LMP-1 Antibody Purification

Growing GST & GST-LMP1 cultures for protein purification.

A. Streak fresh plates of clones.
   1. In the morning start two 10ml cultures from freshly streaked plates. (Don't forget the Amp!)
   2. At end of the same day, inoculate two 100ml cultures + 100ul AMP/culture. Let grow overnight.
   3. Next day, inoculate four 500ml cultures(or how ever many flasks you need.) with 50mls of overnight culture. Add 500uls Amp to each flask. Let grow 2.5 hours, (OD 600 between 0.6-1.0) REMEMBER: remove 1ml of cells before adding IPTG. This is to check for induction. Add 500 uls 1M IPTG to each flask. (1mM final concentration.) Let grow @ 37 C for 4 hours. Spin down pellets and freeze until ready for purification.

GST Purification-KEEP EVERTHING ON ICE

A. Resuspend pellets in 25mls CHILLED PBS + 0.5% triton X100. (625 uls of 20% tritonX/ 25mls PBS.)
B. While chilling PBS, prepare glutathione sepharose.

$$\frac{\text{Xmls slurry needed}}{1.33\text{mls slurry}} \times 1\text{ml beads} = 2\text{mls beads}$$

X= 2.66mls beads needed. (Wash and resuspend in 2mls PBS for final volume of 4mls of 50% slurry.

Wash: Remove 2.66mls of beads. Spin down 2000 RPM 5 min. in TC table Top. Resuspend pellet in 10mls cold PBS, spin again @ 2000 RPM. Remove PBS and resuspend in 2mls fresh cold PBS. Slurry now readies for use.

C. Transfer resuspended pellet to a 50ml conical tube. Sonicate in 7th floor cold room. 15secs. X 4 on setting 7. (use old sonicator.)

D. After sonication, transfer cells back to 500ml bottles and spin 10K 10 min. in Sorvell(rotor code= 30). Transfer supe to new 50 ml tubes, 25mls/tube. REMEMBER to save 200uls for flowthru sample. Add 1 ml of prepared slurry to each tube. Let rock in cold box 3 hrs.

E. After 3 hrs. spin down 3000 RPM 10 min. Remove supe, resuspend in 8mls wash buffer, transfer to 15ml tube, rinse tube with 2ml wash buffer, add to 15ml conical, spin 3000 RPM 5 min. (Save 200uls of first wash.)

Repeat washes 3X with 10mls wash buffer. (total of 4 washes.) After last wash, spin and remove as much buffer as possible with pipetman.

F. Resuspend beads in 1ml elution buffer (located in freezer by Dong Yun) and transfer to 2 ml screw cap tube. Put @ 4C 30 min. in cold box with rocking. After 30 min. spin 14K 3min. and remove supe to new 2ml tube. Repeat 3X for a total of 4 elutions. Combine elution into 2, 2ml tubes. (4mls total)

G. Run 5uls of elutions , pre-induction sample and flowthru sample on 10% Protein gel to check for induction.

H. If induction and purification work, protein can be concentrated by putting in dialysis tubing and placed in Aquacide. Let approx. 1/2 of liquid be removed. Then dialyze overnight in coupling buffer. Once dialyzed into coupling buffer, freeze @ -80C until ready for column purification.

Dialysis/Coupling Buffer: Make Fresh

8.4g NHC03
29.22g NaCl
pH to 8.3 with NaOH or HCl
Bring to 1 liter with H2O

Column Preparation; Coupling protein to Sepharose Beads (CNBr-Activated Sepharose 4B- Sigma C9142, 5 grams.)

E. Once protein is concentrated with aquacide and dialyzed into coupling buffer, it must be quantitated using a Bradford assay. Want ~10mg protein/ml slurry gel. Determine how much CNBr sepharose to prepare using following equation:

$$\text{total mg protein} \times \frac{\text{1ml slurry}}{\text{10mg protein}} \times \frac{\text{1gr dry beads}}{\text{3.5mls slurry}} = \text{grs. beads needed}$$

F. If protein is very concentrated and at a low vol., add coupling buffer to bring vol. up to 10mls. Transfer all protein to be coupled to 15ml conical tube.

G. Coupling:
   1. Weigh out X grs of beads needed. Pour into a small beaker. Add excess vol. of 1mM HCL. Stir well with spatula. Stir every few minutes for 15 min.
   2. Onced swelled, pour beads into sintered glass funnel and rinse beaker multiple times with 1mM HCl. Wash beads with several vols. of 1mM HCl ,(~200mls/g dry sepharose) removing suction to stir slurry with

spatula. After washing with 200mls, dry beads well with suction.

3. QUICKLY add 5mls coupling buffer to beads and dry with suction to a workable paste. IMMEDIATELY scoop paste into tube containing protein to be coupled. Work quickly because the coupling buffer causes coupling to begin.
4. Parafilm tube and let go overnight @ 4C with rotating. Clean out funnel well with H20.
5. Next day, pour beads over funnel and COLLECT supe in tube.(will test this to make sure there is no protein still present. This will determine our coupling efficiency.) Scoop out beads to new tube containing 10mls of blocking reagent = 1M Ethanolamine. Let go overnight @4C.
6. Next day, wash beads in funnel alternatley 4X with coupling buffer and NaAcetate buffer. This removes noncovalently bound protein.
7. Wash in TE, and store in TE. Can add 0.02% sodium azide if going to be stored for long periods.
8. Solutions:

   Coupling Buffer-Make fresh- see above

   <u>1mM HCl 500ml</u>
   0.4ml 1.21M HCl (stock diluted 1:10)
   499.6ml MilliQ H2O

   <u>Ethanolamine for blocking 100mls</u>
   6ml ethanolamine (make sure it's clear)
   60ml coupling buffer
   pH to 8.3 with HCl (cool on ice before if mixing produces heat)
   (solution may turn yellow `pH 8-9)
   Bring volume up to 100ml with coupling buffer

   <u>Sodium Acetate Buffer + 0.5M NaCl 500mls</u>
   6.804 g NaAcetate
   14.61 g NaCl
   pH to 4 with acetic acid (5-10mls_
   Bring volume up to 500ml with MilliQ H2O
9. Run a Bradford assay on coupling flow through to check coupling efficiency. If most protein is gone,(coupled to the beads) continue with protocol. If not, you will have to repeat the coupling reaction.

Running columns for LMP-1 Antibody Purification

A. Spin down rabbit serum to be used @ 3800 RPM 15mins, in TC room to remove particulate matter from serum. Pour to new tube and store @ 4C until ready to apply to columns.
B. Pour prepared coupled beads into BioRad 15ml disposable columns. Wash with 10 bed vols. = 30mls 10mM Tris ph 7.5. This may take all day to wash

through. Can cap off column at any time and continue washing next day. Just leave ~3mls of buffer on top of beads in column so beads do not dry out. Parafilm top and bottom of column overnight.

C. Once column is washed, apply serum to the GST coupled column. Apply 3X to column to assure all GST Antibodies are bound to column.
D. Once serum has been passed over the GST coupled column 3X, pass it over the GST- LMP-1 coupled column 3X. (Because the LMP-1 protein is fused to GST protein, we must first clear the serum of all antibodies to the GST protein, which is accomplished by running it over the GST coupled column. We are now isolating antibodies that only recognize LMP-1 when we run it over the GST-LMP-1 coupled column.)
E. Once serum has been passed over the column 3X, wash with 20 bed vols. = 60mls of 10mM Tris pH 7.5. (Wash both columns the same.)
F. Wash with 20 bed vols. = 60mls 500mM NaCl, 10mM Tris pH7.5.
G. Elute GST antibodies off GST couple column and elute LMP-1 Antibodies off the GST-LMP1 coupled column as follows:
   1. Elute with 10mls 100mM glycine pH 2.5 adding 1ml at a time and collecting each ml separately.
   2. Collect each elution in 1.5ml eppie containing 100uls 1M Tris ph 8.0.
   3. Collect a total of 10-1ml fractions. Check each fraction by running a mini Bradford assay in a 96 well plate. You will be able to see which fractions contain antibodies.
H. Take elutions with most antibody and dialyze overnight in 1X PBS.
I. Run Western gel to check for affinity and quantify as follows:

$$\text{Xmg/ml Antibody} = \frac{\text{A280} \times \text{1mg/ml} \times \text{diln. Factor}}{1.36}$$

J. Aliquot and store @-80C.