Biotinylation of IA^b-pep-BirA and Multimer Formation

I. Purification

Purify the IA^b-pep-BirA on M5/114-Sepharose 4B affinity beads. We use the M5/114 antibody at 5mg ab/mL swollen beads, and we have a column of about 25mL. This amount of beads will purify a 5L Prep in one run, usually. Spin the baculovirus supernatant in a superspeed rotor at about 15,000xg for 30 minutes. We use the Beckman JLA-8.1 rotor with the 1L bottles, but any rotor with reasonably sized bottles will do. The supernatant is filtered through a 0.2u filter to remove any debris (helps keep beads from getting clogged.) Before loading the supernatant, pre-elute the beads with 2 column volumes of 50mM $NaCO_3$ pH 10.8 (10% 50mM $NaHCO_3$/ 90% 50mM Na_2CO_3). This is the buffer that will elute the IA^b-pep-BirA from the beads. Re-equilibrate the column with PBS until the pH is equal to the pH of the PBS. We use pH paper for this. Load the supernatant overnight at 4^{o}C by gravity flow. Wash the column with 500mL of PBS. Set up the tubes for the elution (we use borosilicate glass 12x75mm tubes) with 300uL 1M Tris pH6.9 and 23uL of 100X pepstatin+leupeptin (pepstatin 0.7ug/mL final, leupeptin 1ug/mL final). The Tris will neutralize the pH and the pepstatin and leupeptin will protect the BirA tag from proteolysis. Elute with 2mL of 50mM $NaCO_3$ pH 10.8 (10% 50mM $NaHCO_3$/ 90% 50mM Na_2CO_3) for each fraction. After each fraction is collected, add 2.3uL 1000X PMSF (0.1mM final, another protease inhibitor) and immediately vortex to mix everything. Immediately wash the M5/114-Seph4B column with PBS (for another run) or PBSA. The column is stored in PBSA. Keep the fractions cold from this point on. Read the OD_{280} for each fraction, using the elution buffer + Tris + protease inhibitors as the blank.

The biotinylation reaction requires a buffer exchange and concentration step. Centricon-30's work fine, but recently we have been using Amicon-Ultra 15 (30kD MWCO) concentrators. They work a lot faster with less precipitation. Concentrate the peak fractions, and exchange the buffer to 10mM Tris, pH 8, with pepstatin-leupeptin-PMSF (PLP, same final concentrations as above). It is very important to get the material biotinylated right after it is purified. Delays seem to result in a lower incorporation efficiency of biotin.

II. Incorporation of biotin

The maximum and recommended concentration of the IA^b-pep-BirA for the biotinylation reaction is 38uM, which is about 1.8mg/mL for a 50,000kd protein. Use the BirA enzyme kit purchased from Avidity, in Denver. (2349 Eudora St., Denver, CO 80207 303 320 6063, Fax 303 320 1439. Product #BirA500, Biotin Ligase). We find the following conditions to yield 80-90% biotin labeling.

A. Reaction Conditions
   1. BirA tagged protein at 38uM or lower = 8 parts. Try not to use your protein more dilute than this, because you will only be wasting enzyme.
   2. 1 part biomix A (contained in the Avidity product kit)
   3. 1 part biomix B (contained in the Avidity product kit)
   4. Bir A enzyme at a final concentration of 15-20 ug/mL.
   5. Incubate the reaction mix overnight at room temperature.
   6. Add $1/100^{th}$ volume of 1M Iodoacetamide. (Caps free sulfhydryl groups) Incubate at room temperature 30 minutes.

B. Processing the biotinylated protein
   1. A large volume sample will have to be concentrated to run on a Superdex200HR10/30 column or 10/300 GL. (or other suitable size exclusion column) Best resolution is obtained with sample volumes of 200uL, but we often run 500uL samples for this step.
   2. Run the biotinylation reaction mixture over the sizing column using PBS with 5mM NaN_3. (NaN_3 acts as a preservative) This step will remove any aggregates, and leave only “monomeric” protein, and will remove all the reactants. Add protease inhibitors to the pooled fractions. Concentrate if necessary, and add $1/50^{th}$ volume each of 0.5M EDTA and 0.5M EGTA. Store at 4^oC, or if your protein can tolerate freezing, you can aliquot and freeze at -70^oC. If you are going to freeze aliquots, save out a small amount for testing the percent of biotin incorporation, and a larger aliquot for making some multimer (200 – 400ug is a good amount).

III. Determination of extent of biotinylation, using a capture ELISA

A. Wash avidin-agarose beads (2mg avidin/mL of gel, Vectra Labs) 5 times with ELISA diluent, and resuspend to the same volume. Spin in an eppendorf centrifuge 5000rpm for 2 minutes for each wash.

B. Dilute the biotinylated samples to 1ug/mL in ELISA diluent, making 1 mL of the dilution. 1ug/mL is the concentration of Class II IA and IE that gives a maximal signal in our ELISA.

C. Reserve 0.5mL of each dilution. To the remaining 0.5mL add 50uL of the washed avidin-agarose beads. Tumble at room temperature for 1 hour. Spin down the beads.

D. Test the un-precipitated and the precipitated samples for binding in the capture ELISA by serial dilution of each sample. We use a 1:3 serial dilution for IA^b-pep-BirA. We use M5/114 as the capture antibody and biotinylated 17/227 for the detection. You may also use biotinylated 3F12. Follow the same ELISA protocol, omitting the biotinylated antibody step and going directly to the extravidin-alkaline phosphatase step, to measure directly the presence of biotin on the ClassII.

E. Results of a good incorporation of biotin are a strong signal for Class II that is 80-90% removed by the avidin-agarose beads, and a strong signal for biotin alone, which is completely removed by the avidin-agarose beads. It is wise to use both methods in analysis as a crosscheck.

IV. Multimer Formation

A. The PE is light sensitive, so protect it from light whenever possible. Mix one mole of streptavidin-PE (we are using Prozyme #PJRS25, it gives bright staining and the concentration of the streptavidin is included in their product insert) with 8 moles of biotinylated protein. Eight moles is an excess of material that will ensure saturation of all the binding sites on the streptavidn-PE.

B. Incubate 1 hour to overnight at 4^{o}C. Add extra protease inhibitors based on the volume of streptavidin-PE added.

C. Separate unbound biotinylated protein from multimers on a sizing column. We use Superdex200HR10/30 or 10/300 GL, and PBS with 5mM NaN_3. The multimers come out in the excluded peak, at an approximate MW of 600kD. Any unbound strepatvidin-PE will also elute with the multimer peak, since both are larger than the 200kD exclusion limit of the column. This is another good reason to use an excess of biotinylated protein. There is sometimes a trailing edge to the peak, which is not included in the pool. Monomeric Class II, whether biotinylated or not, will come out at the 50-60kD position. Add protease inhibitors to the fractions containing the multimers and the separated Class II monomers.

D. Pool the desired fractions, and if necessary, concentrate. The final concentration is ideally greater than 200ug/mL.

E. Determine the OD_{280} of the pooled multimer peak. We assume an OD_{280} of 1 is equal to 1mg/mL, although this may not be true. Nevertheless, using the OD_{280} as a measure of protein concentration will allow for internal consistency.

F. Pool the unincorporated monomer peak, and assay it for % biotinylation as in Step III above. We assume that all the biotin-binding sites on the PESA in the multimer-forming reaction were saturated if we are able to find 15% or more biotinylation in the unincorporated monomer peak.

V. Staining T-cell Hybridomas or T-cells with the multimers

A. Use $1x10^5$ hybridomas or $1x10^6$ T-cells per stain, in a volume of 30uL. Diluent for the cells is culture medium for T-cell hybridomas.

B. We have found that a final concentration of 20ug/mL** of the Pe-SAv-$(IAbPep)_4$ is close to saturation, and that a 2hr incubation at 37^oC is also close to saturation. Add 20uL of the Pe-SAv-$(IAbPep)_4$ at a concentration of 50ug/mL, for the final concentration of 20ug/mL. Include sodium azide at a final concentration of 5mM in the reaction mixture. It has been determined that the azide does not have a deleterious effect on viability in the 2 hour time span. Make 12.5mM sodium azide in culture medium to dilute the tetramers: e.g. 12.5uL 0.5M + 500uL medium = 12.5mM azide

C. Incubate in a gassed 37^oC incubator for 2 hours, then wash 2 times with PBSWash Buffer (hybridomas) or BSS WashBuffer (T-cells). Resuspend in a final volume of 300uL and read on a flow cytometer. If you need to stain with FITC-ab also, add in the last 20-30 minutes, and put at 4^oC, followed by washing.

D. Include a stain for T-cell receptor and/or CD4, following the regular staining protocol for your lab.

E. Negative controls for the reagent are an irrelevant Pe-SAv-$(IAbPep)_4$, and/or an irrelevant T-cell Hybridoma or T-cells from a negative animal.

F. To enhance some low-affinity interactions between T-cell hybridomas and their specific Class II's, HAM57-597 (anti-Cb) may be included in the reaction mixture in step B. Decrease the volume of the cells to 20uL and add 10 uL of 5ug/mL HAM57-597 along with the 10uL of Pe-SAv-$(IAbPep)_4$. Final concentration of the HAM57-597 will be 1ug/mL.

**** We assume an extinction coefficient of 1 for these reagents, and use theOD_{280} as the mg/mL.**

VI. Protease Inhibitor Recipes

Leupeptin is made up at 2mg/mL in Water and stored in aliquots at –70^{o}C.
Pepstatin A is made up at 2mg/mL in Methanol and stored in aliquots at –70^{o}C.

The 100X stock is made up from the 2mg/mL stocks.
100X PepstatinA+Leupeptin is 70ug/mL Pepstatin A and 100ug/mL Leupeptin
Per 2mL: 70uL 2mg/mL PepstatinA
100uL 2mg/mL Leupeptin
1.83mL 10mM Tris, pH 8.0

1000X (0.1M) Phenylmethylsulfonylfluoride (PMSF) is made in absolute (100%) ethanol. PMSF is hydrolyzed in water after 20 minutes, so always keep the aliquots free of water. Store at –70^{o}C. The PMSF falls out of solution at –70^{o}C. Allow it to warm to room temperature and mix before using. The absolute ethanol is very volatile, so keep the tube closed whenever it is not in use, plus it smells. PMSF is toxic, so use proper precautions.

VII. Bio Mixes

Bio-Mix A: 0.5M Bicine pH 8.3
MW 163.17g/mole

Store 0.5mL aliquots in gasketed tubes in a non-frost free -20^{o}C freezer.

Bio-Mix B 100mM ATP
100mM MgAcetate
400uM Biotin

ATP Disodium Salt 555.1g/mole
MgAcetate·4H_2O 214.46g/mole
Biotin: we have a large stock of 500uM Biotin from Avidity

Weigh ATP and calculate volume required to make it 100mM. Add that amount of 500uM Biotin solution and the appropriate amount of MgAcetate. (Solution will also containStore 0.5mL aliquots in a non-frost free -20^{o}C freezer.