PURIFICATION, LABELING AND RECONSTITUTION OF GPI PROTEINS FOR BILAYERS

A. **Purification of GPI Proteins**
   1. **Preparation of antibody-sepharose for affinity chromatography**
   2. **Cell lysis/sonication**
   3. **Capture of antigen to sepharose (affinity chromatography)**
   4. **Labeling and elution of captured antigen**

B. **Liposome Preparation (Separate Protocol)**
C. **Bilayer Preparation (Separate Protocol)**

General Comments:

- Isolation of gpi anchored membrane proteins from cell lysates: GPI anchored proteins tend to be insoluble in Triton X-100 and other non-ionic detergents due to inclusion in glycolipid rich domains. Several detergents for extraction of GPI anchored MHC class II have been tested and none worked well at $4^{O}C$. All of the non-ionic detergents solubilized the GPI-anchored MHC class II when the lysate was intensively sonicated. Therefore an inexpensive detergent (Triton X-100) is used with sonication to solubilize these proteins. Elution of labeled protein from affinity chromatography columns is performed in n-Octyl-Beta-D-Glucopyranoside (OG) detergent.

-Throughout the purification and labeling process many centrifugations of small volumes of sepharose media are required – losses of media can be kept to a minimum by filling tubes to their maximum volume with the appropriate buffer at each step for washing, centrifugation and incubations.

-All buffers are routinely sterile filtered although protein preparations are not performed under sterile conditions.

-Routinely protein from approximately 3 grams of cells is isolated.

[A]

1. **PREPARATION OF MAb AND IRRELEVANT Ab SEPHAROSE - Covalent coupling of antibody via primary amines to cyanogen bromide activated sepharose.**

   - An irrelevant antibody should be coupled to sepharose and used as a step prior to using the Mab-sepharose to adsorb sticky (non-specifically binding) proteins from the cell lysate.

   - **An alternative to purchasing pre-activated sepharose is to prepare cyanogen bromide activated sepharose using Sepharose CL-4B.**

Reagents:

-C9210 (Sigma) Cyanogen Activated agarose (cross-linked 4% beaded agarose) –

1g agarose swells to about 3–3.5 ml.

-**Coupling Buffer – 0.1M NaHCO3 – 0.5M NaCl pH8.3-8.5 (3x- 4x stock is prepared)**
Coupling buffer should not contain any amines that may compete for coupling to beads (Tris, etc). High salt is used to prevent ionic interactions.

-Protein - approx 2mg purified antibody is coupled / ml packed agarose. Protein should be in non-amine buffer such as PBS and > 3.0mg/ml.

Usually 1.2 – 1.5 ml of sepharose is washed initially per 3.0g cell prep.

1) Wash agarose with
    a. Cold 1mM HCL (200 ml/ gram dry gel)
    b. Cold distilled water – 10 gel volumes

    Comments:
    Sepharose can be initially washed in an appropriate size sinter glass funnel or in conical plastic tube (15 /50ml) followed by transfer to a 2.0 to 5.0 ml tube for antibody coupling (screwcap microtubes work very well).

    For washing in funnel gently remove almost all liquid – careful not to dry out beads. After washing, transfer moist gel with a spatula to tube in which coupling to antibody will be accomplished. Rinse out funnel if necessary to remove any remaining beads.

    or

    In centrifuge tubes (15 or 50ml) - fill completely to avoid losing agarose beads. Centrifuge at about 1500-2000 RPM (Beckman GS6R swinging bucket) for 5-10min – no brake. Carefully aspirate supernatant liquid to avoid sucking up beads. Transfer to smaller tube for coupling to antibody.

2) Coupling antibody to agarose

    In the smaller volume tubes centrifuge sepharose and remove as much liquid (H_2O) as possible. Combine protein and coupling buffer first and then add to beads as an equal volume. Final concentrations in the mixture are:

    Coupling buffer: 0.1M $NaHCO_3$ -0.5M NaCl pH8.3-8.5
    Protein concentration: couple ~2mg protein/ml packed beads.

    [Sepharose should not be in coupling buffer for any length of time prior to the addition of protein (hydrolysis of reactive groups), therefore in this protocol protein and coupling buffer are added together to beads after they are washed in water]

The protein–sepharose mixture (~2.0 to 2.5ml) is incubated end over end >16hrs 4^oC or 4 hours room temperature. DO NOT USE STIRBAR.

3) Washing and blocking of antibody coupled agarose.

After coupling, centrifuge beads and measure OD_{280} of the supernate. Approx. 98-99% of protein is usually coupled. Wash once in same tube with 1x coupling buffer. Block sepharose by adding 100mM ethanolamine or 0.2 M glycine pH 8.0 in same tube and mix end over end for 2 hrs room temp or 6-8 hours 4^oC.

After blocking, beads should be transferred back to larger tube(15-50ml) for washing with coupling buffer (at least 5x) and then washed with 25mM Tris- 0.15M NaCl pH 8.0 (4^oC) – 0.02%azide for storage until ready for affinity purification of GPI proteins.
[Beads will be washed with either acid or base cycle right before affinity purification].

2. CELL LYSIS AND SONICATION - All of the following is performed in ice or at 4^oC**:**

Lysis buffer for: a) lysing fresh or frozen cell pellets - 20ml/ g cells and
b) column (Ab-sepharose) wash:

1% Triton X-100,
25 mM Tris pH 8.0 - 0.15 M NaCl (pH at 4^oC)
0.02% NaN_3

Add the following (protease inhibitors) to buffer just before adding buffer to cells:

Final Concentration in Lysis Buffer:
5 mM EDTA
5 mM iodoacetamide (prepared fresh)
1:150 dilution Aprotinin (5-10 TIU/ml)
1mM PMSF (very unstable in aqueous solution - add immediately before adding buffer to cells)
(PMSF is prepared as 200mM stock in EtOH)

Sonication Protocol (Bronson Sonifier 450):

Volume > 50 ml: When cell pellet just starts to thaw, quickly add PMSF to lysis buffer and add complete buffer to pellet. Make sure pellet is broken up (spatula). Transfer to glass beaker so that it's about half full. Use flat sonicator probe. With 50 -100 ml volume set to output of 7 cycle to 50%.
With >150 ml set to output of 10. Set cycle to 50%.

Test the probe in similar volume of lysis buffer first to make sure that the power setting does not cause aerosoling or spattering of sample. Probe is immersed about 1 inch. Sonicate sample for 3 x 30 seconds with 30 sec to 1 minute of rest on ice between cycles. (Beaker in ice during entire sonication)

Volume < 50 ml: As above but use microtip - set to output of 7 (max) - cycle to 50%. Test with lysis buffer first and adjust output down if aerosoling or spattering is excessive.

Incubate lysate in cold for 1 hour (including time to sonicate)

Clarification:
Since the nuclei are disrupted by sonication and the DNA is sheared there is no need to do a low speed centrifugation to remove the nuclei.
Ultracentrifuge the solution for 1 hr at >100,000 x g in a fixed angle rotor at 4°C. Collect the supernatant and 0.2μm filter with prefilters to prevent clogging. [Can use Whatman 4.7mm filters (GD/D with GD/F) or 0.45μm with prefilters).

3. CAPTURE OF ANTIGEN (GPI PROTEIN) TO SEPHAROSE

Reagents:

a) Wash Buffer for antigen capture (essentially lysis buffer) 25mM Tris- 0.15M NaCl - 1% Triton X-100 pH8.0

b) Labeling Buffer: 0.05% Triton – 0.1M Bicarb/Carbonate Buffer pH 8.5-9.0 (pH depends on which dye is coupled (with CY dyes pH 8.5 labeling buffer is used). Buffer should be used within one week of preparation or check pH before using.

c) Wash Buffer after Labeling- 25mM Tris-0.15M NaCl pH8.0 - 0.05%Triton-X100

d) Wash Buffer before Elution- 25mM Tris-0.15M NaCl pH8.0 – 1% OG

e) Elution Buffers - 50mM Triethylamine or 50mM Glycine (pH 11.5 or pH 3.0)
0.15M NaCl
1% OG detergent

Protein	Elution pH
ICAM-1	3.0
I-E^{k}	11.5
CD48	12.0
CD16	3.0
CD80	11.5

Prepare specific Mab-Sepharose and irrelevant antibody Sepharose as in preceding section. Routinely the protein from 3g cells is combined with 1ml Mab-sepharose (2mg Ab/ml packed beads). The following steps assume about 60ml lysate from 3g cells.

Prewash both the non-specific and specific MAb-sepharose beads under elution conditions, but with Triton X-100 instead of OG. Elution conditions are usually pH 3.0 or pH 11.5-12.0

depending on the protein being isolated. Wash 3 X at elution conditions and 3X with neutral buffer (25mmTris-0.15M NaCl -1% Triton X-100 pH8.0)

Incubate the cell lysate with 1 ml of non-specific antibody Sepharose to preclear proteins that adsorb non-specifically to Sepharose. This may be repeated twice to optimally remove sticky proteins. This step is performed with continual mixing end over end at 4°C for at least 1 hour each time. Centrifugation is the same as for initial washing of sepharose.

> [For 60 ml lysate – divide 1.0 -1.2 ml Ab-sepharose into two 50ml conical centrifuge tubes and add >30 ml lysate to each tube. Both irrelevant and specific Ab sepharose are never reused in the preparation of gpi proteins which will be reconstituted in liposomes for bilayers].

Save a small fraction of the cleared lysate for analysis of antigen content by capture assay or Western blot.

Transfer the cleared lysate to a tube with 0.5-1 ml of specific mAb-Sepharose. Incubate by mixing end over end at 4°C for 4 hr to overnight. [Again 30ml lysate combined with 0.5ml mAb-sepharose] Save the supernate after centrifugation for analysis of residual specific protein. Usually at this point, after the capture of antigen, beads are combined and transferred to one 15ml conical centrifugation tube from the two 50ml tubes and washed. Wash the beads extensively (at least 5 times) with TS 1% Triton X-100 (lysis buffer) followed by 5 times with 0.05% Triton X-100 in 0.1 M Bicarbonate/Carbonate pH 8.5 to pH 9.0.

During the last wash transfer sepharose (~1 to 1.5ml packed beads) to smaller tubes for labeling of protein. If protein is to be labeled with 2 dyes or eluted unlabeled – it is at this point that sepharose should be divided (usually as 0.5ml packed beads) into separate tubes. [Beads which will not be labeled should be handled in a similar fashion to those being labeled]. Centrifuge and resuspend the beads in 1 volume of labeling buffer (0.05% Triton X-100 in 0.1 M Bicarbonate/Carbonate Buffer pH 9). The beads are now ready for labeling.

LABELING OF GPI PROTEIN (ANTIGEN)

***Resuspend the labeling dye in a small volume of 0.05% Triton X-100 in 0.1 M Bicarbonate/Carbonate Buffer pH 9. Usually use the amount of the dye that is recommended for labeling the amount of antibody that is on the beads (50ug dye/mg antibody protein(0.5ml packed beads)). If there are mobility problems with the prep it may be necessary to decrease the relative amount of dye. The labeling reaction should be complete after 60 min at room temperature with constant agitation.

or revised protocol:

Dye Solvent
Labeling dye is dissolved in DMSO and added to the sepharose-labeling buffer mixture so that the final DMSO concentration is 1-2% by volume. Immediately transfer dye to the beads. The beads and dye are mixed end over end at room temp with aluminum foil covering tube for 1 hour and then the mixture is centrifuged in the same tube. DO NOT USE STIR BAR.

Dye Concentration

Based on recent studies in the lab it appears that the sepharose alone (without protein present) will bind dyes.

Therefore use 2-3 times the amount of dye as recommended above. This equates to about 200-300ug dye per ml packed beads. It may be that the amount necessary for labeling will differ for each dye and each protein and should be determined independently.

****Mobility problems may result from over labeling - see note below.

Washing Labeled/Unlabeled Beads
After labeling transfer beads back to 15ml conical centrifuge tube for washing. Rinse out the smaller labeling tube to remove all beads.

Wash the beads at least 5 times with 0.05% Triton X-100 in TS and then 5 times with 1% OG in TS. All washing and centrifuging of beads is done at 4^oC.

ELUTION OF LABELED PROTEIN:

At 4^oC transfer the beads (0.5 to 1.0ml) to a small disposable column (BioRad). Elute with 0.5-1 ml aliquots of elution buffer.

Typical elution buffers are either
 1% OG - 50 mM Glycine pH 3.0 - 0.15 M NaCl or
 1% OG - 50 mM Triethylamine pH 11.5 - 0.15 M NaCl.

Some proteins may require pH 12 to elute. All tubes in which fractions are collected should have neutralization buffer in them prior to elution. The neutralization buffers in the tubes should be about 0.1 volumes of 1% OG, 1 M Tris pH 8.5 (for pH 3) or 1% OG 1 M Tris pH 7 (for pH 11.5). Mix the fractions as they are collected to neutralize. (Prior to elution check buffers alone to make sure that pH is neutral)

Assay.
Assay the purified proteins by adsorption to polystyrene plates and RIA (I-125) (routinely 5ul fraction + 45ul TS in 96well plate are incubated followed by blocking of plate and then addition of appropriate I-125 antibody). Use an antibody capture assay if available to assay the lysate before and after exposure to the specific mAb-Sepharose.

B. Reconstitution. (See separate Liposome protocol)

Labeled gpi proteins are ultracentrifuged in small microtubes immediately prior to reconstitution.

*** Dustin Lab original protocol.

**** Bilayer mobility problems – if after reconstitution of proteins into liposomes there is little mobility in bilayers try adding about 10% of volume or less of 100% DOPC liposome (or other egg phospholipid liposome). Totally immobile preps can be >60% mobile or more after addition of 100% lipid liposome.