Oligo-Antibody Conjugation

1.Pre-wet one Amicon 30 kDa MWCO filter per antibody with 400 ul 1X BBS

2.Add 25 ug antibody to the liquid in the filter and centrifuge at 14,000 xg for 5 mins

Note: Can use up to 100 ug antibody here or as little as 5 ug (although percentage yield of 5 ug is lower in my experience)

3.Discard the flow through and add another 400 ul 1X BBS and centrifuge again 14,000 x g for 5 mins.

4. Invert the filter into a clean collection tube and spin at 1,000 x g for 2 mins to elute

Note 1: The expected volume at this stage should be anywhere from 20-80 ul
Note 2: Save the filter and put in old collection tube and fill with 400 ul 1X BBS to stay wet. We will use this again

5.If volume is below 45 ul, add 1X BBS to bring volume to at least 45 ul

6.Dilute 1 ul of provided 20 mM mTz-PEG4-NHS in 9 ul DMSO to make 2 mM solution

Note: If conjugating > 2 antibodies or if one of the two antibodies have a volume >45 ul, then more solution will have to be made. Just scale accordingly.

7.For concentrated antibodies in 45 ul, add 5 ul of 2 mM mTz-PEG4-NHS and flick to mix, spin down briefly in a table top minifuge.

Note: For volumes > 45 ul, scale the amount of 2 mM mTz-PEG4-NHS added

8. Incubate labeling reactions for 30 mins at room temperature

9. Add 1 ul of provided 1M glycine to the labeling reaction to quench unreacted groups. Flick to mix and incubate at room temperature for 5 mins

10. Transfer the solution to the saved 30 kDa cutoff filter and spin at 14,000 x g for 5 mins

11.Discard flow through, add 475 ul 1X BBS and spin again at 14,000 x g for 5 mins

12. Invert the filter into a clean collection tube and spin at 1,000 x g for 2 mins to elute

13. Measure volume with p200 pipette and adjust to 50 ul with 1X BBS

Note: The volume will likely be higher than 50 ul – if this is the case, no volume adjustment is necessary

14. For each ug of antibody, add 15 pmol (0.15 ul) of provided TCO-PEG4-oligo, flick to mix and spin down in tabletop microfuge

Note: The prelabeled oligos are provided at a concentration of 100 uM. As an example, for 20 ug of antibody, add 3 ul of provided TCO-PEG4-oligo. Be sure to add only one oligo per antibody and to record which oligo was added to each antibody.

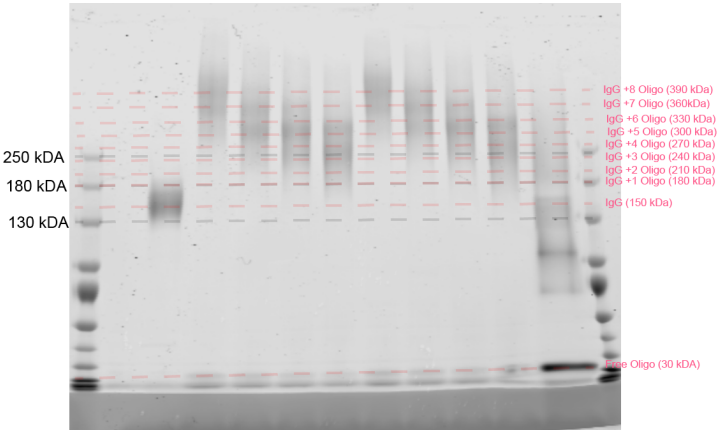
15. Incubate these conjugation reactions overnight at 4C

16. After reaction is complete, add provided 10 mM TCO-PEG4-Gly at a dilution of 1:10 (e.g. 5.55 ul in 50 ul conjugated antibodies). This will quench any leftover reaction sites.

17. Store the conjugated antibodies at 4C until they you are ready to pool them

To verify conjugation:

Take approximately 1 ug of labeled antibody and run on a protein gel, using Coomasie staining (or alternative, like InstantBlue) to stain and image the gel. Use 1ug of any unlabeled antibody as a control. You should observe a size shift on the order of 30 kDa increments representing the amount of oligos labeled per-antibody (See below figure). This protocol aims to add 2-4 oligos per antibody, therefore you should see protein bands at ~210,240,270 kDa (IgG is approx. 150 kDa). Do not be concerned if the sizes don't line up perfectly, as long as there is a size shift. If you see no size shift, redo the conjugation reaction. If you see too much shifting it just means more oligos per antibody and is not concerning. If there is a mixture of labeled and unlabeled antibody (0-2 oligos/antibody) that is ok too.



Conjugated antibody pooling and cleanup

In my experience, conjugated antibodies are stable at 4C for quite a while (I have used conjugated antibodies up to a year post-conjugation). Perform the pooling step at least a day before you plan to run your experiment to save time on experiment day.

1. Run a BCA or equivalent protein quantification assay on your individual conjugated antibodies.

> Note: Because there is significant antibody loss through the filtering steps, it is not safe to assume that the mass output of the conjugation reaction is the same as the input

2. For pooling antibodies for a single experiment, mix together 3 ug of each antibody in low-bind protein 1.5 ml tube

3. Adjust the volume to 60 ul with PBS. If the volume is over 60 ul, there is no need to adjust

4a. **Optional but highly recommended step for larger panels (>10 abs):** Add 40 ul (40% final) saturated ammonium sulfate (~4.32 M),mix well and incubate on ice for 15 minutes

Note 1: This step leads to significant antibody loss, however the amount of free oligo that is removed is much higher than using just molecular weight cut-off filters. For larger panels, this step is necessary because the total amount of free oligo in a pre-cleaned pooled antibody set will be quite high.

Note 2: If pooling lots of antibodies, the volume will likely be much higher than 60 ul, so be sure to add enough ammonium sulfate that the final proportion is 40%

4b. Spin full speed for 15 mins at 4C

Note: Be sure to note the orientation of the tube in the centrifuge. The protein pellet will be totally transparent on the side of the tube, so it is important to note where it is so you down pipette it out in the next step

4c. Carefully pipette out the supernatant and place in a new low-bind tube. Set aside for comparison later

4d. Resuspend the pellet in 200 ul PBS

5. Pre-wet a 50 kDa MWCO filter with 200 ul PBS

Note: In this step, it is seemingly important to use a 50 kDa filter. 30 kDa will lead to less free oligo filtering and for some reason, even though the antibodies are much larger, a 100 kDa MWCO leads to more antibody less in my experience

6. **If not doing Ammonium Sulfate Step, continue here:** If the antibody solution is not at 200 ul, add PBS to bring up to 200 ul

7. Add the antibody pool to the filter and spin 14,000 x g for 5 mins

8. After spinning, remove the flow through from the collection tube and add to a separate tube indicating which flowthrough it is (e.g. Flow-1)

9. Add 475 ul PBS to the filter and repeat the 14,000 x g for 5 mins spin. Collect flow through and put in a different tube

10. Quantify the amount of DNA in the flowthrough on a nanodrop or equivalent instrument. You should see the quantity of DNA drop significantly between each spin

11. Repeat washes until the flowthrough DNA quantity falls to ~0.2 ng/ul or lower

Note: As this point, any leftover free oligo will be difficult to get rid of, and performing more spins with the same filter will just lead to antibody loss without reducing the relative free oligo significantly

12. Invert the filter in a new collection tube and spin at 1000 x g for 2 mins

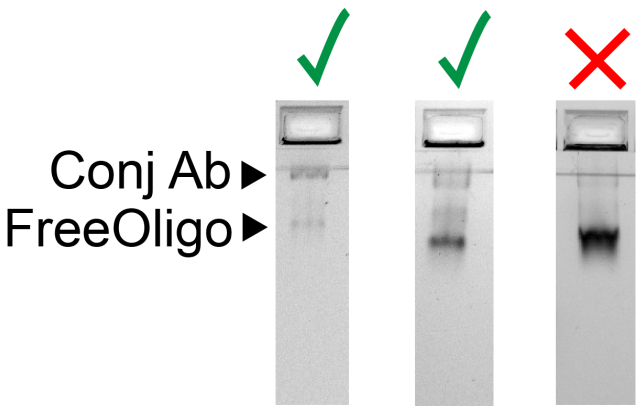
13. Run 1 ul of the collected antibody pool on an e-gel or other electrophoresis method. You will see a band corresponding to free oligo, then a heavier smear corresponding to conjugated antibody (see figure below)

14. If there is still significant free oligo leftover, add the pool antibodies to a FRESH 50 kDa filter in 400 ul PBS

Note: You can reuse the old filter, however I find that it is not effective anymore at filtering and tends to lead to antibody loss

15. Proceed with at least 2x more PBS washes, invert the filter and collect again and reassess the remaining oligo on a gel.

Note: There will almost always be some oligo leftover with this protocol, it is almost impossible to get rid of it all. Adding proper blocking and an overabundance of SSB during the Phospho-seq procedure will negate the effects of these leftover free oligos



16. Run a BCA or other protein quantification method on the antibody pool to determine the total amount of antibody. Divide by the number of antibodies in the pool to get the best estimate of concentration per antibody. For each phospho-seq experiment add the equivalent of 0.25-0.50 ug/ antibody to the cells in the staining step.