PROTEIN PURIFICATION

AIM: **To purify histidine-tagged protein prolidase and irisin from Rosetta and E.coli BL21(DE3) strain.**

Recipe for 100 mL of 2xYT media

- Bacto-tryptone = 1.6 g
- Yeast extract = 1 g
- NaCl = 0.5 g

Recipe for 100 mL of LB media

Add 2.5 grams of pre-mixed formulation of Luria-Bertani powder containing tryptone, NaCl and yeast extract in 100 mL of distilled water.

Stock solutions

- 1M Tris base 7.5 pH 100mL volume(Make 60ml first, adjust pH with HCl and add water to make it up to 100ml)
    I. Measure 12.114g of tris base powder and pour it into a 150 mL bottle
    II. Add distilled water leaving some space for HCl to adjust the pH
    III. Add HCl dropwise and adjust the pH to 7.5 using a pH meter
- 1M imidazole, volume 200ml
    I. Measure 13.6154 g of imidazole powder in a 250 mL bottle
    II. Add 200 mL of distilled water and dissolve it to make a clear solution
- 3M NaCl solution, volume 100 mL
    I. Measure 17.532 g of NaCl powder in a 150 mL bottle
    II. Add distilled water up to 100 mL mark and dissolve it
- 0.5 M IPTG, volume 10 mL
    I. Measure 1.19155 g IPTG in 10mL of Milli-Q water
    II. Filter sterilize it using a 0.22 u filter and store it at -20 deg celsius
- 1 M IPTG, volume 1 mL
    I. Measure 0.238 g IPTG in 1mL of Milli-Q water
    II. Filter sterilize it using a 0.22 u filter and store it at -20 deg celsius
- 100% glycerol, 100 mL
- 100 mg/mL ampicillin
    I. For 2mL, weigh 200 mg of ampicillin salt. Add 2 mL of Milli Q water.
    II. Store it at -20 degree celsius.
- 34 mg/mL chloramphenicol
    I. For 2mL, weigh 68 mg of chloramphenicol. Add 2mL of Molecular Biology Grade Ethanol.
    II. Store it at -20 degrees Celsius

Recipe of buffers for prolidase

- Lysis buffer (250mL)

Reagent	Concentration	Mass/Volume from the stock
Tris-HCl, pH = 8	25mM	6.25 mL
NaCl	300 mM	25 mL
Glycerol	5% (v/v)	12.5 mL
Imidazole	5 mM	1.25 mL
PMSF	1mM	43.55 mg

- Wash 0 buffer (1L)

Reagent	Concentration	Mass/Volume from the stock
Imidazole	10mM	10 mL
NaCl	300mM	100mL
Tris HCl pH = 8	25mM	25mL
Glycerol	5%	50mL

- Wash buffer 1 (100mL)

Reagent	Concentration	Mass/Volume from the stock
Imidazole	50 mM	5 mL
NaCl	300mM	10mL
Tris HCl , pH =8	25mM	2.5mL
Glycerol	5%	5mL

- Wash buffer 2 (50mL)

Reagent	Concentration	Mass/Volume from the stock

Imidazole	120 mM	6 mL
NaCl	300mM	5mL
Tris HCl, pH=8	25mM	1.25mL
Glycerol	5%	2.5mL

- Elution buffer for prolidase (100mL)

Reagent	**Concentration**	**Mass/Volume from the stock**
Imidazole	300 mM	15 mL
NaCl	300 mM	10 mL
Tris HCl, pH=8	25 mM	2.5 mL
Glycerol	5%	5 mL

Recipe of buffers for irisin:

For irisin, adjust the pH of all the buffers to **7.5** using dilute HCl.
In lysis buffer for irisin, add 0.5 mM beta-mercaptoethanol

PROLIDASE

Procedure for test expression

Induction

- Take 20 mL of 2xYT media in a falcon tube.
- Add 20 uL of 100 mg/mL ampicillin and 20 uL of 34 mg/mL chloramphenicol.
- Incubate in a shaking incubator at 37-degree celsius, 200 rpm till the O.D. reaches between 0.4 to 0.6
- Take the falcon out and keep it in an ice bath for 10 minutes.
- Pipette out 1 mL of the above-uninduced sample and keep it as a control to run it on the gel. Centrifuge the control at 5,000 rpm, 4 degrees celsius, for 10 minutes. Discard the supernatant and store the pellet at 4 degrees Celsius.
- Add around 20 uL of 1M IPTG. After adding IPTG, keep the falcon in an ice bath for 10 minutes.
- Keep it in a shaking incubator at 18 degrees Celsius, 200 rpm, for 10-12 hours.

Cell lysis

- After overnight induction, take 1 mL of the induced sample in Eppendorf, centrifuge at 5000 rpm, 4 degrees celsius for 10 minutes and store the pellet at 4 degrees Celsius.

- Centrifuge the rest of the 15 mL sample at 5000 rpm, 4 degrees celsius, for 10 minutes. Discard the supernatant and store the pellet at 4 degrees Celsius.
- Resuspend the pellet in 3 mL lysis buffer and 5mg of lysozyme.
- Vortex properly and incubate it on an ice bath (3-D rocker) for 30 minutes.
- Sonication - take the 15 mL tube (containing cells). Clean the probe with Milli Q water. Insert the probe into the falcon till it touches the bottom, and move the probe up by 1 to 2 cm.
- Amplitude = 25 %, pulse on for 5 seconds, pulse off for 10 seconds. Time = 2 minutes, total cycles = 3. Sonicate it till it gets transparent. (from opaque to clear solution)
- Transfer this 3 mL solution into 2 x 1.5 mL eppendorfs.
- Centrifuge it at 14,000 rpm for 20 minutes at 4 degrees (pre-cool at 4 deg for some time)
- After centrifugation, take the controls (10 uL) of supernatant (lysis pellet has cell debris)

Resin equilibration

- Take 300 uL Ni-NTA resin (invert the resin bottle properly) in 2 x 2mL Eppendorf. Add 1.5 mL milli Q water in each eppendorf. Invert the tube 2-3 times. Let the resin settle down at the bottom—centrifuge at 1,200 rpm, 4 deg for 20 minutes. Discard the supernatant.
- Repeat the above step with a lysis/binding buffer (25mM Tris-HCl, 300 mM NaCl, 5% v/v glycerol, 5mM imidazole, 1 mM PMSF)

Binding

- Add 1.5 mL of lysis supernatant to each Eppendorf containing equilibrated resin.
- Allow it to bind to resin for 30 minutes on ice and put it on a 3-D rocker

Washing and Elution

- Centrifuge it at 1,200 rpm for 15 minutes at 4 degrees Celsius.
- Separate the supernatant and add 1 mL of wash 0 buffer. Again centrifuge at 1,200 rpm for 15 minutes at 4 degrees Celsius.
- Add 200 uL of wash 1 buffer, and centrifuge. (same conditions as above)
- Add 200 uL of elution buffer and centrifuge.
- Store all the samples and controls at -4 degrees celsius.

Procedure for bulk expression

Induction

- Inoculation from amp+chloram prolidase agar plates.
- Take 7 mL of 2xYT media in a 15 mL falcon tube. Add 7uL of ampicillin (conc. 100 mg/mL) and 7 uL of chloramphenicol (conc. 34 mg/mL) in the falcon tube containing the media. Final conc of ampicillin in tube = 100 ug/mL and chloramphenicol = 34 ug/mL.
- Using a 1mL pipette tip, gently pick one colony and immerse it in the media. Mix it properly in the media. Keep the falcon in a 37-degree celsius incubator overnight at 200 rpm.
- Add 2mL of ampicillin and chloramphenicol in expression media (2L media). Add 7mL of the primary medium in secondary media. Keep in a 37-degree celsius incubator at 200 rpm.

- When the O.D. reaches between 0.4 to 0.6, (take a few milliliters of control) and induce it with 1mM of IPTG.
- Keep the culture overnight at 18 degrees celsius in a shaking incubator at 200 rpm.

Centrifugation and snap-freezing

- Take the culture out of the incubator and pour it into two large centrifuge bottles (1L each)
- Balance them using measuring balance
- Centrifuge the bottles at 4,000 rpm for 15 minutes at 4 degrees. (supernatant = 2xyt media, pellet = bacterial cells). Decant the supernatant, add 50 mL bleach, keep it for an hour and discard the supernatant.
- Take a small amount of cell pellet in 15 mL falcon and store it at -20 deg as control.
- Add Milli-Q water in centrifuge bottles and dissolve the pellet. Add the solution to two 15mL falcons—centrifuge at 4,000 rpm, 15 mins at 4 deg. Discard the clear solution (excess water).
- Take liquid nitrogen in an ice bucket. After centrifugation, put the falcons in Liq N2 and wait for 2-3 minutes till the liquid nitrogen evaporates. Immediately store them at -80 degrees. (snap freezing)

Cell Lysis and sonication

- Take the pellet out of -80 and wait till the colour of the pellet changes from white to yellowish
- Add 14 mL lysis buffer (add 10% glycerol and 1% triton x) in each falcon.
- Add 3 mg of lysozyme to each falcon. Vortex and incubate in ice on a 3-D rocker for half an hour.
- Add the solution of both the falcons in a steel glass (better conductor). Put the steel glass in ice to bring the temperature down.
- Sonicate the sample for 2 minutes (5 seconds pulse on and 10 seconds pulse off) and amplitude 40% (for thick probe) or 25% (for thin probe). Repeat 4-5 cycles.
- Give 5-minute break between each cycle. Keep the sample in ice while sonicating and for further purification steps.
- Sonicate till the cloudy appearance of lysate changes to a transparent solution 9less viscous).
- Pour the lysate into a 50 mL falcon tube.
- Centrifuge at 12,000 rpm at 4 degrees Celsius for 1 hour. Pre-set the centrifuge before using it. Transfer the lysis supernatant into another falcon tube. Store the lysis pellet.

Resin Equilibration

- Gently invert the Ni-NTA resin to mix the slurry. When it becomes homogeneous, take 1 mL and pour it into a 15 mL falcon tube. Add 10 mL of Milli Q water and centrifuge at 1,200 rpm, 4 degrees celsius, for 15 minutes.
- Discard the Milli Q and repeat this step with the binding/lysis buffer (25mM Tris-HCl, 300 mM NaCl, 5% v/v glycerol, 5mM imidazole, 1 mM PMSF). Centrifuge at the same conditions as above and discard the supernatant.

Binding

- Transfer the equilibrated resin from a 15 mL falcon tube to a 50 mL falcon.
- Pour the lysis supernatant into the falcon containing equilibrated resin.
- Allow it to bind to resin for 30 minutes on ice and put it on a 3-D rocker.
- After incubating the resin and lysis supernatant, let the resin settle at the bottom.
- Collect the supernatant after binding and slowly pass the resin through the Ni-NTA column. (Before using the column, wash it with Milli-Q and ethanol)

Washing and elution

- Keep all the buffers in ice to avoid heat shock to protein.
- Pass 30 mL of wash 0 buffer slowly. Collect the wash 0 flow through.
- Pass 5 mL of wash 1 buffer and collect flow through.
- Pass 1 mL of wash 2 buffer and collect flow through.
- Add elution buffer dropwise and simultaneously collect the flow through in Eppendorf.

Bradford test

- Use 96 well plates and add 100 uL of Bradford reagent in each well.
- Collect 0.5 mL elutes in 3 eppendorfs and start collecting 1.2 mL of elutes in 1.5 mL eppendorfs.
- Add 10uL of eluting fractions to it and observe the colour change from reddish brown to blue.

Procedure for concentrating and desalting

- Use a 10 kDa concentrator of 15 mL capacity.
- Add Milli-Q water (around 15 mL). Centrifuge at 3,000 rpm at 4 deg for 15 mins. Repeat this step.
- Repeat the above step with the desalting buffer (25 mM tris ph = 8, 300 mM NaCl, make 200 mL buffer).
- Discard the flow through.
- Pool the wash 1,2, and elutes 1 and 2 and load it in the concentrator—centrifuge at 3,000 rpm, 4 deg for 15 mins.
- Collect the flow through (1) and use nanodrop to check the concentration of the protein.
- Next, pool elutes (3 to 7), load it in a concentrator, centrifuge and collect the flow through (2).
- Finally, load the elutes 4 to 11 and collect the flow through (3).
- Run an empty spin at 3,000 rpm, 4 deg for 15 mins and collect the flow through (4).
- Carefully collect the bound protein sample in the concentrator in a 1.5 mL Eppendorf (using a pipette). Do not touch the membrane of the column.
- Rinse the PD10 column with 20 mL Milli-Q and 20mL desalting buffer.
- Pass the protein sample in the column and let it pass through the resin bed completely.
- Collect the flow through (concentrate). The protein binds to the PD10 membrane.
- Add a desalting buffer and start collecting the flow through (around 1 mL in each Eppendorf).

- Use Bradford dye to confirm the presence of the protein.
- Check the concentration of protein using nanodrop.
- Make 200 uL aliquots of the elutes, snap freeze and store at -80 degrees.

IRISIN

Procedure

The procedure for test and bulk purification is similar to that of prolidase.
The primary and secondary cultures are set up in LB media that is supplemented with 100 ug/mL of ampicillin, and induction is done with 0.5 mM of IPTG.