OBJECTIVE: Plasmid DNA Isolation from *E. coli* culture (Manual Method).

Chemical required: Glucose, Tris-Cl, EDTA, potassium acetate, glacial acetic acid, NaOH, Sodium dodecyl sulfate, Iso-propanol, RNase (10mg/ml, Qiagen) ddH_2O/MilliQ. LE Agarose (EEO: 0.09-0.13)

Plastic ware/ glassware: Measuring cylinder, autoclaved micro centrifuge tubes, 1.0 ml tips, 0.02ml-0.2 ml tips, enzyme tips, 100ml/250 ml flasks, reagent bottle, 0.2 µ syringe filter.

Equipment required: Horizontal electrophoresis unit, power supply, casting tray, combs, minispin (centrifuge), pH meter.

Recipe for solutions:

1. Solution I (100 ml)

50 mM glucose	1.8 ml of 50 % glucose
25 mM Tris HCl (pH 8.0)	2.5 ml of 1M Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)	2.0 ml of 0.5 M EDTA (pH 8.0)
	93.7 ml of H_2O (autoclaved)

2. Solution II (100 ml)

(This should be freshly prepared)

0.2 N NaOH	10 ml 2N NaOH
1.0 % SDS	10 ml 10% SDS
	80 ml of H_2O (autoclaved)

3. Solution III (100 ml)

Potassium acetate (5M, pH 4.8)	60 ml 5M Potassium Acetate
Glacial acetic acid	11.5 ml Glacial acetic acid
Sterile distilled water	28.5 ml of H_2O (autoclaved)

4. TE (100 ml)

10 mM Tris- HCL (pH 8.0)	1 ml 1 M Tris-HCl (pH 8.0)
1 mM EDTA-sodium salt	0.5 ml 0.5 M EDTA (pH 8.0)

Reagents

98.5 ml of H_2O (autoclaved)

PROTOCOL

Inoculate a single colony of *E. coli* DH5α in 5ml LB and incubate at 37°C, 180 rpm overnight

Harvest cells from overnight grown culture (~O.D$_{600nm}$ 1.0- 3.0) at 12000 rpm for 2min.

Wash the pellet with MilliQ

Resuspend pellet in 100 µl of Solution I and keep on ice for 5 min.

Add 200 µl solution II and thoroughly mix by inverting tube 4-5 times.

Add 150 µl ice-cold solution III and mix by inverting tube 4-5 times, incubate for 5- 10 min on ice

Centrifuge mixture at 12000 rpm for 15 min. Collect supernatant in separate tube and add 0.8 volume of isopropanol followed by incubation on ice for 10-15 min.

To get plasmid DNA centrifuge mixture at 12000 rpm for 15 min.

Wash the pellet with 1 ml 70% ethanol followed by centrifugation at 12000 rpm for 2 min.

Air dry the pellet and dissolve in 40 µl of TE buffer.

Add RNase 100 µg/ml to final concentration and incubate for 1 hr at 37°C.

Check the plasmid DNA on 1 % agarose gel.

TE

M9

Ratnesh

Day 2 Mantel

2/3

OBJECTIVE: Agarose gel electrophoresis

Chemicals required: LE Agarose (EEO: 0.09-0.13), ethidium bromide, tris base, EDTA, glacial acetic acid, bromophenol blue, sucrose, ddH_2O/ Milli Q, NaOH, HCl.

Plastic ware/glassware: measuring cylinder, autoclaved micro centrifuge tubes, 1.0 ml tips, 0.02ml-0.2 ml tips, enzyme tips, 100ml/250 ml flasks, reagent bottle.

Equipments required: Horizontal electrophoresis unit, power supply, casting tray, combs, pH meter.

Recipe for buffer

1. **50X TAE (200 ml)**: 48.4 g of Tris base, 7.44 g of EDTA ,11.42 ml of glacial acetic acid
2. Volume make up to 200 ml by Milli Q. pH adjusted to 8.0.
3. **6X Loading dye**: glycerol 60%, Tris-HCl (pH 7.6) 10 mM, EDTA 60 mM, bromophenol blue 0.03%, xylene cyanol 0.03%.
4. **Ethidium bromide(10mg/ml)**: weigh 10 mg ethidium bromide and add 1ml ddH20/milliQ. Allow it to mix properly (use orbital shaker).

PROTOCOL

1. Prepare 0.8% agarose gel (0.4g in 50 ml 1X TAE), boil it until it dissolves. Cool to RT and add ethidium bromide to final concentration of 1 µg/ml.
2. Cast gel by pouring it into a casting tray with a comb placed in it.
3. Let the gel solidify, place the tray in buffer tank and remove comb.
4. Load DNA (2µl ~~plasmid~~ DNA+1µl 6X loading dye+ 3 µl MilliQ) adjacent to the ladder.
5. Apply voltage of 5-8 V/cm. Visualize the DNA bands under UV light.

Day (2) Manual (3/3) Refresh

OBJECTIVE: Preparation of competent cells from *E. coli* DH5α cells

Chemicals required: $MgCl_2$, $CaCl_2$, glycerol, luria agar (HiMedia), luria broth (HiMedia), ddH2O/MilliQ

Plastic ware/ glassware: measuring cylinder, autoclaved micro centrifuge tubes, 1.0 ml tips, 0.02ml-0.2 ml tips, enzyme tips, 100ml/250 ml flasks, reagent bottles, Test tubes, , Cuvettes.

Equipment required: refrigerated centrifuge, UV spectrophotometer

Recipe for solutions:

Luria broth: 2.0 g in 100 ml (autoclaved)

Solution I (100 ml)

S.No.	Chemical(s)	Concentration	Weight
1.	$MgCl_2$	80 mM	1.642 g
2	$CaCl_2$	20 mM	0.294 g

Solution II (100 ml)

S.No.	Chemical(s)	Concentration	Weight
1.	$CaCl_2$	0.1 M	1.4702 g

Solution III (100 ml)

S.No.	Chemical(s)	Concentration	Weight
1	$CaCl_2$	0.1 M	1.4702 g
2	Glycerol	15%	

Solutions were prepared and autoclaved

PROTOCOL:

1. Inoculate a single colony of *E. coli* DH5α in 5ml LB (primary inoculum) and incubate at 37°C. 180 rpm overnight.

2. Transfer 1 percent of primary inoculum in 100 ml LB and grow at 37°C. 180 rpm until OD_{600nm} reaches ~0.5-0.6.

Why this OD?

5ml - 1ml = 1X
4X 4ml

Ratnish

OBJECTIVE: Restriction enzyme digestion

Day 3

Chemicals required: Plasmids, Respective restriction enzyme, Restriction buffer, MQ (Sterile water), Agarose, 6X loading dye, Ethidium Bromide, 1X TAE buffer

Plastic ware/glassware: autoclaved micro centrifuge tubes, 0.2ml/0.2 ml tips, enzyme tips

Equipment required: Dry bath

PROTOCOL

MQ	- 6.7 ul	MQ -	8 ml
p43	-15 ul	Plasmid -	8 ml
10X CutSmart	- 2.5 ul	10X Buffer -	2 ml
SfoI enzyme	– 0.3 ul	NsiI Enzyme 1 -	1 ml
NdeI enzyme	– 0.5ul	XbaI Enzyme 2 -	1 ml
			20 ml

Total Volume- 25 ul

1 hour

1. Mix slowly by gentle pipetting and incubate at 37° C for 3 hour. → 11:15 AM - starting time
2. After incubation mix 10 µl of the sample and 4 µl of the loading dye.
3. Prepare 1 % agarose gel and load the samples including 1 kb DNA ladder, undigested plasmid and digested plasmid.
4. Perform agarose gel electrophoreses of the sample.
5. Visualize the gel under UV transilluminator.

→ Gel documentation unit

Gel running

11:40 PM

Polymerase Chain Reaction

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

Materials Required: 5X Phusion buffer, dNTPs, milliQ, forward primer, reverse primer, genomic DNA template, Phusion polymerase, PCR tubes, Thermal cycler

Protocol:

1. Assemble the following components in 3 different PCR tubes:

10 X Standard Taq Buffer = 2 µl
dNTP = 1.5 µl
Forward primer (5 pM) = 1 µl
Reverse primer (5 pM) = 1 µl
MilliQ = 13.3 µl
Taq Polymerase enzyme = 0.2 µl
Template DNA = 2 µl
Total = 20 µl

2. Set the following conditions in a thermal cycler:

Initial denaturation (95°) = 3 minutes

Denaturation (95°) = 30 seconds
Annealing (58°) = 30 seconds
Extension (72°) = 30 seconds
} 30 cycles

Final extension (72°) = 10 minutes

Hold = ∞

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

4. Prepare 1% agarose gel (as done previously).

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

6. Analyse your gel results.

3/4

Refresh
17/08/2022

Day 3

Purification of PCR product

Objective:

To purify the PCR product by Phenol:Chloroform method

Materials required:

MiliQ water, Elution Buffer, Phenol: Chloroform (1:1), ice cold 100% ethanol.

Procedure:

1. Transfer the contents of the PCR vial to a new micro-centrifuge tube (MCT).
2. Add 140ul of Mili-Q water to it.
3. Then add 200 ul Phenol Chloroform (1:1) to the same MCT.
4. Mix the above by gently inverting.
5. Centrifuge the tube at 12000 rpm for 15 minutes.
6. Collect the upper layer carefully in a fresh MCT. Approximate the volume.
7. Add two volumes of 100% Ethanol to the same vial.
8. Keep the MCT in -20 degrees refrigerator for an hour.
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 30µl Elution Buffer (EB).

4/4

Rathesh

Day 3

OBJECTIVE: Recovery of DNA from agarose gels

Chemicals required: TE Buffer warmed to 67°C, buffer equilibrated phenol, chloroform, Phenol/chloroform (1:1), 100% ethanol (ice cold), 70% ethanol (room temperature), TE Buffer pH 8.0, 10 M ammonium acetate

Plastic ware/glassware: autoclaved micro centrifuge tubes, 1.0 ml tips, 0.02ml-0.2 ml tips enzyme tips, Scalpel or razor blade, glass or plastic stir rod

Equipments required: vortex mixer, microcentrifuge

PROTOCOL:

1. Electrophorese DNA in an agarose gel prepared in 1X TAE Buffer. ←
2. Excise gel slice containing DNA and put it in a microcentrifuge tube
3. Weigh the gel slice and calculate volume based on weight. ← 0.0917 g
4. Mash the gel slice with the stir rod.
5. Add an equal volume of buffer-equilibrated phenol and vortex for 10 seconds 300 µl TE Buffer + 300µl phenol.
6. Freeze at -70°C for 5 to 15 minutes
7. Centrifuge for 15 minutes at room temperature and remove the supernatant which contains the DNA. Place the supernatant in a clean microcentrifuge tube.
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 microcentrifuge
12. Carefully collect the supernatant and place in a clean micro centrifuge tube
13. Measure the volume of the sample and add 0.2 volumes of 10 M ammonium acetate to the sample. Add 2 volumes of 100% ice-cold ethanol
14. Briefly vortex
15. Store the mixture for 30 minutes to overnight at room temperature.
16. Centrifuge for 30 minutes at 12,000 rpm.
17. Decant the supernatant.
18. Wash the pellet with 70% ethanol
19. Allow to air-dry at room temperature on the bench top.
20. Dissolve the DNA in TE Buffer

1/2 Refresh 24/05/2022

Ligation and transformation of ligated product to *E. coli* DH5α competent cells

Objective: To clone PCR product/antibiotic cassette and transform in *E. coli* DH5α competent cells.

Materials required: Ligase, Insert, vector, buffer, mQ, LA (Kanamycin (50mg/ml stock)/Ampicillin (100mg/ml stock)), Laminar Hood, Pipettes, tips, X-gal (stock 2%), IPTG (1M stock)

Protocol:

1. Set up the following reaction:

	TEST	CONTROL
a.	10X Ligase buffer = 1 µl	10X Ligase Buffer - 1.0
b.	Insert = 3 µl	Insert - 6.0
c.	Vector = 1 µl	Vector - 1.0
d.	mQ = 4.5 µl	MQ - 7.5
e.	T4 DNA ligase = 0.5 µl	T4 Ligase - 0.5
f.	Total = 10 µl	Total - 10.0

2. Incubate the mixture in thermal cycler at 22□ for 3 hours, followed by 16□ for 2 hours.
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□ for 90 seconds and immediately keep on ice for 5 minutes.
6. Inside laminar, add 1 ml LB to the cells.
7. Keep it at 37□ with shaking for 1 hour.
8. Meanwhile, spread 30 µl X-gal and 30 µl IPTG on plates and let it dry.(this step is only for p19 vector)
9. After 1 hour incubation, spin the culture at 5000 rpm, for 5 minutes.
10. Inside laminar, discard 70% supernatant, resuspend pellet in remaining media.
11. Spread the mixture in the desired antibiotic plate.
12. Invert the plate, wrap parafilm, label and store at 37□ overnight.
13. Next day, visualize the plates for transformed colonies.

2/2

Refresh
24/05/2022

Objective: To identify the overexpressed proteins using SDS-PAGE analysis.

Chemicals required: Acrylamide, bis acrylamide, SDS, Tris-HCl, Glycine, HCl.

Plastic ware/glassware: measuring cylinder, autoclaved micro centrifuge tubes, 1.0 ml tips, 0.02ml-0.2 ml tips, enzyme tips, 100ml/250 ml flasks, reagent bottle.

Equipments required: Vertical electrophoresis unit, power supply, casting tray, combs, pH meter.

Solution/buffer preparation:

1X running buffer: 3g Tris, 1g SDS, 14.4g Glycine, volume make upto 1L.

30% Acrylamide solution: 29.2g Acrylamide and 0.8 g bis acrylamide, volume make upto 100 ml.

12% separating/ Resolving gel:

components	For 10 ml
Milli Q	3.5 ml
30% acrylamide	4 ml
Tris (pH 8.8)	2.5 ml
10% SDS	100µl
10% APS	100µl
TEMED	10µl

5% stacking gel.

components	For 5 ml
Milli Q	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

Protocol:

1. Take approximately 1 ml of bacterial culture from each of the sample and centrifuge at 12,000 rpm for 2 min.
2. Discard supernatant and wash the pellet with MilliQ.
3. Centrifuge the suspension then discard the supernatant.
4. Add 50µl 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.