Preparation of Antibody-Oligo conjugates using Steptavidin-Biotin interaction

Antibody-oligo conjugates can be prepared by various methods which form covalent links between antibodies and oligos (*e.g.* iEDDA-, Maleimide- and NHS- click chemistries) or by indirect streptavidin-biotin-linkage.
For our proof-of-principle experiments (Stoeckius et al., 2017, Nature Methods) we used streptavidin-biotin-linkage to couple oligos to antibodies, and include a cleavable linker in the oligos (see protocol 1 below). We note that a cleavable linker adds no benefit in the streptavidin-biotin-linkage strategy, and in the case of direct conjugation, appears to be detrimental. For experiments involving conjugation via a streptavidin-biotin-linkage, we therefore recommend simply ordering biotinylated oligos.
We have successfully tested direct antibody-oligo conjugation chemistry (iEDDA, essentially as described in Van Buggenum *et al.*, Scientific Reports, 2016; Stoeckius et al., 2017, bioRxiv) and a commercially available kit (Thunder-Link PLUS, Innova Biosciences) without a cleavable disulfide linker for CITE-seq (see protocol 2 below).
CITE-seq antibody-oligo conjugates and 'cell hashing' antibodies are also available from BioLegend (USA): https://www.biolegend.com/totalseq

1. Preparation of Antibody-Oligo conjugates using Streptavidin-Biotin

Obtain biotinylated oligos

- Order 5'Biotinylated oligos with a specific barcode (for examples see page 8):
    - Small synthesis scales of ~25 nmoles are sufficient for many antibody conjugations.
- Adjust concentration to 100 µM with TE.
- Heat at 55 ˚C for 10 minutes.
- Centrifuge full speed (~18,000*g*) in table top centrifuge for 5 minutes at room temperature.
- Carefully transfer supernatant to new tube if there is visible pellet. Pellet is residual resin from oligonucleotide synthesis.
- Quantify oligo, if needed adjust concentration to 100 µM with TE and store at -20˚C.

Streptavidin labelling of antibodies

- *Only use flow cytometry optimized monoclonal antibody clones.*
- *Verify antibody concentration, 15 µg of antibody are needed for conjugation.*
- Clean 15 µg of antibody on 50 kDa cutoff column per manufacturer protocol to exchange buffer and remove contaminants:
    - Pre-wet 50 kDa cutoff column by adding 200 µl PBS pH 8.5.
    - Combine 15 µl antibody with 200 µl PBS pH 8.5 and transfer to column.
    - Spin at room temperature 4 minutes 14,000*g*.
    - Discard flowthrough.
    - Add 400 µl PBS pH 8.5 to column.
    - Spin at room temperature ~4 minutes at 14,000*g* until all liquid has drained to ~20 µl mark on column.
    - Recover concentrated purified antibody by placing column upside down in new tube and spin for 2 minutes at 3,000*g*.
    - Adjust volume of recovered purified antibody to 30 µl with PBS pH 8.5.
- Streptavidin label antibodies using a 10 µg streptavidin kit (see materials) per manufacturer's protocol with the following modifications:
    - *Note: 10µg streptavidin kit conjugates ~ 2 streptavidin tetramers to each antibody on average when using 15 µg of Antibody input.*
    - Add 3 µl of modifier solution (from kit) to 30 µl recovered purified antibody.
    - Add purified antibody solution containing modifier directly onto the lyophilized reactive 10µg streptavidin.
    - Mix by flicking the tube carefully.
    - Incubate for at least 3 hours (or overnight) at room temperature.
    - Quench reaction by adding 3 µl quenching solution (from kit).
    - Add 4 µl 5M NaCl to increase the NaCl concentration to ~0.5M.
    - Add 4 µl Tween 20 (0.1% in H_2O) to get final of ~0.01% Tween.
- *Antibodies are now ready to be attached to biotinylated oligos without additional cleanup steps (see below).*

by Marlon Stoeckius mstoeckius@nygenome.org & Peter Smibert psmibert@nygenome.org

Merge streptavidin-antibodies with biotinylated-oligos in PBS/0.5M NaCl.

- *Note: Each antibody should be labelled with ~2 streptavidin molecules according to the kit specifications. 10µg streptavidin = ~200pmol X 4 = 800pmol (biotin binding sites) If all binding sites are saturated, each antibody will have 8 oligos on average.*

- Add ~800 pmoles of biotinylated oligo directly into streptavidin antibody reaction tube.
- Incubate overnight at room temperature.
- Wash oligo-labelled antibodies on 50 kDa cutoff column per manufacturer's protocol.
  - Pre wet 50 kDa cutoff column with ~300 µl PBS.
  - Transfer to oligo-labelled antibody to 50 kDa cutoff column.
  - Spin at room temperature for 4 minutes at 14,000*g.*
  - Discard flow through.
  - Wash antibody-oligo-conjugate 7 times in PBS on column (Spin at RT ~4min ~14,000*g*, per cutoff column protocol).
  - Perform the final wash with PBS.
  - Spin at room temperature ~4 minutes at 14,000*g* until all liquid has drained to ~20 µl mark on column.
  - Recover concentrated purified antibody by placing column upside down in new tube and spin for 2 minutes at 3,000*g*.
  - Adjust volume of recovered purified antibody to 30 µl with PBS.
- Validate oligo-conjugation:
  - Run ~1 µg of antibody complex and 100 pmol of oligo on 4% Agarose gel (E-gel) for 4 minutes.
  - Cool gel before visualization at 4˚C for at least 10 minutes (see Figure 1).
- Store Antibodies in storage buffer at 4˚C until use (PBS, 1 µg/µl BSA, 0.05% Sodium Azide).
- Keep barcoded antibodies as pure stocks. Pool with other labeled antibodies only directly before use.
  - Note that we have not extensively tested the shelf life of these conjugates. We recommend using the antibody-oligo complexes within a few weeks.
  - If antibodies were not used for a prolonged period of time (> 3 months) it is advisable to run an aliquot on a 4% agarose gel (Figure 2) to verify oligos are still attached.

Please note that we include this protocol to enable reproducibility. In our own experiments, we have entirely switched to the iEDDA click chemistry conjugates.

by Marlon Stoeckius mstoeckius@nygenome.org & Peter Smibert psmibert@nygenome.org

Preparation of antibody panels:

For streptavidin-biotin antibody-oligo conjugates:

- Steptavidin-biotin-antibody-oligo conjugates have to be stored separately and should only be pooled shortly before the run.
- Make antibody panel by pooling all streptavidin-biotin antibodies and clean pooled panel on 50kDa cutoff column per manufacturer's protocol to remove unbound oligos shortly before CITE-seq run:
    - Use 1-2 µg of each antibody-oligo complex, comparable to what is recommended for flow cytometry per test
        - Optionally, optimal antibody concentration can be titrated by testing different concentrations.
    - Merge appropriate amounts of all antibodies for one CITE-seq run in ~300 µl 0.5M NaCl/PBS containing 2 µl of 10 mM biotin to **block** unoccupied biotin-binding sites in streptavidin.
    - Incubate for ~5 minutes at room temperature.
    - Pre-wet 50kDa cutoff column with ~100 µl PBS.
    - Transfer biotin-blocked antibody panel to 50kDa cutoff column.
    - Spin at RT 5 minutes 14,000*g.*
    - Discard flow through.
    - Wash antibody-panel 2 times in 400 µl PBS on column (Spin at RT ~4min ~14,000*g*, per cutoff column protocol).
    - Perform the final wash with 400 µl PBS.
    - Spin at RT ~5 minutes at 14,000*g* until liquid has drained to ~20 µl mark on column.
    - Recover concentrated purified antibody by placing column upside down in new tube and spin for 2 minutes at 3,000*g*.
    - Adjust volume to 100 µl with cell staining buffer (2%BSA/0.02%Tween, PBS).
- Streptavidin-biotin antibody-oligo pools have to be used for cell labelling immediately. Do not store merged antibody-oligo pool.

by Marlon Stoeckius mstoeckius@nygenome.org & Peter Smibert psmibert@nygenome.org

Figures

Figure 1. Verification of antibody-oligo conjugation.

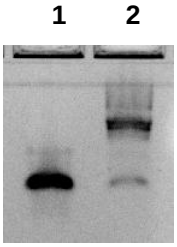

Figure 1. Verification of antibody-oligo conjugation. Efficient antibody-oligo conjugation and minimal un-conjugated oligo carryover should be verified by running ~1 ug of antibody-oligo complexes on a 4% Agarose gel. Successfully conjugated and purified antibody-oligo complexes should appear as a high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

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 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGACCATCCAABAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO2 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACATGTTACCGTBAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO3 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGCTTACTATCCBAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO4 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCGATAATGCGABAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO5 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGGCTGAGCTABAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO6 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTGTGACGTATTBAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO7 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTGTCTAACGGBAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO8 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTATCACATCGGTBAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO9 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCACATAATGACGBAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**
- HTO10 **GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAACGACGTGGTBAAAAAAAAAAAAAAAAAAAAAAAAA*A*A**

by Marlon Stoeckius mstoeckius@nygenome.org & Peter Smibert psmibert@nygenome.org

Materials and Kits needed:

- Flow-cytometry grade monoclonal antibodies, unlabeled and purified (*e.g.* BioLegend)
- Antibody streptavidin conjugation kit
  (*e.g.* Bio-Rad LYNX kit #LNK163STR, or, Innova Biosciences Lightning-Link kit # 708-0030)
- Centrifugal Filters 50kDa cutoff (*e.g.* Millipore Amicon Ultra – 0.5mL Centrifugal Filters #UFC505024)
- Desalting columns (*e.g.* Bio-Rad, Micro Bio-Spin 6 Columns. #732-6221)
- E-gel 4% (Invitrogen, USA)
- Low-bind 1.5 mL tubes
- Qubit (Invitrogen, USA)
- DMSO (Common lab suppliers).
- PBS (Common lab suppliers)
- Na-Azide (Common lab suppliers)
- NaCl (Common lab suppliers)
- Tween20 (Common lab suppliers)
- Biotin (Common lab suppliers)
- TE pH 8.0 (Common lab suppliers)
- BSA (Common lab suppliers)

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