new means of detecting DNA polymorphisms, or sequence variations, SSCP analysis offers an inexpensive, convenient, and sensitive method for determining genetic variation (Sunnucks et al., 2000).

Like restriction fragment length polymorphisms (RFLPs), SSCPs are allelic variants of inherited, genetic traits that can be used as genetic markers. Unlike RFLP analysis, however, SSCP analysis can detect DNA polymorphisms and mutations at multiple places in DNA fragments (Orita et al., 1989). As a mutation scanning technique, though, SSCP is more often used to analyze the polymorphisms at single loci, especially when used for medical diagnoses (Sunnucks et al., 2000). Single-stranded DNA mobilities are dependent on temperature. For best results, gel electrophoresis must be run in a constant temperature. Sensitivity of SSCP is affected by pH. Double-stranded DNA fragments are usually denatured by exposure to basic conditions: a high pH. Kukita et al. found that adding glycerol to the polyacrylamide gel lowers the pH of the electrophoresis buffer--more specifically, the Tris-borate buffer--and the result is increased SSCP sensitivity and clearer data (1997). Fragment length also affects SSCP analysis. For optimal results, DNA fragment size should fall within the range of 150 to 300 bp, although SSCP analysis of RNA allows for a larger fragment size (Wagner, 2002). Tthe presence of glycerol in the gel may also allow a larger DNA fragment size at acceptable sensitivity (Kukita et al., 1997). Under optimal conditions, approximately 80 to 90% of the potential base exchanges are detectable by SSCP (Wagner, 2002). If the specific nucleotide responsible for the mobility difference is known, a similar technique called Single Nucleotide Polymorphism (SNP) may be applied.

The procedure used during the development of SSCP was as follows:

- digestion of genomic DNA with restriction endonucleases
- denaturation in an alkaline (basic) solution
- electrophoresis on a neutral polyacrylamide gel

- transfer to a nylon membrane
- hybridization with either DNA fragments or more clearly with RNA copies synthesized on each strand as probes (Orita et al., 1989).

- Since then, more convenient procedures have been developed, taking into account other molecular techniques, although sometimes it is simpler to amplify the double strand and then denature it into single strands instead of trying to find suitable primers for the below PCR method if the targeted sequence is unknown.

- Most experiments involving SSCP are designed to evaluate polymorphisms at single loci and compare the results from different individuals.

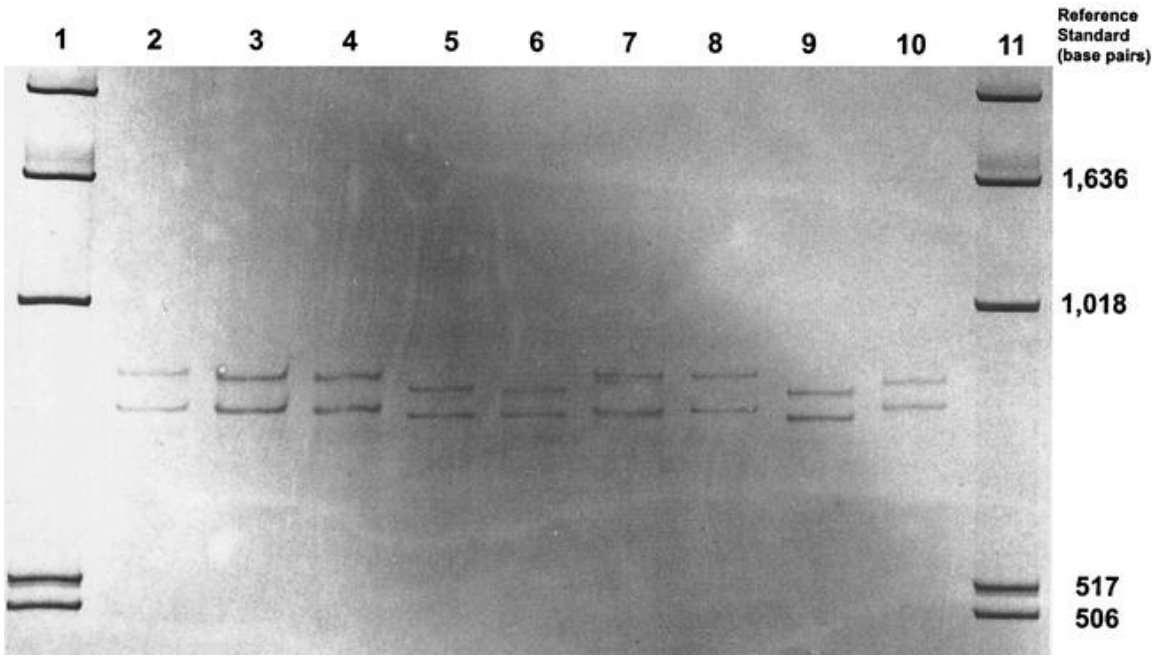


***Figure 1: Sample SSCP Gel Result and Interpretation.*** **DNA was isolated and amplified from sand flies (*Lutzomyialongipalpis*). SCCP analysis of the DNA shows multiple haplotypes, or sets of alleles usually inherited as a unit. Lanes 3 and 4 were identical haplotypes from two individuals. The difference in band migration in adjacent lanes is associated with the number of nucleotide differences (in parentheses): lanes 2-3 (2), lanes 3-4 (0), lanes 4-5 (3), lanes 5-6 (1), lanes 6-7 (3), lanes 7-8 (1), lanes 8-9 (1), and lanes 9-10 (4).** Source: Hodgkinson, et al,. 2002

## 8.3 Procedure

*Preparation of SSCP Gel*

The percentage of gels used for SSCP analysis varies from 8-20 percent but most of the primers were optimized with good results in 12% PAGE solution.
Composition of 12% nondenaturing PAGE Solution (For 100 ml)

| Component | Volume |
|---|---|
| 50% Acrylamide-bisacrylamide | 24 ml |
| 10 X TBE | 5 ml |
| Glycerol | 10 ml |
| Autoclaved HPLC water | 61 ml |

The gel mixture is kept dissolved completely and stored at -20□ Ctill it is used.

*PCR-SSCP*

The PCR-SSCP procedure includes following steps viz; PCR amplification of the gene fragments, resolution in nondenaturing PAGE and visualization using silver staining. PCR conditions are optimised for PCR-SSCP by testing a number of variables such as concentration of DNA, Taq polymerase, dNTPs, $MgCl_2$ and temperature profile. The PCR amplification protocol for all the SSCP primers used is same except the annealing temperature, which varies between primers.

1. The Single Strand Conformation Polymorphism analysis of amplified gene fragments is carried out using Bio-Rad Protein II xi Cell vertical gel electrophoresis unit (Bio-Rad laboratories). The two glass plates are washed thoroughly using tap water with detergent and rinsed initially under running tap water till no remains of detergent are left. The plates are wiped two times with tissue paper soaked in

distilled water first, 70 percent alcohol and then air-dried. The similar thorough cleaning treatment is given to spacers and comb to ensure proper alignment of 20 cm glass plates.

2. The gel sandwich is assembled on a clean surface laying down the long rectangular plate first, then two spacers of equal thickness along the long edges of plate and the short plate is placed on the rectangular plate. The two glass plates with spacers between them are fitted well with proper alignment by tightening the bulldog clamps. The sandwiched gel plates are fitted in the stand with screw clamps. The cleaned comb (20 wells) is inserted from the topside of the gel sandwich and immediately bulldog clamps are applied over the plates containing comb to create sharp wells.
3. The bottom side of the gel sandwich is sealed using 10 ml of 12% gel mix. The gel sandwich is kept in slanting position and the solution mixed with 50 µl APS and 20 µl TEMED is injected between the two glass plates using syringe fitted with 10 µl tip and allowed to polymerize for 10 minutes.
4. After polymerisation the assembled gel sandwich is placed in alignment slot of casting stand. The 12% native PAGE gel mix (25 ml) is prepared by adding APS (100 ul) and TEMED (40 µl) at a time and mixed well. This gel mix is filled from upper side of gel sandwich using syringe smoothly without any bubble and clamps are immediately applied over the comb to ensure sharp wells. The gel is kept undisturbed at least 45 minutes for polymerisation.
5. After polymerisation the comb is removed and wells are flushed with 0.5X buffer. The gel sandwich is placed in electrophoresis tank with notched plate facing towards the buffer reservoir. The reservoir of the electrophoresis tank is filled with 0.5X TBE and the gel is given pre-run at 200 volts at constant temperature for minimum 45 minutes. Ice cooled water circulation with electric pump is applied to central cooling core of assembly to maintain constant temperature.
6. About 4 µl PCR product and 12 µl of a formamide dye is prepared in PCR tube and denatured at 95°C for 10 minutes in the Biometra PCR machine. After denaturation the samples are immediately kept in ice-chilled box and kept in -20°C deep freeze for 10 minutes.

7. After completion of pre-run the wells are flushed again using buffer. The samples are loaded on a nondenaturing 12% acry1amide: bis-acrylamide (49:l) gel with gel loading tip and immediately electrophoresis is performed in 0.5 X Tris borate (pH 8.3)-EDTA buffer at 10-12.5 volts/cm for 3-24 hr at room temperature depending on the optimised conditions for each primer.
8. After completion of the electrophoresis for required time the glass plates are removed from the assembly. There after gel is subjected to silver staining to visualize SSCP band patterns.
9. In order to stain the gel it is immersed in a tray of appropriate size filled with 10% acetic acid (500ml) for at least 30 minutes for fixing DNA bands in gel so as to prevent diffusion of the DNA bands (Care has to be taken so that gel remains dipped well in solution). The gel is agitated slowly for 30 minutes or until the tracking dye is no longer visible.
10. The acetic acid is decanted and 500 ml of distilled water is poured in the tray and rinsed thoroughly by placing the tray on oscillatory automatic shaker for 20 minutes.
11. Meanwhile 500 ml of 0.1% silver nitrate solution is prepared in amber color bottle and 750 µl of 37% formaldehyde is added and mixed. Distilled water is gently decanted from tray. The gel is stained for 45 minutes in silver nitrate solution with constant shaking in a dark room or covering the tray with black cloth.
12. Then the gel is rinsed briefly for 25 seconds in distilled water.
13. Working quickly distilled water is decanted from the tray. The freshly prepared and chilled 3% sodium carbonate solution (3% $Na_2CO_3$ and 750µl of 37% formaldehyde + 1% sodium thiosulfate) is transferred to the tray. The gel is kept immersed until bands get developed sharply.
14. The gel is given 10 percent acetic acid (stop solution) treatment for 10 minutes. Then 500 ml distilled water is added to tray.
15. The gel is transferred gently on the transparency; excess water is soaked with tissue paper, and air dried for half an hour.

*Observations*:

The silver stained gel is kept on transilluminator and SSCP variants are recorded. Then gel is labeled and scanned for computer image analysis and documentation.

## 8.4 References


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# 9. Dot Blot

## 9.1 Introduction

Dot and slot blotting are simple techniques for immobilizing bulk unfractionated DNA on a nitrocellulose or nylon membrane. Hybridization analysis can then be carried out to determine the relative abundance of target sequences in the blotted DNA preparations. Dot and slot blots differ only in the geometry of the blot, a series of spots giving a hybridization pattern that is amenable to analysis by densitometric scanning. Samples are usually applied to the membrane using a manifold attached to a suction device.

The technique offers significant savings in time, as chromatography or gel electrophoresis, and the complex blotting procedures for the gel are not required. However, it offers no information on the size of the target biomolecule. Furthermore, if two molecules of different sizes are detected, they will still appear as a single dot. Dot blots therefore can only confirm the presence or absence of a biomolecule or biomolecules which can be detected by the DNA probes or the antibody.

A hybridization experiment can be divided into three stages. First, the membrane isincubated in a prehybridization solution containing reagents that block nonspecific DNAbinding sites on its surface, thereby reducing background hybridization. In this protocol,the blocking agents are Denhardt solution and denatured salmon sperm DNA. In the second stage, the prehybridization solution isreplaced by fresh buffer containing the labeled probe, and an overnight incubation iscarried out to allow the probe to bind to target sequences in the immobilized DNA. Duringthis hybridization step, the probe pairs not only with target sites that have 100%ncomplementarity with the probe, but also with related sequences. In the final stage of theexperiment the membrane is washed with a series of solutions that gradually removebound probe molecules until only highly matched hybrids remain.

**9.2 Materials**

DNA to be used as probe

**20x TAE MW g/L**

0.8 M Tris 121.1 96.9

0.4 M base sodium acetate 82.04 32.8

20 mM EDTA 372.2 7.45

pH to 7.4 with glacial acetic acid

**5x TBE MW g/L**

0.5 M boric acid 61.8 30.9

0.5 M Tris base 121.1 60.5

10 mM EDTA 372.2 3.73

**20x SSC MW g/L**

3 M NaCl 58.44 175.0

0.3 M trisodium citrate 294.1 88.2

**20x SSPE MW g/L**

3.6 M NaCl 58.44 210.0

0.2 M Na2HPO4•7 H2O 268.07 53.6

20 mM EDTA 372.2 7.44

**TE**

10 mMTris-HCl, pH 8.0

1 mM EDTA, pH 8.0

**100x Denhardt's Solution MW g/100 ml**

2% bovine serum albumin 2

2% polyvinylpyrrolidone 360,000 2

2% Ficoll 400,000 2

**20% SDS MW g/L**

20% sodium dodecyl sulfate 288.38 200

Heat to 65°C to get into solution

**1 M Na2HPO4, pH 7.2 MW g/L**

1 M Na2HPO4•7 H2O 268.07 268.07

Add 4 ml 85% H3PO4 [1 M in Na+, see Reference 4]

### 9.3 Instruments

- Hybridization oven (e.g., Hybridiser HB-1, Techne) *or* 68° C water bathor incubator
- Hybridization tube *or* sealable bag and heat sealer
- Additional reagents and equipment for DNA labeling by nick translation or random oligonucleotide priming

- ***CAUTION: Wear gloves to protect your hands from the alkali solution and to protect the membrane from contamination. Avoid handling nitrocellulose and nylon membranes even with gloved hands—use clean blunt-ended forceps instead.***

### A. Application of DNA Dots for hybridization

1. Cut a strip of uncharged nylon membrane to the desired size and mark out a grid of 0.5-cm x 0.5-cm squares with a blunt pencil. Pour 6x SSC to a depth of ~0.5 cm in a glass dish; place membrane on the surface and allow to submerge. Leave 10 min.

*A nitrocellulose membrane should be wetted in 20x instead of 6x SSC, and a positively charged nylon membrane should be wetted in distilled water.*

2. To each DNA sample, add 1/2 vol of 20x SSC to give a final concentration of 6x SSC in the minimum possible volume. Denature the DNA by placing in a water bath or oven for 10 min at 100° C, then place in ice.

*The amount of DNA that should be blotted will depend on the relative abundance of the target sequence that will subsequently be sought by hybridization probing. The sample the volume should be no more than 30 ul and if possible much less. If necessary, reduce volume by ethanol precipitation before adding SSC.*

*If using positively charged nylon, add 1 M NaOH and 200 mM EDTA, pH 8.2, to each sample to give a final concentration of 0.4 M NaOH/10 mM EDTA, then heat as described.*

*If using a nitrocellulose membrane, add an equal volume of 20□ SSC to each sample after placing on ice.*

3. Place the wetted membrane over the top of an open plastic box so that the bulk of the membrane is freely suspended.

4. Spin each sample in a microcentrifuge for 5 sec, spot onto the membrane using a pipet, and allow to dry.

*Do not touch the membrane with the pipet when applying the samples. Up to 2 ul can be spotted in one application. If the sample volume is >2 ul, it should be applied in successive 2-ul aliquots, with each spot being allowed to dry before the next aliquot is applied on top. Drying can be aided with a hair dryer, but be careful that the blower does not spread the sample over the surface of the membrane. Try to keep the diameter of each dot to <4 mm.*

**B. Fixing the membrane for hybridization**

Use only one method of the following

**-To fix by baking in a vacuum oven**

a. Remove the membrane from the 6x SSC and allow excess fluid to drain away. Place the membraneflat on a paper towel to dry for at least 30 minutes at room temperature.

b. Sandwich the membrane between two sheets of dry blotting paper. Bake for 30 minutes to 2 hoursat 80°C in a vacuum oven.

*Overbaking can cause nitrocellulose membranes to become brittle. If the gel was not completely neutralized before the DNA was transferred, nitrocellulose membranes will turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.*

**-To fix by baking in a microwave oven**

a. Place the damp membrane on a dry piece of blotting paper.

b. Heat the membrane for 2-3 minutes at full power in a microwave oven (750-900 W).*Proceed directly to hybridization or dry the membrane and store itbetween sheets of blotting paper until it is needed.*

**To cross-link by UV irradiation**

a-Place the damp membrane on a dry piece of blotting paper.

b- Irradiate at 254 nm to cross-link the DNA to the membrane.

*Make sure that the side of the membrane carrying the DNA faces the UV light source.*

## 9.4 METHOD

1. Float the membrane containing the target DNA on the surface of a tray of 6x SSC (or 6x SSPE) until the membrane becomes thoroughly wetted from beneath. Submerge the membrane for 2 minutes.

2. Prehybridize the membrane by one of the following methods.

**For hybridization in a heat-sealable bag**

a. Slip the wet membrane into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent), and add 0.2ml of prehybridization solution for each square centimeter of membrane. Squeeze as much air as
possible from the bag.

b. Seal the open end of the bag with a heat sealer and then make a second seal. Test the strength andintegrity of the seal by gently squeezing the bag. Incubate the bag for 1-2 hours submerged in a
water bath set to the appropriate temperature (68°C for aqueous solvents; 42°C for solventscontaining 50% formamide; 65°C for phosphate-SDS solvents).

**For hybridization in a roller bottle**

a. Gently roll the wetted membrane into the shape of a cylinder and place it inside a hybridization rollerbottle together with the plastic mesh provided by the manufacturer. Add 0.1 ml of prehybridization
solution for each square centimeter of membrane. Close the bottle tightly.

b. Place the hybridization tube inside a prewarmed hybridization oven at the appropriate temperature(68°C for aqueous solvents; 42°C for solvents containing 50% formamide; 65°C for phosphate-SDSsolvents).

**For hybridization in a plastic container**

a. Place the wetted membrane in a plastic (e.g., Tupperware) container, and add 0.2 ml ofprehybridization solution for each square centimeter of membrane.

b. Seal the box with the lid and place the box on a rocking platform in an air incubator set at theappropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide;
65°C for phosphate-SDS solvents).

3. If the probe is double-stranded DNA, denature it by heating for 5 minutes at 100°C. Chill the probe rapidly in ice water.

*Alternatively, denature DNA probes by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, chill the probe to 0°C in an ice-water bath, and add 0.05 volume of 1 M Tris-Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.*

*Single-stranded DNA and RNA probes need not be denatured.*

4. To hybridize the probe to a blot containing genomic DNA, carry out one of the following methods.

**For hybridization in a roller bottle**

a. Pour off the prehybridization solution from the hybridization bottle and replace with freshhybridization solution containing probe.

b. Seal bottle and replace in hybridization oven. Incubate for the required period of hybridization.

**For hybridization in a plastic container**

a. Transfer the membrane from the container to a sealable bag or a hybridization bottle.

b. Immediately treat as described above.

5. After hybridization, wash the membrane.

**For hybridization in a heat-sealable bag**

a. Wearing gloves, remove the bag from the water bath, remove the outer bag, and immediately cut offone corner of the inner bag. Pour out the hybridization solution into a

container suitable for disposalof radioactivity, and then cut the bag along the length of three sides.

b. Remove the membrane and immediately submerge it in a tray containing several hundred millilitresof 2x SSC and 0.5% SDS (i.e., approx. 1 ml/cm2 membrane) at room temperature. Agitate the traygently on a slowly rotating platform.

**For hybridization in a roller bottle**

a. Remove the membrane from the hybridization bottle, and briefly drain excess hybridization solutionfrom the membrane by holding the corner of the membrane to the lip of the bottle or container.

b. Place the membrane in a tray containing several hundred milliliters of 2x SSC and 0.5% SDS (i.e.,approx. 1 ml/cm2 membrane) at room temperature. Agitate the tray gently on a slowly rotatingplatform.

*When hybridizing in phosphate-SDS solution, remove the membrane from the hybridization chamber as described in Step 5 and place it in several hundred milliliters (i.e., approx. 1 ml/cm2 membrane) of Phosphate-SDS washing solution 1 at 65°C. Agitate the tray. Repeat this rinse once.*

- **IMPORTANT** *Do not allow the membrane to dry out at any stage during the washing procedure.*

6. After 5 minutes, pour off the first rinse solution into a radioactivity disposal container and add several hundred milliliters of 2x SSC and 0.1% SDS to the tray. Incubate for 15 minutes at room temperature with occasional gentle agitation.

*If hybridization was carried out in a phosphate-SDS buffer, rinse the membrane a total of eight times for 5 minutes each in several hundred milliliters of Phosphate-SDS washing solution 2 at 65°C. Skip to Step 9 after the eighth rinse.*

7. Replace the rinse solution with several hundred milliliters of fresh 0.1x SSC with 0.1% SDS. Incubate the membrane for 30 minutes to 4 hours at 65°C with gentle agitation. *During the washing step, periodically monitor the amount of radioactivity on the membrane using a handheldminimonitor. The parts of the membrane that do not contain DNA should not emit a*

*detectablesignal. Do not expect to pick up a signal on the minimonitor from membranes containing mammalianDNA that has been hybridized to single-copy probes.*

8. Briefly wash the membrane with 0.1x SSC at room temperature.

9. Remove most of the liquid from the membrane by placing it on a pad of paper towels. Place the damp membrane on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink (orphosphorescent dots) to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the membrane. Cover the labels with Scotch Tape. This prevents contaminationof the film holder or intensifying screen with the radioactive ink.
*Alternatively, dry the membrane in the air and glue it to a piece of 3MM paper using a water-soluble glue.*

10. Cover the membrane with a sheet of Saran Wrap, and expose the membrane to X-ray film for 16-24 hours at -70°C with an intensifying screen to obtain an autoradiographic image.
*Alternatively, cover the hybridized and rinsed membrane with Sarap Wrap, and expose it to a phosphorimager plate. An exposure time of 1-4 hours is usually long enough to detect single-copy gene sequences in a Southern blot of mammalian genomic DNA.*

## 9.5 REFERENCES

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## 10. Hemagglutination

Hemagglutination is an assay based on the ability of antibodies binding to their antigen to alter the physical state of the bound antigen. In contrast to the ELISA, that works on the principle of directly measuring antibody to antigen binding, hemagglutination detects secondary interactions, as the clumping/agglutination of large particles, red blood cells (hema-) containing antigen on their surface by the respective specific antibodies. Binding of the antibodies to their antigen found on the surface of the red blood cells causeshemagglutination. Hemagglutination is usually utilized to determine the ABO blood group of blood donors and transfusion recipients. Cells agglutinate when crosslinked by antibodies, requiring antibodies to have at least two identical antigen-binding sites. Hemagglutination is similar to the precipitin reaction. Lattice formation between antibody (multivalent) and multivalent antigen containing cells leads to agglutination, with no agglutination in areas of excess antibody or antigen. Agglutination can be caused by the primary, first antibody, in direct agglutination, or by a second antibody directed against the first, in indirect agglutination. Agglutination using antibodies to antigenic components adsorbed to red cells (or inert particles) is called passive hemagglutination (passive agglutination) (1,3,4).

Simple Hemagglutination Experiment

In a simple experiment, TNP-specific conjugated sheep red blood cells can be utilized to test whether antibodies present in the supernatant can mediate hemagglutination. This experiment is performed in both the presence and absence of anti-IgM antibodies, which provide further information on the valency of the IgM molecules. Agglutinated red blood cells will occur if the antibody can crosslink them. Monomeric IgM is incapable of hemagglutination at the level of efficiency that polymeric IgM is capable. Addition of the anti-IgM will aggregate the monomeric IgM, allowing for the agglutination of the red blood cells like a polymeric IgM. You are therefore capable of assessing, which IgM supernatants contain the ability to form pentamers and/or hexamers and which could not. NonpolymericIgM will form red "bottoms" on the microtitre, while polymeric IgM will not, but form the agglutinate at the side of the well. The addition/subtraction of anti-IgM would depict IgM that need antibodies to aggregate them into polymerics to form the hemagglutination reaction.

Agglutination Reaction

The agglutination reaction has become less used for research and diagnosis, because it is not possible to precisely quantitate the reaction (with modern technology, such as light diffraction it is possible). Moreover, there is a high percentage of error, as failure to observe the presence or absence of agglutination in one tube or one doubling dilution is representative of a 100% error. Visualization of precipitates can be erroneous. Therefore, agglutination is seldom used, being replaced more often by various types of immunoassays (3).

Anti-Mu Antibody

Addition of anti-mu antibody, an appropriate amount, would be able to aggregate any IgM, which is monomeric, rather than polymeric, enabling for agglutination of the red blood cells. This would allow us to reveal if any secreted IgM, that could not agglutinate or fix complement efficiently like polymeric IgM, to be due to a mutation that affected IgM from polymerizing, rather than due to a V-region mutation, because no agglutination would be visible in monomeric, with added non anti-mu, IgM.

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## 11. Diagnosis of hypoalbuminaemia using radial immunodiffusion (RID)

### 11.1Principle:

The radial immunodiffusion (RID) is a technique which involves the detection of any substance based on antigen-antibody reaction. Any substance to which an antibody is available can be estimated by this technique. The antibody is incorporated into an agar gel in a petri disc. Tiny holes are made in the disc and the antigen (any substance to be estimated) is added in a measured volume in the holes and the petri disc is covered. It is kept at room temperature. After incubation, diffusion proceeds and the antigen which has been allowed to diffuse into the agar reacts with specific antibody, produces a ring of precipitation that will form at the point where the antigen and antibody have reached equivalence. However, as diffusion proceeds radially from the well, an excess of antigen develops in the area of the precipitate causing it to dissolve only to form once again a greater distance from the site of origin. Precipitate will occur only at the zone of equivalence. The greater the concentration of the antigen in the well, the faster precipitation will take place. Diffusion of antigen will proceed from the well with a build-up of precipitate at the outer edge of the ring, where the antigen will be encountering additional antibody. The system is initially in a dynamic state, as the rings increase with time. A static state of precipitation is reached when the entire antigen has diffused into the gel and precipitation is complete.

The diameter ofthe precipitin ring is proportional to the concentration of the antigen present in the test sample. Bycomparing the diameter of the test specimen precipitin ring to known standards, the estimation of the concentration of specific antigen can be achieved.

Radial immunodiffusion is a reliable quantitative method and is particularly useful for difficult samples,e.g., that are turbid and for which other methods are inappropriate. This test is

commonly used in the clinical laboratory forthe determination of immunoglobulin levels in patient samples.

The technique is relatively simple, rapid to perform and of low cost because it requires no specialequipment.

**11.2Reagents**

RID kit for albumin.

Agarose 0.75 g 1.5 g 3 g

5X Assay buffer

Antiserum 2.5 ml 4.5 ml 9 ml

Standard Antigen 0.5 ml 1 ml 2 ml

Test Antigen 200 µl 400 µl 800 µl

Alcohol

Distilled water.

(All the reagents should be stored at 2-8°C when not in use).

**11.3Glassware:**

Conical flask,

Measuring cylinder.

**11.4Other Requirements:**

Micropipette,

Tips,

Moist chamber (box with wet cotton),

Gel puncher or bore mouth 1 ml micro tip, Petridish.

**11.5PREPARATION OF REAGENTS**

**Assay Buffer:** Prepare 1X **Assay buffer** by diluting it with distilled water (Add 20 ml distilled water to 5ml 5X Assay buffer. The diluted buffer can be stored at 4°C for further use.

**Preparation of Standards (serial dilutions)**

**Concentration of given Standard antigen is 2 mg/ml**

1. Label four micro test tubes: 1:2, 1:4, 1:8, and 1:16.

2. Using a micropipette, add 50 µl of 1X Assay buffer to each tube.

3. With a fresh pipette tip, add 50 µl of Standard antigen to the tube labeled 1:2. Mix well.

4. With a fresh pipette tip, transfer 50 µl of the 1:2 dilutions to the tube labeled 1:4. Mix well.

5. With a fresh pipette tip, transfer 50 µl of the 1:4 dilutions to the tube labeled 1:8. Mix well.

6. With a fresh pipette tip, transfer 50 µl of the 1:8 dilutions to the tube labeled 1:16. Mix well.

7. There are now five antigen samples for the standard curve (see table).

**Tube No. Dilution Concentration**

1 Undiluted 2 mg/ml

2 1:2 1 mg/ml

3 1:4 0.5 mg/ml

4 1:8 0.25 mg/ml

5 1:16 0.125 mg/ml

**11.6Procedure:**

**11.6.1 Preparation of Agarose Gel**

1. Prepare 17 ml of 1.0% agarose (0.17 g) in 1X Assay buffer by heating slowly till agarose dissolvescompletely. Take care not to froth the solution.

2. Allow the molten agarose to cool to approx 55°C. Save approximately 2 ml of molten agarosesolution for sealing the wells.

3. Add 450 µl of Antiserum to 15 ml of agarose solution. Mix by gentle swirling for uniform distribution ofantibody.

4. Pour agarose solution containing the antiserum onto a clean Petridish and allow it to solidify for 15-20 minutes.

5. After solidification, the gel will appear slightly opaque.

6. Now punch the wells using gel puncher or with back of 1 ml micropipette tip corresponding to thetemplate given below. The distances between the wells are important. Try to follow the template asaccurately as possible.

7. Seal the wells with 20 µl of molten agarose solution per well and ensure that the distribution isuniform.

8. Allow them to solidify for 15-20 minutes.

### 11.6.2 Loading of sample

a) Before loading label the wells on the bottom of the plate by marker as 1, 2, 3, 4, 5, 6, 7, and 8.

b) In well # 1, load 30 µl of the undiluted standard sample.

c) In well # 2, load 30 µlof the 1:2 standard antigen dilution.

d) In well # 3, load 30 µlof the 1:4 standard antigen dilution.

e) In well # 4, load 30 µlof the 1:8 standard antigen dilution.

f) In well # 5, load 30 µlof the 1:16 standard antigen dilution.

g) Load 30 µlof test antigen in the well 6-8.

h) Label the cover of the Petridish. Place the dish (do not invert) inside the moist chamber (boxcontaining wet cotton) and incubate at 37°C for overnight or at room temperature for 24 to 48 h.

### 11.6.3 Reading the results

The precipitin rings will be visible in 24 to 48 hours. Carefully hold a plate up so that the overheadroom lights shine through it. You should be able to see opaque circles around each well whereantigen and antibody have precipitated.

With a ruler, measure the diameter (through the centers of the wells) of the precipitin ring inmillimeters. Note down your observations.

Plot a graph of diameter of ring (on Y-axis) versus concentration of antigen (on X-axis) on a semiloggraph sheet. Calculate the value of the unknown antigen concentration from the graph.

**11.7Results:**

## 11.8Discussion

**11.9Questions:**

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