

## Experiment # 1

Objective: - To calculate the percentage of error in pipette.

Materials required :-

Pipettes, Tips, MCT, and Water

Apparatus required :-

Weighing balance

Procedure :

1. Empty MCT was weighed.

2. Using the pipette 1 ml water was added to the MCT.

3. MCT containing water was weighed.

Calculation and observation :

$$\text{Weight of MCT (empty)} = 1.1123 \text{ g}$$

$$\text{Weight of MCT (with water)} = 2.1031 \text{ g}$$

f. Actual weight of water = 0.9908

Calculated weight of water

$$= 1 \text{ ml} \times 1 \text{ g/ml} = 1 \text{ g}$$

$$\% \text{ error} = \frac{| \text{actual weight of water} - \text{calculated weight of water} |}{\text{calculated weight of water}} \times 100\%$$

actual weight of  
water

$$= \left| \frac{0.9908 - 1}{0.9908} \right| \times 100\%$$

$$= 0.9285\%$$

## Experiment #2

Isolation of genomic DNA from E. coli.

Objective :- To isolate the genomic DNA  
E. coli

Materials required :- Microcentrifuge tubes, Pipettes,  
pipette tips.

Solutions required :-

- 1) LB Broth
- 2) TE buffer (pH 8)  
100 mM Tris base  
10 mM EDTA
- 3) RNase A : 10 mg /ml

4) Solution I :

TE buffer : 900 ul  
RNase A : 100 ul

5) 10% SDS

6) Proteinase K : 10 mg /ml

7) Phenol - Chloroform mixture

8) 5M sodium acetate (pH 5.5)

9) Isopropanol

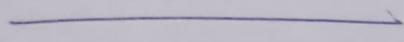
10) 70% Ethanol

Apparatus required :- Centrifuge, Water bath.

Procedure :

1. Inoculate a single colony of E. coli in LB broth.
2. Take 5ml of overnight grown E. coli culture was taken and cells were harvested at 12000 RPM for 2 minutes.
3. Wash the pellet with 1ml MilliQ and centrifuge at 12000 RPM for 2 min.
4. 875ml of solution I was added and cells were resuspended.
5. 100  $\mu$ l of 10% SDS and 5  $\mu$ l of Proteinase K was added to the cells.
6. The mixture was well mixed and then incubated at 37°C for an hour.
7. 1 ml of phenol-chloroform mixture was added and mixed. Kept at room temperature for 5 minutes.

8. Centrifuge the contents at 5,000 RPM for 10 minutes at 4°C.
9. Collect the highly viscous jelly like supernatant.
10. Repeated the process once again with phenol-chloroform mixture.
11. Added 100ul of 5M sodium acetate and then mixed gently.
12. Added 2 volume of isopropanol.
13. Centrifuge the contents at 5000 RPM for 10 minutes.
14. Remove the supernatant and add 1 ml 70% ethanol.
15. Centrifuge the above contents at 5,000 RPM for 10 minutes.
16. After air drying for 5 minutes, add 2ml of TE buffers.



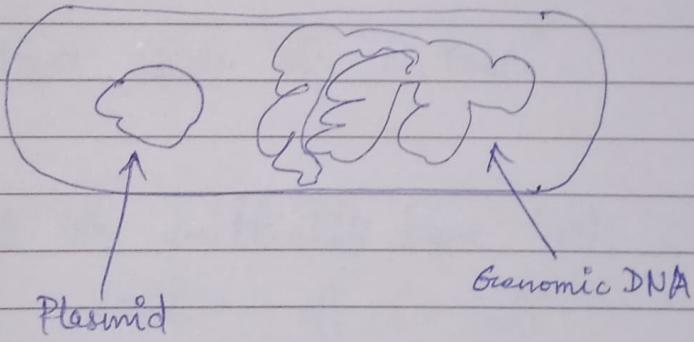
## Experiment #3

— ~~filtration~~ — 242 222  
— ~~homogenization~~ — ~~one minute~~

Objective: Plasmid DNA Isolation from  
*E. coli* culture. (Manual method)

### Methodology

- ① Plasmids are small circular DNA which are found in prokaryotic organisms (i.e bacteria).



- ② Why we want to isolate plasmid?

Ans:- Many a times we wish to see, if there is plasmid inside our the strain grown

or not: In order to confirm this we need to isolate the plasmid and observe.

Q3. How can plasmid be isolated?

Ans:- As we know that plasmids are found inside the bacterial cell. The number of plasmids in the cytosol can be one or two to even hundreds.

In order to isolate the plasmid and get the plasmid separated, we need to get rid of everything else. So, this is the main philosophy we follow for getting the plasmid.

A4. Here we have isolated plasmid from "DH5 $\alpha$  strain of E. coli".

(A) We initially have 5ml culture of E. coli



(B) In order to get the cells only from the culture

⇒ Centrifuge at 12000 RPM.  
for 2 minutes

④ To get rid of media completely and have only the bacteria cells

⇒ Wash the pellet with Milli Q

⑤ Again centrifuge at 12000 RPM for 2 min

↓  
We only have the cells  
⇒ Pure form of cells

⑤ Then we have 3 solutions

Solution I

Solution II

Solution III

Glucose +  
Tris HCl +  
EDTA

→ Buffering action

Chelates Metal ions

and saves DNA/RNA ,

NaOH

+

SDS

→ lysis of cell wall, so,

then plasmid

DNA can come out

Potassium Acetate

+

Glacial acetic acid

Attaches to Proteins

KAc precipitates

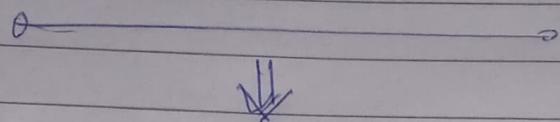
this

+ Neutralisation of solution

so that plasmid DNA can renature

6. Now → Centrifuge at 12000 RPM

for 15 min.



Plasmid DNA will be in supernatant

and everything else will be precipitated.



'Get the supernatant.'

7. How to get the plasmid from supernatant?

→ Precipitate it



For this we add 0.8 volume of Isopropanol.

⇒ Plasmid DNA is not soluble in Isopropanol.



Centrifuge at 12000 RPM for 15 minutes.

Pellet



(8.) Now, we have the plasmid. So,

we only need to clean it and get the pure form.

(A) Wash the pellet with

70% ethanol

⇒ 2 min centrifuge

at 12000 RPM.

(B) Air dry the pellet.

(C) Add TE buffer to preserve  
this at specific pH

(9.) In this process RNA also comes out  
with the DNA (small plasmid DNA).

\* We will have to degrade  
these

⇒ Add RNase and keep this for  
1 hour at 37°C.

(10)

Now, we only have the plasmid in the remaining solution.

⇒ This can be checked by gel Electrophoresis or Absorbance measurement.

o o o o

### Question for Discussion

How the DNA is denatured including plasmid DNA?

What about renaturation process?

Here we are not using heat or any enzyme like helicase and ATP?

## Experiment #4

### Agarose gel electrophoresis

Objective :- To run the genomic DNA and plasmid DNA into agarose gel and understand the principles.

### Methodology and Experimental set up

① The principle of agarose gel electrophoresis is when you macromolecules like DNA, RNA etc. into a viscous fluid,

"The macro molecules are separated according to there size"

⇒ Since bigger molecules face more drag force than smaller molecules.

⇒ Thus bigger molecules lag behind and smaller ones go ahead.

## (2) Agarose gel

Agarose is solid material. There we have this in powder form. Agarose comes from a seaweed named agarose.

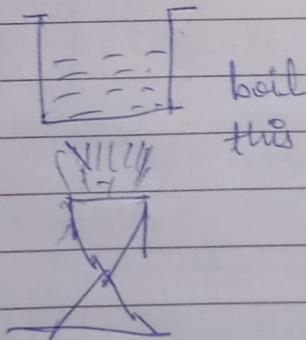
Now, to prepare the gel,

0.8% gel

0.8%  $\Rightarrow$  Agarose gel

|  $\rightarrow$  solvent  $\Rightarrow$  TAE buffer

$\hookrightarrow$  To dissolve the agarose in  
TAE buffer



boil the  
agarose in TAE mixture



Add EtBr  $\Rightarrow$  Why?



EtBr intercalates in the grooves of  
DNA

(3.) Why EtBr is added?

To visualize DNA under UV light,

The DNA should be unbound (at least a bit)



EtBr intercalates between the grooves of DNA and then

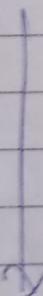
↓ → This surrounds the DNA

If we expose this to UV light the DNA can be visualised.

(4.) The Agarose is boiled into TAE until it dissolves.



Then we cool it to room temperature.



→ In order for it to let solidify.

We pour this in Gel casting tray

→ In which a comb is placed



which forms wells.

⑤ DNA can be loaded into the ~~rem~~ wells.

↳ After removing the collotape  
and pouring the TAE into the  
remaining spaces around this.

Wells  $\Rightarrow$  To load DNA



We load our samples and

↳ A ladder for the reference

Buffer in the tank

↳ This is very very important for  
generating electric field.

⑥ loading dye

The DNA is mixed with the loading  
dye before we place it into the  
wells  $\Rightarrow$  loading dye is there for

knowing us where the  
molecules travelling.

7 Voltage of 5-8 V/cm is applied.

↓  
This is for the DNA to run  
inside the gel.

8 Gel documentation unit / UV transiluminator.

↳ After the gel has been run  
↓

We can visualize the DNA under  
UV light.

### \* Questions for discussion →

- 1 The difference between TAE/TBE/TE buffer.
- 2 Applied voltage and gel % relationship.
- 3 Design an experiment to see that DNA is negatively charged.

## Experiment #5

Aim : Preparation of competent cells  
from E. coli DH5 $\alpha$  cells.

### Methodology

#### Pipeline

- ①. Grow the cells  
↓
- ②. Make it competent  
↓
- ③. Preserve it

(1) In order to grow the cells

↳ We can a single colony of E. coli

and then place it the growth media

→ 37°C  
Overnight ] 180 rpm Q2

Q

Primary inoculum / Secondary inoculum ??

- ②. The 1% of primary inoculum is transferred into 100 ml of LB and grown at  $37^{\circ}\text{C}$ , 180 rpm

→ until O.D.<sub>600nm</sub> reaches

0.5 - 0.6. ?

Q.

Why O.D. 0.5 - 0.6?

- ③. After attaining appropriate absorbance keep the culture on ice for 20 min.

→ To the culture to homogenize.

④.

Cref the cells (And washing)

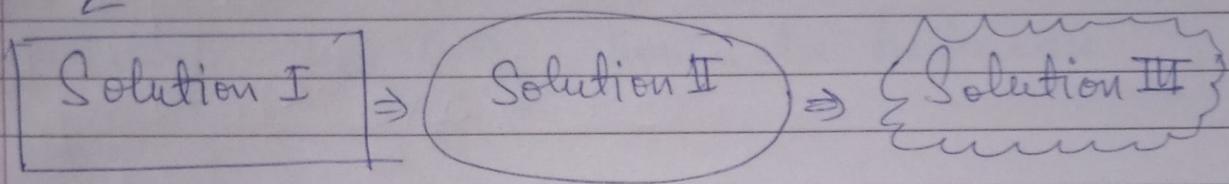
→ 4000 rpm, 5 min at  $4^{\circ}\text{C}$  Centrifuge

→ Again centrifuge

→ To wash.

5.

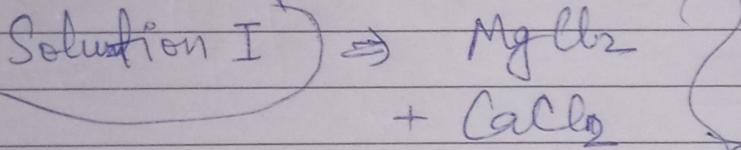
Name In order to make cells  
Competent



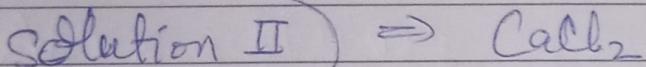
↓

Added sequentially

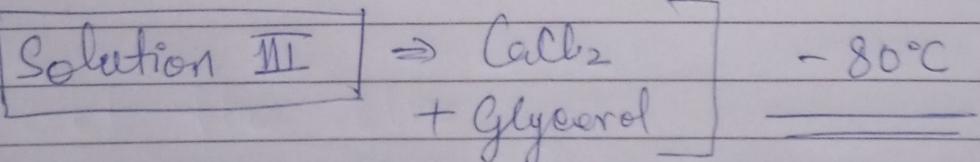
1400 rpm  
5 min, 4°C



Basically



they increase  
the ~~flexibility~~  
flexibility of plasma  
membrane.



- 80°C

↓  
glycerol is to preserve the cells.

## Questions for discussion

- ① Why do we do all these operations at 4°C
- ② What is the difference between roles of  $MgCl_2$  and  $CaCl_2$
- ③ Why the O.D. should be between  $_{600nm}$ .  
0.5 - 0.6. ?

## Experiment # 6

### Restriction Enzyme digestion

~~(\*)~~ Methodology :

#### Restriction enzymes

A restriction enzyme is a protein found in bacteria.

→ It cleaves the DNA at specific sequences

Each restriction enzyme has its special sequence where it cleaves.

#### Restriction Digestion

Mix DNA with Restriction Enzyme in a test tube  $\Rightarrow$  It will cut.

~~(\*)~~ MQ = 8 μl

Plasmid = 8 μl

10x buffer = 2 μl

Enzyme 1 = 1 μl

Enzyme 2 = 1 μl

↓

Mix the mixture by gentle pipetting  
and incubate at 37°C for 1 h

⇒ Why not 3 h?

↳ It will be fully digested.  
↓

So what's wrong with that?

Why 37°C ?

↳ Because the enzyme which we  
are using work best at 37°C.

Why 1 h ?

↳ It takes time for the reaction  
to take place.

(\*) Then we will be doing agarose  
gel electrophoresis. Why?

↓  
In order to visualize the fragments.

Q What is CetSMART buffer?

50 mM KAc  
20 mM Tris-Ac  
10 mM MgAc  
100 µg/ml BSA

Basically it  
has buffer action  
↓

So, the enzyme will  
function at its optimal  
condition.

↳ Maintains pH and provides favourable  
Environment.

\* ~~N~~ S I restriction site



ATGCA / T

\* Xba I restriction site

T / CTA GA

## Discussion

Q

What is the plasmid we are cutting  
actually? (sequence/size).

# Experiment #7

## Polymerase chain reaction

Aim : To amplify a gene using specific primers by polymerase chain reaction.

Materials required :

5 x Phusion buffer, dNTPs, milliQ, forward primer, reverse primer, genomic DNA template, Phusion polymerase, PCR tubes, Thermal cycler.

Protocol :

- ① Assemble the following components in 2 different PCR tubes.

10x Standard Tag Buffers  
= 2.0ul

dNTP = 1.5 ul.

Forward primer f. = 1ul

Reverse primer = .1ul

MgCl<sub>2</sub> = 13.3 ul

Taq polymerase enzyme = 0.2 ul

Template 43 = 2.0ul

Q What is template 43?

Q What is the gene we are going to amplify?  
⇒ Sequence?

Q Forward primer sequence?  
length?

Q Reverse primer sequence?  
length?

② Set the following conditions in a thermal cycler:

Initial denaturation ( $95^{\circ}\text{C}$ ) = 3 min

Denaturation ( $95^{\circ}\text{C}$ ) = 30 sec

Annealing ( $58^{\circ}\text{C}$ ) = 30 seconds.

Q There is specific temperature

required for annealing of different primers. So, why we are annealing

at  $58^{\circ}\text{C}$ ?

Extension ( $72^{\circ}\text{C}$ ) = 30 seconds.

\* This is done for 30 cycles.

→ The ~~opti~~ amplification will

be at the orders of  $10^3$  times.

Final extension ( $72^{\circ}\text{C}$ ) = 10 minutes.

⇒ Why do we require final extension?

⇒  $\boxed{\text{Hold} = \infty}$  ??

③ Keep the PCR mix containing the mixture in the thermal cycler.

④ Prepare 1% agarose gel.

⑤ After PCR is over, take 5  $\mu\text{l}$  of sample add 1  $\mu\text{l}$  of dye and load the product on agarose gel.

⑥ Analyze your result? ) )

What kind of bands, we expect to get? why?

## Exp #8

Aim :

To purify the PCR product by Phenol/Chloroform method.

Materials required :

① MilliQ water

↳ For washing, dilution etc.

② Elution buffer

↳ To ~~wash~~ keep } What is this?

③ Phenol : Chloroform (1 : 1)

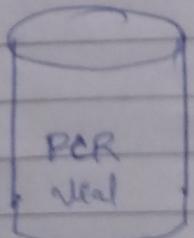
↳ For separating proteins and DNA

④ Ice cold 100% ethanol.

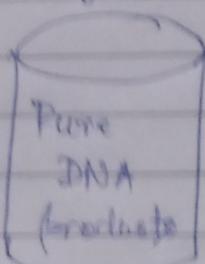
↳ To wash

Methadology

What we have



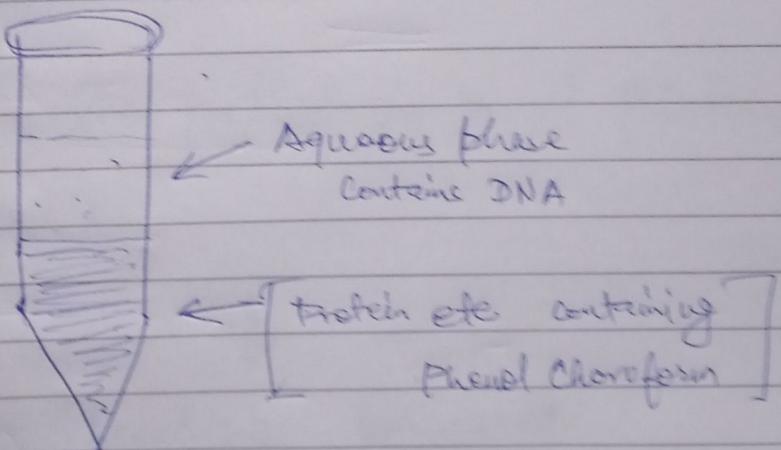
To get



## Procedure : (As written in the manual )

- ① Transfer the contents of the PER vial to a new MCT.
- ② Add 240  $\mu$ l of MilliQ water to it
- ③ Then 200  $\mu$ l of Phenol Chloroform (1:1) to the same MCT.
- ④ Mix the above by gently separating.
- ⑤ Centrifuge the tube at 15000 rpm for 15 minutes.

$\Rightarrow$  Result will be



(6.) Collect the upper layer carefully  
in a fresh MCT.

Approximate the volume.

(7.) Add two volumes of 100% Ethanol  
algy? → to the same vial.

(8.) Keep the MCT at -20°C for an hour.  
algy? →

(9.) Centrifuge the MCT for 20 minutes at  
12000 RPM.

(10.) Discard the supernatant.

(11.) Air dry the pellet till ethanol  
evaporates.

(12.) Dissolve the pellet in 30 ml Electon  
Buffer (EB).



For keeping preserved.

## Question for discussion

① Here, we don't get proteins etc in our PCR mix.

Then, what's the reason we need to add chloroform phenol?

② Why is it necessary to keep the mixture of ethanol at -20°C?

## Experiment #8

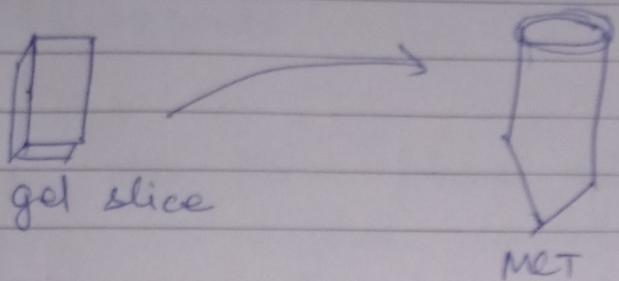
To recover DNA from agarose gel

- ① Electrophorese DNA in an agarose gel prepared in 1X TAE Buffer.

⇒ Result

⇒ Result ⇒

- ② Excise gel slice containing DNA and put it in a MCT.



- ③ Weigh the gel slice and calculate volume based on weight. Why?

Why?

Ans: → By slice was of  
0.0917 g

?? → Density of 1x TAE Agarose gel (0.8%)

↳ ??

➤ This step was skipped for everyone.

- ④ Mash the gel slice with a stir rod

↑

(We did this using a pipette tip.)

5. Add an equal volume of buffer-equilibrated phenol and vortex for 10 seconds

⇒ Here phenol will dissolve the organic contents.

6. Freeze at  $\sim 70^{\circ}\text{C}$  for 5 to 15 minutes

⇒ Why to freeze?

7. Centrifuge 1200 RPM

15 minutes

Supernatant

contains DNA

Take this in a fresh MCT.

8. Phenol-chloroform extract the supernatant

9. Follow with a chloroform extraction

10. Chill the supernatant on ice for 15 minutes.

11. Centrifuge the sample for 15 minutes at maximum speed in a MCT.

12. Carefully collect the supernatant and place in a clean MCT.

13. Measure the volume of the sample and add 0.2 volumes of 10M ammonium acetate to the sample.

Add  $\textcircled{A}$  2 volumes of 100% ice-cold ethanol.

14. Briefly vortex

15. Store the mixture for 30 minutes  $\textcircled{B}$  at room temperature.

(16.) Centrifuge for 80 minutes at 12000 rpm.

(17.) Decant the supernatant.

(18.) Wash the pellet with 70% ethanol.

(19.) Allow air dry at room temperature on the bench top.

(20.) Dissolve the DNA in TE Buffer.



This is to preserve

You can store this at  $-70^{\circ}\text{C}$  now

to keep it preserved  
for long time.

Question for discussion ???

1. How to check ~~the~~ whether there is DNA in our vial or not?

# Experiment #9

Ligation and transformation of  
ligated product to E. coli

DH5 $\alpha$  competent cells.

Ques Prior to Lab?

(1) What is the gene sequence to  
want to insert.

(2) What are sticky ends in them.

Relations to previous lab

We already have done

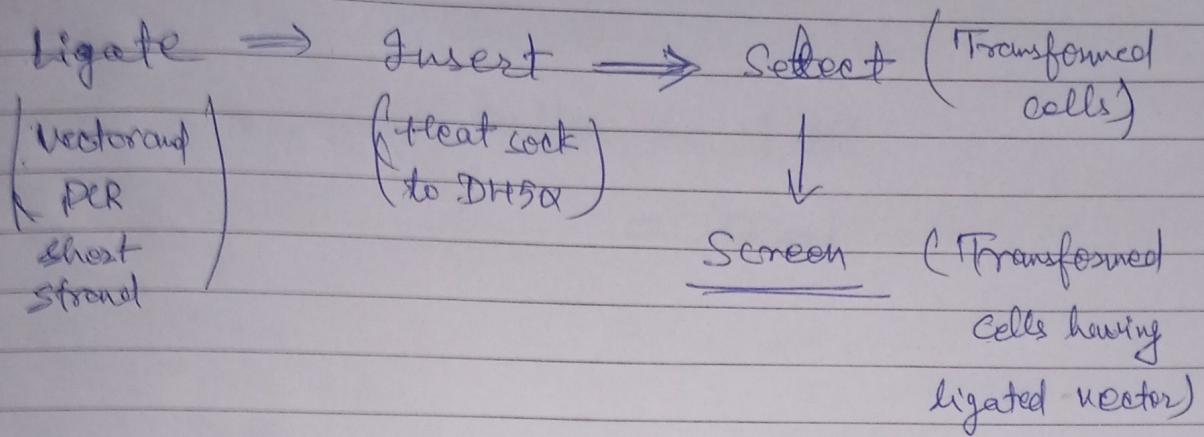
PCR | Comp cell preparation | Restriction  
Digestion

Gene

E. coli  
DH5 $\alpha$ .

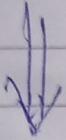
Vector

## Ripeline



② Interesting observation:

No one in entire batch got the gene ligated into DNA vector.



③ Inference  $\Rightarrow$  The ligation of DNA with a vector is a very very rare event.



# Protocol (As per written in the manual)

① Set up the following reaction.

a. 10x Ligase Buffer = 1  $\mu$ l

b. Insert = 3  $\mu$ l

c. Vector = 1  $\mu$ l.

d. M<sub>R</sub> = 4.5  $\mu$ l.

e. T4 DNA ligase = 0.5  $\mu$ l.

f. To Total = 10  $\mu$ l.

Set up a control? Why?



To get acquainted with the  
differences in the result

while having ligation.

control

Control will have same composition  
but no insert

## Control composition:

10x ligase buffer - 1  $\mu$ l

Insert — 0  $\mu$ l

vector — 1  $\mu$ l

Mg — 7.5  $\mu$ l

T4 ligase — 0.5  $\mu$ l

Total — 10.0  $\mu$ l.

Question : Why to add 10x ligase buffer  
in control.

Isn't it costly?

- ② Incubate the mixture in thermal cycler  
at  $22^{\circ}\text{C}$  for 3 hours, followed by  $16^{\circ}\text{C}$   
for 2 hours.



Chef. 3h and then 2 hours?



Is this necessary for the sticky  
ends to join?

Ligation complete



- ③ Inside laminar, add entire ligation mixture to Competent cells and tap to mix.



- ④ Store in ice for 30 minutes.



- ⑤ Give heat shock at  $42^{\circ}\text{C}$  for 90 seconds



Again immediately keep on ice  
for 5 minutes

- ⑥ Inside laminar, add 1ml LB to cells

- ⑦ Keep it at  $37^{\circ}\text{C}$  with shaking for 1 hour



→ For growing the cells and getting the culture of transformed cells

Only the bacteria have  
issues.



### Question?

Qn 4 The antibiotic are  
too high because of  
which the cells died  
and we could not get colonies.

Ans: We had  
the colonies?

This transformation has happened for sure. [

8.

↓  
Get the cells  $\Rightarrow$  spin at 5000 RPM  
for 5 minute

Cells will be pelleted

9.

Discard supernatant (70%)  
and resuspend pellet in remaining  
media.

10.

Now in order to get  
the transformed cells

$\Rightarrow$  Antibiotic Screening is done

$\hookrightarrow$  Kanamycin (50mg/ml)

$\hookrightarrow$  Ampicillin (100mg/ml)

11.

Incubate overnight at 37°C

$\hookrightarrow$  Next day visualize.

② Question for discussion? ?

① Can't we use normal incubator or water bath to keep the ~~at~~ ligation mixture at 37°C for 3 h and then 2 hours.

② Plasmid? Vector? Insert?

Everything else is written →

in the manual

- ① Chemical required,
  - ② Plastic ware/ glassware ,
  - ③ Equipment required etc .
-

## Experiment # 10

Aim:- To identify the overexpressed protein using SDS-PAGE analysis.

Oversexpression: When we ~~not~~ induce which produces the protein using some special inducers, what we get is, ~~said~~ we get much more amount of protein than we can get naturally. Such a phenomena is called oversexpression of protein.

SDS-PAGE:

Sodium dodecyl sulphate

- Polyacrylamide gel electrophoresis

SDS  $\rightarrow$  SDS breaks up the two and three dimensional structure of proteins by adding negative charge to amino acids.

## Polymerisation of acrylamide :

Polyacrylamide gels are formed by copolymerization of acrylamide and bis-acrylamide.

The reaction is a vinyl addition polymerisation initiated by a free radical-generating system.

Polymerisation is initiated by ammonium persulfate and TEMED.

(<sup>↓</sup>Tetra methyl ethylenediamine)

## Stacking gel

\* Stacking gel (5%) pH 6.8

This helps proteins to come in one line so that they can be separated according to their size.

## 5% stacking gel

Components For 5 ml

MQ 3 ml

30% acrylamide 0.734 ml

Tris (pH 6.8) 1.25 ml

10% SDS 50 µl

10% APS 50 µl

TEMED 5 µl

o o

## (\*) Separating gel (12%) pH 8.8

⇒ Separating gel is denser and the pH is high. So, the proteins have negatively charge distributed on it and they repel each other.

Components

For 10 ml

MQ 3.5 ml

30% acrylamide 4 ml

Tris (6.8 pH) 2.5 ml

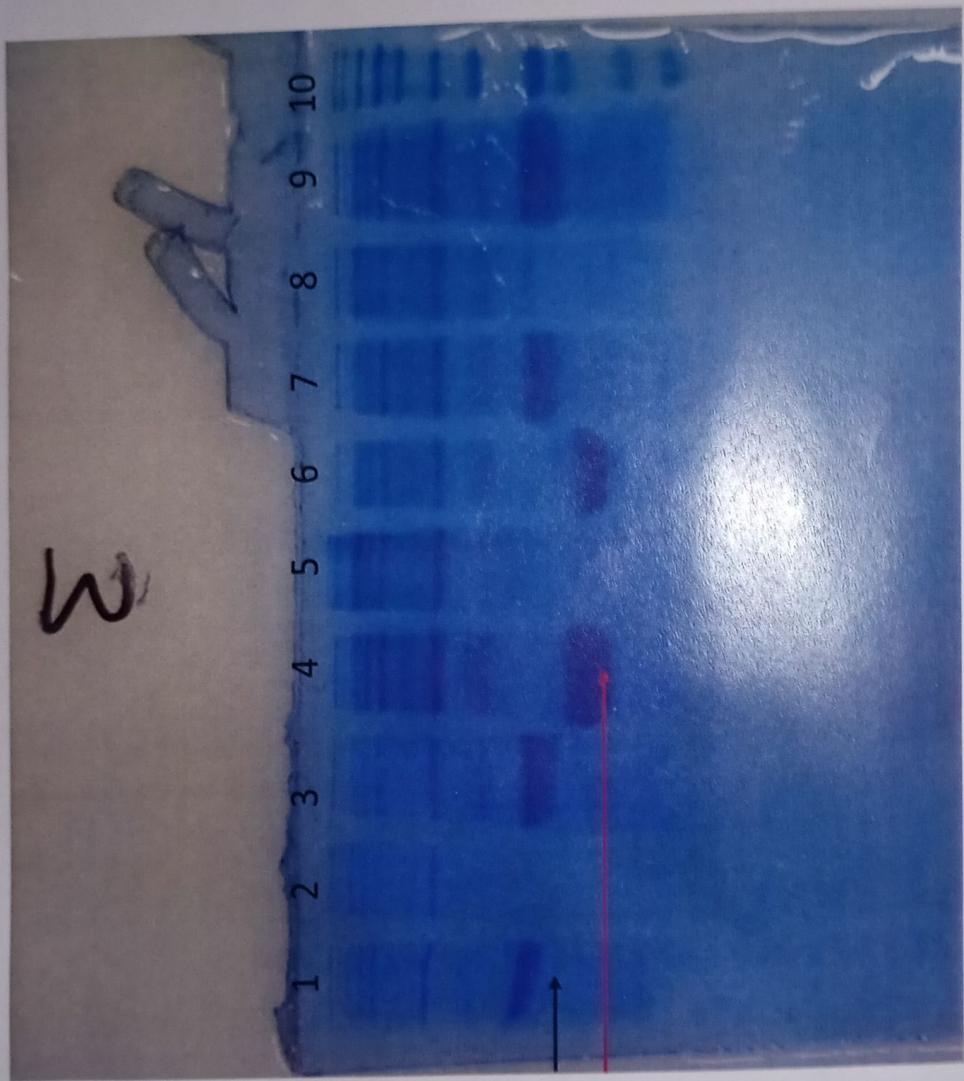
10% SDS 100 µl

10% APS 100 µl

TEMED 10 µl

(\*) Protocol (Exactly the same written in manual)

- ① Take approximately 1ml of bacterial culture from each of the sample and centrifuge at 12000 RPM for 2 min.
- ② Discard supernatant and wash the pellet with MilliQ.
- ③ Centrifuge the suspension then discard the supernatant.
- ④ Add 50 μl of SDS - lysis buffer to the pellet, resuspend the pellet gently in the lysis buffer.
- ⑤ Boil the sample at 95°C for 10 min.
- ⑥ Centrifuge the suspension at 12000 RPM for 5 min.
- ⑦ 15 μl of supernatant was loaded on a SDS-PAGE gel.
- ⑧ Run the gel until dye reaches  $\frac{3}{4}$  th of the gel at 80V.



25 kDa  
18 kDa

(9) Stain the gel with Coomassie R-250  
solution for 15 min.

(10) De-stain the gel with de-staining  
solution.

Q What was the de-staining solution?

Result

(Ponceau)

I got 25kDa protein band.

## Exp # 11

### Colony PCR

Aim: - To screen whether the gene has been ligated into to vector or not using colony PCR.

#### Materials required:

4x Phusion Buffer,

dNTPs

MQ

Forward primer

Reverse primer

Template

Enzyme Phusion

PCR Tubes

Thermal cycler

## Protocol :

① Prepare the reaction mixture —

4x Phusion Buffer — 4 ul

FP — 1 ul

RP — 1 ul

dNTP — 1.5 ul

Template — 3 ul

Enzyme Phusion — 0.2 ul

MA — 9.3 ul.

Total — 20 ul.

② Set the following conditions in thermal cycles.

Initial denaturation ( $95^{\circ}\text{C}$ ) = 10 min

Denaturation ( $95^{\circ}\text{C}$ ) = 1 min

Annealing ( $58^{\circ}\text{C}$ ) = 1 min

Extension ( $72^{\circ}\text{C}$ ) = 2 min

Final extension ( $72^{\circ}\text{C}$ ) = 10 min

Hold ( $4^{\circ}\text{C}$ ) —  $\infty$ .

(3.) Keep the PCR vial containing the mixture in the thermal cycles.

(4.) Prepare 1% agarose gel.

(5.) After PCR is over, take 5ul of sample add 1ul of dye and load the product on agarose gel

(6.) Analyse your gel results.

Observation :

←

Result:

→ We did not get the insert ligated.

## Question

① Why do we keep the initial denaturation for 10 min instead of 3 minutes?

Ans :-

This is for cells to burst out.

② What can be the reason for not being ligation of the insert?

Ans : Sticky ends may not be similar.