CITE-seq Hyper Antibody-Oligo Conjugation

This protocol describes the covalent and irreversible conjugation of purified antibodies to oligonucleotides by iEDDA-click chemistry, essentially as previously described in Van Buggenum *et al.*, 2016.

TCO-PEG4-Oligo Labeling

Required reagents:

- 10X Borate Buffered Saline pH 7.6 (BBS): 0.5 M borate, 1.5 M NaCl, pH 7.6. Dilution of this buffer to 1X concentration results in a pH of 8.5.
- Dimethyl sulfoxide (DMSO)
- 100 mM TCO-PEG4-NHS (Click Chemistry Tools) in DMSO
- 1 M glycine pH 8.5
- Micro Bio-Spin P-6 Gel Columns, Tris Buffer (Bio-Rad)

1. Dissolve the oligo (100 nmole scale) in 100 µl water and heat at 55 ˚C for 10 minutes.
2. Spin the oligo at full speed for 10 minutes to pellet any insoluble debris from the synthesis. Transfer the supernatant to a clean microfuge tube.
3. Add 10 µl 5 M NaCl, mix well, and 300 µl ethanol. Invert to mix – a precipitate readily forms.
4. Chill the sample at -80 ˚C for 5 minutes (or -20 ˚C for an extended period of time) and spin at full speed for 15 minutes to pellet the oligo. Discard the supernatant and wash the pellet with 2 x 0.5 ml 80% ethanol with 10 minute spins between each wash.
    - At this concentration of oligo, the cold incubation can be skipped with minimal impact on recovery.
5. After the last wash, quick spin the sample and remove any residual liquid. Air dry the pellets for 10 minutes.
6. Dissolve the oligo in 40 µl water, using heat if needed.
7. Dilute 1 µl of the oligo with 49 µl water (1:50 dilution) and determine the concentration via NanoDrop.
    - The oligo concentration (in µM) is calculated as (A260/ε) x Dilution Factor x 10e6.
8. Set up the following 70 µl conjugation reaction:
    - ~35 nmol amine-modified oligo (this can be a bit less depending on synthesis scale) (0.5 mM final concentration)

- Water to 49 µl
- 7 µl 10X BBS (10% of final volume)
- 7 µl DMSO (10% of final volume)
- 2 x 3.5 uL of 100 mM TCO-PEG4-NHS in DMSO, added in sequential additions every 15 minutes.
    - Note: The reaction can be scaled up or down as needed.

9. React one aliquot of the TCO linker with the amine-labeled oligo for 15 minutes. Add the second aliquot and react for an additional 15 minutes.
    - Note: the TCO-PEG4-NHS may become slightly turbid or cloudy after the second addition, but this does not affect conjugation efficiency or purification of the modified oligo.
10. Add 1.45 µl 1 M glycine pH 8.5 (final concentration ~20 mM) and incubate at room temperature for 5 minutes to quench residual NHS groups.
11. Desalt the reactions using Bio-Rad Micro Bio-Spin P-6 columns.
    - Briefly, gravity drain the column and spin at 1,000*g* for 2 minutes to remove the storage buffer (Tris). Apply the sample to the center of the column and spin for 4 minutes at 1,000*g* to desalt.
    - Save the eluate from the desalting column as a blank for the NanoDrop.
12. Adjust the concentration to what you expect to be 200uM based on the QC from the IDT tube.
13. Quantify a 1:20 dilution (by diluting 1 µl of each sample with 19 µl water) and determine the concentration via NanoDrop.
    - The oligo concentration (in µM) is calculated as (A260/ε) x Dilution Factor x 10e6.
14. Adjust concentration to ~100uM with 1xBBS. Store oligo at -20˚C.
15. Verify modification of oligo by running ~100 fmol on a Bioanalyzer Small RNA chip (see Figure 1).

mTz-PEG4-Antibody Labeling

Required reagents:

- 10X Borate Buffered Saline (BBS) pH 7.6: 0.5 M boric acid, 1.5 M NaCl, pH 7.6. Dilution of this buffer to 1X concentration results in a pH of ~8.5.
- Dimethyl sulfoxide (DMSO)
- 20 mM mTz-PEG4-NHS (Click Chemistry Tools) in DMSO
- 1 M glycine pH 8.5
- Amicon Ultra-0.5 Centrifugal Filter Unit with 30 kDa MWCO membrane (EMD Millipore)

Notes:

- The protocol outlined below is for 50 µg of antibody.
- mTz-PEG4-antibody conjugation can be scaled up or down as needed. However, ensure that the final concentration of the antibody in the conjugation reaction is 1 mg/ml, and 1:10 final volume of mTz-PEG4-NHS 2mM (final 0.2mM), for the most efficient and reproducible results.

1. Pre-wet an Amicon Ultra-0.5 30 kDa MWCO filter unit with 400 µl 1X BBS.
2. Add 50 µg antibody to the liquid in the filter and spin the unit at 14,000*g* for 5 minutes.
3. Discard the flow through and add 400 µl 1X BBS. Spin the sample for 5 minutes at 14,000*g*.
4. Invert the filter into a clean collection tube and spin at 1,000*g* for 2 minutes to elute.
   - Keep 30kDa cutoff filter for subsequent wash. Add 400 uL 1x BBS buffer to avoid drying.
5. Adjust the volume of the concentrated antibody to 45 µl with 1X BBS.
6. Dilute 1 µl of mTz-PEG4-NHS (20 mM in DMSO) with 9 µl DMSO to make a 2 mM solution.
7. Add 5 µl of the diluted mTz-PEG4-NHS (2 mM) solution to the antibody and flick to mix.
   - Concentration of antibody is now 1 µg/µl
8. Incubate the labeling reactions at room temperature for 30 minutes.
9. Add 1 µl 1 M glycine pH 8.5 (final concentration ~20 mM) to the antibody to quench any unreacted NHS groups. Flick to mix, and incubate at room temperature for an additional 5 minutes.
10. Transfer to 30 kDa cutoff filter (reuse filter from above) and spin the unit at 14,000*g* for 5 minutes.
11. Discard the flow through and add 475 µl 1X BBS. Spin the sample for 5 minutes at 14,000*g*.
12. Invert the filter into a clean collection tube and spin at 1,000*g* for 2 minutes to elute.
13. Adjust volume to ~50 µL using 1X BBS.

Oligo-Antibody Conjugation

Required reagents:

- mTz-PEG4-antibody (~1 ug/ul, concentration is not crititcal)
- TCO-PEG4-oligo (~100 µM concentrated)
- TCO-PEG4-gly (10 mM): mix 10 µl 100 mM TCO-PEG4-NHS, 2 µl 1 M glycine pH 8.5, and 88 µl water. Incubate at room temperature for 1 hour to quench all NHS groups. Store at -20 ˚C until needed.
- Phosphate buffered saline (PBS)

Notes:

- The final volume of the oligo-antibody conjugation reaction is not critical, and the reaction will proceed efficiently over a wide range of concentrations. However, the *amount* of TCO-PEG4-oligo is extremely important, and is calculated in the following manner.

1. For each 1 µg of mTz-PEG4-antibody add 30 pmol of TCO-PEG4-oligo and flick to mix.
    - E.g. Mix 50 µg of mTz-PEG4-antibody with 1,500 pmol of oligo.
2. Allow the conjugation reaction to proceed at room temperature (or 4˚C) over night.
3. After the reaction is complete, add 1/10th of the reaction volume of 10 mM TCO-PEG4-gly to quench any residual tetrazine reaction sites on the antibody. Remove ~ 2 µg of the labeled/quenched antibody for PAGE-gel analysis.
4. After verification of good conjugation (see Figure 2) adjust concentration to 0.5 ug/ul with PBS containing 0.06 % Sodium-Azide (final) and store the reagent at 4 ˚C until use or proceed to antibody pooling and cleanup.

Antibody-oligo panel pooling and cleanup

Required reagents:

- Saturated Ammonium Sulfate solution(~4.32M)
- PBS
- Amicon Ultra-0.5 Centrifugal Filter Unit with 50 kDa MWCO membrane (EMD Millipore)

1. Pool the appropriate amounts of labeled antibodies (typically 1 µg antibody per experiment) to create your panel of interest in a low-bind 1.5 mL tube.
2. Adjust volume to 60 ul with PBS
3. Add 40uL (40% final) saturated ammonium sulfate (~4.32M) and incubate on ice for 15 minutes.
4. Spin full speed for 15 minutes at 4˚C
   - Oligo pellet will be a white dense pellet, protein pellet will be almost transparent.
5. Carefully discard supernatant.
6. Resuspend pellet in 60 ul PBS.
7. Add 40uL (40% final) saturated ammonium sulfate (~4.32M) and incubate on ice for 15 minutes.
8. Spin full speed for 15 minutes at 4˚C
9. Repeat for 1 or 2 more times depending on the size of the white oligo pellet.
10. Resuspend in 200 ul PBS.
11. Pre-wet an Amicon Ultra-0.5 50 kDa MWCO filter unit with 200 µl PBS.
12. Add the antibody pool to the liquid in the filter and spin the unit at 14,000*g* for 5 minutes.
13. Discard the flow through and add 475 µl PBS. Spin the sample for 5 minutes at 14,000*g*.
14. Repeat until 7 x PBS washes have been performed.
15. After the last wash invert the filter into a clean collection tube and spin at 1,000*g* for 2 minutes to elute.
16. Verify free oligo depletion on E-gel (see Figure 3).
17. Adjust concentration of panel with PBS and add 1 µg/µl BSA (final), 0.06% Sodium Azide (final)

Oligonuleotide sequences:

CITE-seq antibody-oligos (ADTs):

These contain standard small TruSeq RNA read 2 sequences and can be amplified using Illumina's Truseq Small RNA primer sets (RPIx – primers, see example RPI1 below). Depending on the Antibody-conjugation chemistry used these have to be ordered as 5'Biotinylated (`/5Biosg/`) or 5'Amine (`/5AmMC12/`). See examples below with 12nt barcodes:

- ADT1 **CCTTGGCACCCGAGAATTCCACATGATTGGCTCBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT2 **CCTTGGCACCCGAGAATTCCAGAGGCGATTGATBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT3 **CCTTGGCACCCGAGAATTCCATGTCCGGCAATABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT4 **CCTTGGCACCCGAGAATTCCATGGTGAACCTGGBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT5 **CCTTGGCACCCGAGAATTCCAGATCGTAATACCBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT6 **CCTTGGCACCCGAGAATTCCAAAGCGCTTGGCABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT7 **CCTTGGCACCCGAGAATTCCACATCGGTGTACABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT8 **CCTTGGCACCCGAGAATTCCAGTCTAGACTTCGBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT9 **CCTTGGCACCCGAGAATTCCACGAAGAAGGAGTBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT10 **CCTTGGCACCCGAGAATTCCACGGAGTAGTAATBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT11 **CCTTGGCACCCGAGAATTCCAGTTGTTGGTCCGBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT12 **CCTTGGCACCCGAGAATTCCACACCGCAACGTABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT13 **CCTTGGCACCCGAGAATTCCAGTCCAACAGCCABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT14 **CCTTGGCACCCGAGAATTCCAAGCGAAGACGATBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT15 **CCTTGGCACCCGAGAATTCCATCTGGAGGACAABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT16 **CCTTGGCACCCGAGAATTCCATTGATCGACCGTBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT17 **CCTTGGCACCCGAGAATTCCACGTGATTGAAGGBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- ADT18 **CCTTGGCACCCGAGAATTCCAACCGATCTCAGCBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**

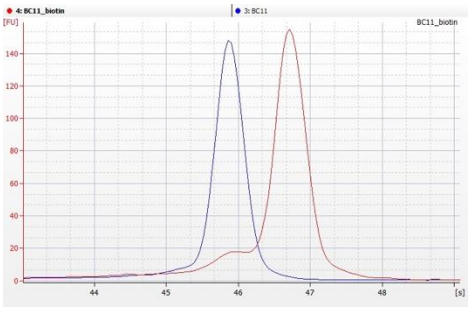
Hashtag barcoding antibody-oligos (HTOs):

These contain standard TruSeq DNA read 2 sequences and can be amplified using truncated versions of Illumina's TruSeq DNA primer sets (see example D701_s below). Depending on the Antibody-conjugation chemistry used these have to be ordered as 5'Biotinylated (`/5Biosg/`) or 5'Amine (`/5AmMC12/`). See examples below with 12nt barcodes:

- HTO1 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGACCATCCAABAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO2 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACATGTTACCGTBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO3 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGCTTACTATCCBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO4 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCGATAATGCGABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO5 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGGCTGAGCTABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO6 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTGTGACGTATTBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO7 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTGTCTAACGGBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO8 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTATCACATCGGTBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO9 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCACATAATGACGBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO10 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAACGACGTGGTBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**

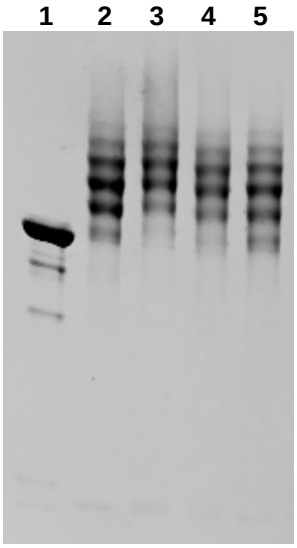
QC Figures.

Figure 1. Verification of TCO-Oligo modification



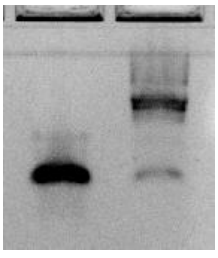
100 fmol of modified (red trace) and unmodified (blue trace) oligo are run on a Bioanalyzer Small RNA chip. A clear size shift should be visible and over 90% of oligo should be modified.

Figure 2. Verification of antibody-oligo conjugation.



Verification of antibody-oligo purification. Efficient antibody-oligo conjugation should be verified by running ~2 ug of antibody-oligo complexes on a **non-reducing (!)** SDS 4-12 polyacrylamid gel to visualize size shift from non-conjugated to conjugated antibody.
1. Not-conjugated antibody control (~150kDa), **2-5.** Conjugated antibodies.

Figure 3. Verification of antibody-oligo pool purification.



Verification of antibody-oligo purification. Efficient antibody-oligo cleanup from un-conjugated oligo should be verified by running ~1 ug of antibody-oligo complexes on a 4% Agarose gel (E-gel). Successfully conjugated and purified antibody-oligo complexes should appear as a high molecular weight smear with some laddering and very little residual oligo (lane 2). **1**. Oligo only control, **2**. Antibody-Oligo Complex.