PROTOCOLS

1. *E. coli* Media Recipes –

a. Low Salt LB (Luria-Bertani) Medium –

Low Salt LB medium is needed for use with the Ampicillin antibiotic. For 1 liter, dissolve the following reagents in 950 ml deionized water: 10 g tryptone, 5 g yeast extract, 5 g NaCl. Autoclave for 20 minutes at 121°C. Let cool to ~55°C and add desired antibiotics at this point. For Low Salt LB medium with Ampicillin, add Ampicillin to 50 μg/ml final concentrations.

b. Low Salt LB Agar Plates -

Make Low Salt LB Medium as above and add 15 g/liter agar before autoclaving.

2. *Yeast* expression media –

a. BMGY: Buffered Glycerol-complex Medium (100 ml)

Component	**Volume (ml)**
Yeast Nitrogen Base (10X)	10
1 M potassium phosphate buffer, pH 6.0	10
Biotin (500X)	0.2
Glycerol (10X)	10
Autoclaved distilled water	80

Store at 4°C. The shelf life of this solution is approximately two months

b. BMMH: Buffered minimal Methanol (1000ml)

Dissolve 10g Yeast Extract and 20g peptone in distilled water and make up volume 790 ml with distilled water and autoclave for 20 min at 15 psi pressure. Cool to room temperature and add following reagents -

Component	**Volume (ml)**
Yeast Nitrogen Base (10X)	100
Potassium Phosphate Buffer (1M, pH6)	100
Methanol (filter sterilized)	10
Biotin (500X)	2

c. Stock solutions for BMMH and BMGY media –

Yeast Nitrogen Base (10X) -

Add 134 g YNB to 500 ml DW. Mix and heat till it dissolves (Do not over heat as it is heat labile). Make up volume to 1000 ml and filter sterilized using 0.45 μ filters. Store at 4°C. The shelf life of this solution is approximately one year.

Biotin (500X) –

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at 4°C.The shelf life of this solution is approximately one year.

1 M potassium phosphate buffer, pH 6.0:

Combine 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and confirm that the pH is 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature. The shelf life of this solution is greater than one year.

Glycerol (10X)

Mix 100 ml of glycerol with 900 ml of water. Sterilize by autoclaving and store at room temperature. The shelf life of this solution is greater than one year.

10X M (5% Methanol)

Mix 5 ml of methanol with 95 ml of water. Filter sterilizes and store at 4°C.The shelf life of this solution is approximately two months.

3. *Pichia* Media Recipes –

a. Yeast Extract Peptone Dextrose Medium (YPD) (1 liter) -

1% yeast extract, 2% peptone, 2% dextrose (glucose). Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. Autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D. Store YPD slants or plates are at 4°C. The shelf life is several months.

b. Minimal Dextrose Medium + Histidine (1 liter)

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle.
2. Cool to about 60°C and then add:

100 ml of 10X YNB

2 ml of 500X B

100 ml of 10X D

3. To make MDH, add 10 ml of 100 X H stock solutions. Mix and store at 4°C.
4. For plates, add 15 g agar to the water in Step 1 and proceed.
5. If preparing plates, pour the plates immediately. MD stores well for several months at 4°C.

c. Minimal Methanol + Histidine (1 liter)-

1. For medium, autoclave 800 ml of water for 20 minutes on liquid cycle
2. Cool autoclaved water to 60°C and add:

100 ml of 10X YNB

2 ml of 500X B

100 ml of 10X M

3. To make MMH, add 10 ml of 100 X H stock solutions. Mix and store at 4°C.

4. For plates, add 15 g agar to the water in Step 1 and proceed.

5. After mixing, pour the plates immediately. MM and MMH stores well for several months at 4°C.

4. Preparation of Competent cells

1. Single colony was used to inoculate in 25mL LB media and incubated at 37°C for 16hrs.
2. Then for 25mL culture volume, 1% of the culture was used and was grown till 0.3 optical density.
3. Growth was ceased by incubating in ice for 20min.
4. Then cells were recovered by centrifugation at 2710g, 4°C, 10min and supernatant was discarded.
5. Then 15mL of 0.08M $MgCl_2$+0.02M $CaCl_2$ was added.
6. Now the pellet was resuspended gently and was subjected to centrifugation at 2710 g, 4°C, 10min for cell recovery.
7. Now supernatant was discarded and 1mL of 0.1M $CaCl_2$ was added.200µL was aliquoted in each tube and 10-15% glycerol was added to the final concentration for preservation.

5. Isolation of Plasmid using Alkaline Lysis Method

1. 5mL of the medium was inoculated with a single colony and incubated at 37oC overnight.
2. Culture was then subjected to centrifugation at 13000 rpm for about 30s.
3. The supernatant was discarded and the pellet was resuspended in 200µL of ice cold AL-1 (50mM glucose, 25mM Tris-Cl at pH-8.0, 10mM EDTA) by vigorous vortexing, followed by the addition of 400µL of AL-2 which should be freshly prepared (0.2N NaOH, 1% W/V SDS) and 400µL of AL-3 (5M CH_3COOK, Glacial Acetic acid and water) and kept in ice for 5min and the mixture was subjected to centrifugation at 13500 rpm for 20 min at 4ºC .
4. The supernatant was separated and was mixed with equal amount of chloroform and isoamylase mixture (24:1) and the mixture was centrifuged at 13500 rpm for 10min at 4ºC.
5. The organic layer was then separated and mixed with two volume of 100% ethanol at RT and incubated for 1hr at -20ºC.
6. The mixture was subjected to centrifugation at 13500 rpm for 20 min at 4 ºC the ethanol was aspirated gently, the pellet was washed with 70% ethanol to remove the debris the mixture was centrifuged at at 13500 rpm for 25 min at 4ºC.
7. Pellet was allowed to dry for complete removal of ethanol followed by the addition of 30µL of elution buffer and 2µL of RNase.
8. The plasmid obtained was labeled and stored at -20ºC for further scrutiny.2µL of the plasmid obtained was run on the Agarose gel for authentication.

6. SDS-PAGE

Principle

SDS-PAGE- sodium dodecyl sulphate Polyacrylamide gel electrophoresis, describes a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate proteins according to their electrophoretic mobility (a function of length of a polypeptide chain and its charges). SDS is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate sixe during electrophoresis.

Components	**15%Resolving gel (7mL)**	**5% Stacking gel (2 mL)**
Distilled water	1.67	1.45
Resolving/Stacking Buffer	1.75	0.24
30% acrylamide	3.8	0.26
10%SDS	0.07	0.02
10%APS	0.07	0.02
TEMED	0.005	0.002

Procedure

1. ß mercaptoethanol was mixed with 4X SDS loading dye in ratio of 1:5 and 5 µL of above mixture was added in 20 µL sample and was heated to 95°C for 5 min.
2. Sample was loaded and run in Tris Glycine SDS (TGS) buffer at 70V until the tracking dye reached at the end of the plate.
3. Visualization of protein was done using Comassive Brilliant Blue staining (RAMA) followed by destaining with Distilled water.

7. Protein estimation by Bradford method

Equipment: Spectrophotometer. Glass or polystyrene cuvettes

Chemicals/reagents:

- Bradford reagent
- Bovine serum albumin (BSA)

Glass wares and others:

- Test-tubes
- Pipettes
- Micro centrifuge tubes

b)Reagent Preparation:

BSA stock solution: 2.0mg/ml in extraction buffer

c) Procedures:

1. Take 100µl of Protein extract containing approximately 10-100µg. As you do not know the protein content of the extract, you will be obliged to run a preliminary assay. Dilute two different concentrations of the extract i.e 20µl and 5µl make up the volume to 100µl with extraction buffer. Add 5ml of dye reagent and mix well. At the same time, prepare a set of standards containing 5, 10, 20, 30, 40, 50, and 100µl of Bovine Serum Albumin (BSA 2.0mg/ml stock in extraction buffer) in separate tubes. Add extraction buffer to each tube to bring the volume to 100 µl. To these tubes also add 5ml of dye reagent and mix well by vortexing. After 5 minutes and before one hour, read the absorbance at 595 nm (OD595) against a reagent blank (100 µl of extraction buffer with 1 ml of dye reagent)

2. Calculate the protein concentration in the extract by comparison with the standard curve for BSA. If the OD595 for the diluted extract is too high or too low, prepare a more suitable dilution.

3. Different proteins show considerable variation in their dye-binding capacities and so give different responses in the assay. In particular, bovine serum albumin gives a high OD595 value and so it is not totally representative of proteins. It is used here for convenience with total leaf extract. If you wish to measure the concentration of a specific protein, it is advised to use a purified form of the same protein as standard.

Reference:

-Method of Bradford, Anal. Biochem.72:248 (1976); see also Anal. Biochem. 86: 142 (1978)

8. PCR

Procedure :

1. Place 10 µl of a Pichia pastoris culture into a 1.5 ml microcentrifuge tube. For relatively dense cultures, dilute 1 µl of the culture into 9 µl water. Alternatively, pick a single colony and resuspend in 10 µl of water.
2. Add 5 µl of a 5 U/µl solution of lyticase and incubate at 30°C for 10 minutes.
3. Freeze the sample at –80°C for 10 minutes or immerse in liquid nitrogen for 1 minute. 4. Set up a 50 µl PCR for a hot start: 10X Reaction Buffer 5 µl 25 mM MgCl2 5 µl 25 mM dNTPs 1 µl 5´ AOX1 primer (10 pmol/µl) 1 µl 3´ AOX1 primer (10 pmol/µl) 1 µl Sterile water 27 µl Cell lysate 5 µl Total Volume 45 µl
5. Place the solution in the thermocycler and incubate at 95°C for 5 minutes.
6. Add 5 µl of a 0.16 U/µl solution of Taq polymerase (0.8 units).
7. Cycle 30 times using the following parameters:

Step	Temperature	Time	Cycle
Denaturation	95°C	1 minute	30X
Annealing	54°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1X

8. Analyze a 10 µl aliquot by agarose gel electrophoresis.

-Reference :
EasySelect™ Pichia Expression Kit
For Expression of Recombinant Proteins Using pPICZ and pPICZα in Pichia pastoris-MAN0000042