Int Column Purification Protocol

Preparation for Cultures

Verify the strain number for pT7-Int+ (EDT 867)
Streak out from frozen pT7-Int+ on a fresh Amp 100 plate, incubate @37°C overnight
Check plate for uniform morphology
Start two 5mL cultures in LB Amp 100, shake @ 37°C overnight
Do a plasmid prep from a kit that delivers DNA suitable for sequencing. Precipitate DNA and resuspend in 10 ul sterile nano water and then combine samples
Transformation:

- Thaw out BL21 $CaCl_2$ cells
- In a sterile tube, add 60ul of comp cells to 5 ul of DNA
- Incubate on ice for 10 minutes
- Incubate at 42°C for 2 minutes
- Add 500 ul LB
- Shake @ 37°C for 30 minutes
- Plate out 100 ul onto a fresh Amp 100 plate
- Incubate 37°C overnight
- Streak purify one transformant on Amp 100, incubate @ 37°C overnight

Growing of Cultures

Start an overnight in LB Amp 100 from the BL21 transformant and incubate @ 37°C overnight.
Start a big enough overnight so that you will have enough to do a 1:50 subculture the next day. For example, if I plan on growing up 15 liters of cells on one day, the day before I start a 300 mL overnight culture.
The next morning, subculture the overnight 1:50 into fresh LB Amp 100.
Shake at 37°C until cells are at midlog (an OD650 of 0.65) This should take about 3 hours.
Induce the cells with IPTG so that the final IPTG concentration is 0.5mM
Shake the cultures at room temperature for another 4 hours.
Transfer cultures into sterile 500mL centerfuge bottles, spin down at 9000 rpm for 30 minutes at 4°C.
Decant supernatant
More culture can be added on top of the pellet and spun again, giving a bigger pellet and using fewer centerfuge bottles. I had 13 bottles with 4 liters worth of pellet each.
Store pellets at -20°C indefinitely.

Cell Extracts *all steps on ice or cold room*

Thaw pellets <u>slightly</u> on ice (less than 5 minutes).
Try to break the pellet off of the bottle in a solid piece with a spatula.
Combine all pellets in a large beaker (for 51 liters, I got about 150 mLs of pellet).
Add 1 to 2 volumes of cold TS (50mM Tris pH7.4, 10% sucrose). I ended up using 1.6 volumes.
This needs to get homogenized and the fastest and easiest way is with a handheld electric mixer. The slowest speed should be used and it should take about 5 minutes. (This can also be accomplished with a manual homogenizer or even a Waring blender)
Add protease inhibitors:

- Leupeptin – final of 1-2 ug/ml

Pepstatin A – final of 1ug/ml
PMSF – final of 100 ug/ml
Soybean trypsin inhibitor – final of 1ug/ml

Add 1/20 volume of 10 mg/ml lysozyme in 0.25M Tris, pH7.4
Mix briefly to distribute inhibitors and lysozyme.
Aliquot 40 mLs into 50 mL centerfuge bottles (I got 12 bottles)
Incubate in ice for 40 minutes
Spin at 15,000 rpm for 45 minutes at 4°C
Pour off supernatants into a common beaker, take a 100ul sample aliquot. The supernatant is very snotty/viscous and has a yellow brown color.
Aliquot 40 mLs into 50 mL conical tubes and quick freeze (I got about 300 mLs or 7 X 40 mLs). This is extract 1 (E1).
Resuspend each pellet in 12.5 mL of TS, add 1/20 volume lysozyme and protease inhibitors, incubate on ice 35 more minutes, spin again and collect supernatants. This is extract 2 (E2).
Take a 100ul aliquot of E2 and aliquot the rest into conical tubes and quick freeze all. (I got about 135 mLs or 3 X 45 mLs).
Store all samples at -80°C
Do an activity/bandshift titration assay of E1 and E2

High Speed Spin 1

Thaw out four of the 40 mL E1 tubes in a 31°C water bath, swirling occasionally, put on ice immediately when thaw.
Combine in a common beaker and add 100 mL of 0.05M Tris, pH7.4 (This brings down the salt concentration and this volume works perfectly to fill up all the sample bottles in the next step)
Fill up the 1" X 3.5" polyallomer tubes (8 X 32.4 mLs) and carefully balance.
Plug and cap the tubes.
Spin in a pre-cooled Beckman 60 Ti rotor at 49,000 rpm at 4°C for 200 minutes
Use a pipet to remove the first 3 mLs of supernatant, then cut off top and pour off the rest of the supernatant. Take a 100ul aliquot of the supernatant. Parafilm the top of the cut tube.
Quick freeze the supernatant (FxIIS), the pellet (FxIIP), and the aliqout (FxIISa) and store at -80°C.
(Int should be in the pellet)

High Speed Spin 2

Partially thaw pellets (FxIIP)
Homogenize pellet with liquid already on the bottom of the tube.
Add Buffer X with 0.6M KCl to the pellet in 1mL increments up to 5mL. Scrape wall thoroughly after each addition.
Pour contents into a homogenizer
Add 5 mL of Buffer X 0.6M to the empty tube.
Scrape thoroughly to get all remainder
Pour contents into same homogenizer
Homogenize until even consistency
Fill into the polyallomer tubes (8 pellets from previous step, resuspended volume is about 125 mL or 4 polyallomer tube)
Spin in a pre-cooled Beckman 60 Ti rotor at 49,000 rpm at 4°C for 200 minutes.

Use a pipet to remove the first 3 mLs of supernatant, then cut off top and pipette off the rest of the supernatant. Take a 100ul aliquot of the supernatant. Parafilm the top of the cut tube. Quick freeze the supernatant (FxIIIS), the pellet (FxIIIP), and the aliqout (FxIIISa) and store at -80°C.
(Int should be in the supernatant)
Do a bandshift titration assay for FxIIS and FxIIIS. FxIIS should only have about 10% of the activity per volume of FxIIIS.

Phosphocellulose Column Purification
Capacity is about 1mL of packed bed per 2 liters worth of sample
This prep is about 30 liters, so I need about 15 mL of resin
Resuspend P-cell stock and transfer 30 mLs into a conical tube.
Spin at setting 2 of the clinical centerfuge for a minute or two.
Pipette off supernatant into waste.
Add 15 ml of Buffer X 0.6M KCl and resuspend.
Repeat spin and resuspension steps 4 times.
After final resuspension, resin is ready for use.
Thaw out FxIIIS and combine in a beaker and add the resin, mix thoroughly.
Divide into 50 mL conical tubes and rotate in cold room for about 30 minutes.
Spin at slowest speed in tabletop centerfuge for 2-3 minutes.
Pipette off supernatant, making sure not to pull up any resin.
Add an equal amount of BufferX 0.6M KCl and resuspend by inverting.
Put a stopcock on end of column and add 1 ml of Buffer X 0.6M KCl
Add the slurry to the column and let the first few mls pack by gravity.
Open up the stopcock and let the rest of the column pack.
Rinse with one bed volume buffer.
Hook column up to peristaltic pump and flow adapter.
Wash with 2-3 bed volumes buffer X 0.6M KCl at a rate of 0.33 mL per minute
Hook up the gradient mixer and run a gradient of 0.6M to 1.6M – 50 mL each
Collect 50 of 2 mL fractions at a rate of 0.33 mL per minute
Take a 30 ul aliquot of every other fraction and quick freeze all.

Assays:
Use the conductivity meter to determine the salt concentration of each of the aliquots.
Do a dot blot on each of the aliqouts to see find the peak fraction and side fractions
Do an activity/bandshift assay for the peak fractions
Do a Coomassie staining of the peak fractions to check purity

S Resin Column Purification
This resin has a high binding capacity (55mg/ml), but I need 5 mL to reach the bottom of the flow adapter.
Resuspend S resin stock and transfer 10 mLs into a conical tube.
Spin at setting 2 of the clinical centerfuge for a minute or two.
Pipette off supernatant into waste.
Add 5 ml of Buffer X 0.5M KCl and resuspend.
Repeat spin and resuspension steps 4 times.

After final resuspension, resin is ready for use.
Add 1 ml of buffer to bottom of column.
Add resin slurry to column and let the first few mls pack by gravity.
Open up stopcock and finish packing column.
Hook column up to peristaltic pump and wash resin with 4 bed volumes of buffer at a rate of 0.33 ml per minute.
Thaw and combine the peak fractions and measure the conductivity to determine salt concentration.
In this case, I pooled fractions 15-30 and it was in 1.05 M KCl
Dilute with Buffer X with no KCl to a final concentration of 0.5M KCl
Load sample onto column at a rate of 0.33 ml/min.
Wash column with 2 bed volumes buffer
Hook up gradient mixer and run a gradient of 0.5M to 2.0M – 25 mL each
Collect 50 of 1 mL fractions at a rate of 0.33 mL per minute
Take a 30 ul aliquot of every other fraction and quick freeze all.

Assays:
Use the conductivity meter to determine the salt concentration of each of the aliquots.
Do a dot blot on each of the aliqouts to see find the peak fraction and side fractions
Do an activity/bandshift assay for the peak fractions
Do a Coomassie staining of the peak fractions to check purity