Date: _____ *Exp:* _____ *Protein:*_____ **B cell tetramer production**

From Justin Taylor Lab, FHCRC

I. Biotinylation of protein

Materials:

EZ-link Sulfo-NHS-LC-Biotynlation Kit (Pierce 21327); Amicon Ultra (or equivalent) spin columns; 1x DPBS, Mg and Ca free; Ultrapure H20

Procedure:

1. Record relevant info about protein:
   Source: _____Purity: _____
   Molecular Weight (MW) of protein: _____
   Molar extinction coefficient of your protein (Ex Co): _____μM^{-1} cm^{-1}
   A280: _____
   Concentration of your protein (A280 / Ex Co): _____ µM
   Media Protein is in: _____
2. If media contains components that will inhibit biotinylation (Aizde, Tris, etc.), exchange media to PBS using dialysis or a spin column with a MW cut off that is smaller than your protein.
   Brand / size / MW cutoff used: _____
   Molar amount of protein added (concentration x volume _____ µL): _____ pmoles
   A280: _____ Concentration: _____ µM
   Molar amount of protein recovered (concentration x volume _____ µL): _____ pmoles
   % protein recovered: _____ (usually ~80%)
3. Calculate the amount of biotin to use.
   Molar amount of protein to be biotinylated: _____ pmoles
   Molar amount of Biotin to add (usually 1 biotin : 1.3 Protein*): _____
   Volume of 10 mM of Biotin added: _____
   *Note: This ratio works well for proteins ranging in concentration of 5 µM to 500 µM. For lower protein concentrations you might need to add a slightly higher ratio of biotin to protein.
4. Remove a 1 mg vial of Sulfo-NHS-LC-Biotin from freezer and equilibrate to room temperature.
5. Immediately before you intend to add biotin to your protein, add 179.7 µL of ultrapure H2O to the vial of biotin, which makes a 10 mM solution. Further dilute biotin in ultrapure H2O if necessary.
   Additional biotin dilution (if necessary):_____
6. Add biotin to protein, mix well and incubate for 2 hours on ice.
7. Add entire reaction to a spin column that has a MW cutoff that is smaller than your protein.
   Brand / size / MW cutoff used: _____
8. Top off spin column with 1 x DPBS. Centrifuge at 2000 x g for 10-20 min or until little media is left on top of the filter.
9. Pour media that passed through the spin column into a new tube. Repeat step 6 & 7 twice, pooling the media that passes through the column. This media will eventually get discarded but if the spin column was broken or you used the wrong cutoff you can recover your protein from this fraction.
10. Collect the sample that remained on top of the filter, rinse the filter with 50 – 250 µL of 1x DPBS. Pipette vigorously to remove any protein that is loosely bound to the membrane.
11. Measure OD280 and determine the molar concentration of your protein using your extinction coefficient.
    A280: _____ Concentration: (A280 / Ex Co) _____ µM
    Molar amount of biotinylated protein (concentration x volume _____ µL): _____ pmoles
    % Protein recovered: _____ (usually ~80%)

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II. Assessing the amount of biotinylated protein
Materials:
SA-PE (Prozyme PJRS25); 4-12% Bis Tris-HCL gels (Life Tech BG04127); 2x native sample buffer (Life Tech LC2673); 20X Bolt SDS Running buffer (Life Tech B0002); SeeBlue Plus2 Protein Standard (Life Tech LC5925); SA-AF680 (Invitrogen S21378); 1x DPBS; Tween-20; Positive control: any biotinylated protein

Procedure:
1. Prepare 40 µl of protein at 2 µM. Lower concentrations can be used, but will need to be accounted for when you determine the biotin:Protein ratio below.
   Protein concentration: _____µM. Dilution Factor: _____
2. Prepare 30 µl of 1 µM SA-PE (using the concentration of SA in the conjugate. Perform a 2-fold dilution series with PBS starting 10 µl of 1 µM SA-PE until you reach 0.03125 µM.
3. Add 5 µl of the 2 µM protein to each tube, then 5 µl of the SA-PE dilutions as shown in the table below. Incubate for at least 30 minutes at room temperature, or indefinitely at 4C.

Tube /lane	Total amount of protein/tube	Volume of protein to be added	Total amount of SA-PE/tube	Volume of SA-PE to be added
1	10 pmol	5 µl of 2 µM	None	0
2	10 pmol	5 µl of 2 µM	5 pmol	5 µl of 1 µM
3	10 pmol	5 µl of 2 µM	2.5 pmol	5 µl of 0.5 µM
4	10 pmol	5 µl of 2 µM	1.25 pmol	5 µl of 0.25 µM
5	10 pmol	5 µl of 2 µM	0.625 pmol	5 µl of 0.125 µM
6	10 pmol	5 µl of 2 µM	0.31 pmol	5 µl of 0.0625 µM
7	10 pmol	5 µl of 2 µM	0.156 pmol	5 µl of 0.03125 µM
8	None	0	5 pmol	5 µl of 1 µM
9	20 pmol Pos control			

4. Add 4 µl of 2x native sample buffer, then run on a 4-12% Bis Tris-HCL gel using native PAGE running buffer. You can use 10 µl of protein standard in lane 10, but it is not really necessary.
5. Run at 150V for 15 minutes.
6. Transfer the proteins to nitrocellulose. We use an iBlot2, 20 volts for 7 minutes.
7. Wash nitrocellulose 1x with PBS with 0.2% Tween-20 (PBST), then incubate with SA-AF680 diluted 1 – 10,000 in PBST 30 minutes at room temperature with rocking.
8. Wash 2x with PBST, then use the Odyssey Infrared Imager to detect AF680 at 700 nm.
9. Lane #1 should have a band or try re-running the assay with more protein.
10. No band should be present in lane #2 because all of the biotinylated protein should be bound to SA-PE. If there is a band present here than either the protein concentration was incorrect, or you have more than one biotin per protein molecule. This latter case can confound tetramer production. Repeat biotinylation procedure with more protein or less biotin.
11. As the SA-PE concentration falls the band should eventually reappear. If the band never reappears, repeat with lower concentrations of SA-PE. Use the concentration of SA in the lane that the band reappears below.
    _____ µM of SA x 4 biotin binding sites x ___ dilution factor (from step II-1) = _____ µM Biotin
    Protein concentration (from step II-1): _____ µM
    Biotin:Protein Ratio (0.25 – 0.75 optimal): _____

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III. Tetramerize the protein

Materials:

SA-PE (Prozyme PJRS25) or SA-APC (Prozyme PJ27S); 1x DPBS; 100 kDa Amicon Ultra centrifugation filters (Millipore UFC910024)

Procedure:

1. For the tetramer to work well, it needs to be fully armed, which means there needs to be ***at least*** 4:1 ratio of biotinylated protein to SA-PE. 100 µl of SA-PE makes a good amount of tetramer.
2. To calculate the amount of biotinylated protein to add to SA-PE, use the below formula.

   _____ µM (pmoles / µl) SA concentration of SA-PE or SA-APC
   x _____ µl of SA-PE or SA-APC added
   = _____ pmoles SA
   x 4 biotin binding sites
   = _____ pmoles of biotin needed to saturate SA
   / _____ µM (pmoles / µl) Biotin concentration of protein
   = _____ µl of protein to add

3. Incubate at room temperature for at least 30 minutes, or 4C indefinitely.
4. Purify the tetramer from unincorporated protein. If your protein is under 100 kDa, use a 100 kDa Amicon Ultra spin filter, washing the fraction that remains on top twice with PBS. If your protein is larger than 100 kDa you can try spin columns with higher cutoffs, but we haven't had great success with these. Size exclusion chromatography is a better option if available. Alternatively, unpurified tetramer usually works fine.
   Brand / size / MW cutoff used (if not 100 kDa): _____
5. Estimate the tetramer concentration by determining the concentration of fluorochrome.
   PE tetramer: A566: _____ / 1.96 cm^{-1} μM^{-1} = _____ µM
   APC tetramer: A650: _____ / 0.7 cm^{-1} μM^{-1} = _____ µM
6. Dilute the concentration of PE or APC to 1.0 µM with 1x DPBS and store at 4°C.