CITE-seq protocol

Preparation of CITE-seq oligos and antibodies:

Biotinylation of oligos

- Order 5'amine-labelled oligos with a C12 spacer and a specific barcode (for examples see page 6):
  - Small synthesis scales of ~25 nmoles are sufficient for many antibody conjugations.
- Clean oligo by Ethanol precipitation to remove residual synthesis contaminants:
  - Resuspend all 25 nmoles of lyophilized oligo in 200 µl 0.5M NaCl.
  - 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.
  - Add 3 volumes (600 µl) of 100% EtOH.
  - Incubate at -80˚C for at least 30 minutes.
  - Centrifuge full speed (~18,000*g*) in table top centrifuge for 30 minutes at 4˚C.
  - Wash pellet in 80% EtOH.
  - Centrifuge full speed (~18,000*g)* in table top centrifuge for 30 minutes at 4˚C.
  - Wash pellet in 80% EtOH.
  - Centrifuge full speed (~18,000*g*) in table top centrifuge for 30 minutes at 4˚C.
  - Air dry pellet.
- Resuspend oligo pellet in 20 µL PBS pH 8.5.
- Biotinylate oligo with EZ-link Sulpho-NHS S-S Biotin per manufacturer protocol (see materials):
  - Resuspend Single Use EZ-link NHS S-S Biotin in 164 µl DMSO to make 10mM solution.
  - Add 10 µl of Biotin-NHS to oligo (100 nmoles ~4 fold excess over oligo).
  - Incubate at RT for 5 minutes.
  - Repeat 4 more additions of 10 µl Biotin-NHS to oligo and incubations for 5 minutes. Final volume now 70 µl.
  - Purify oligo by Ethanol precipitation:
    - Increase volume to 400 µl (add 330 µl water) to reduce DMSO concentration to <10%.
    - Add 1:10 vol. 5M NaCl (40 µl).
    - Add 3 vol. 100% Ethanol (1.2 mL).
    - Incubate for at least 30 minutes at -80˚C.
    - Centrifuge full speed (~18,000*g*) in table top centrifuge for 30 minutes at 4˚C.
    - Wash pellet in 500 µl 80% EtOH.
    - Centrifuge full speed (~18,000*g*) in table top centrifuge for 30 minutes at 4˚C.
  - Resuspend pelleted oligo in 20 µl PBS pH 8.5.
  - Add 10 µl of Biotin-NHS to oligo (100 nmoles ~4 fold excess over oligo).
  - Incubate at room temperature for 5 minutes.
  - Repeat 4 more additions of 10 µl Biotin-NHS to oligo and incubations for 5 minutes. Final volume now 70 µl.
- Clean oligos vigorously to eliminate biotin carryover by two steps:
  1. Ethanol precipitation:
     - Increase volume to 400 µl (add 330 µl water) to reduce DMSO concentration to <10%.
     - Add 1:10 vol. 5M NaCl (40 µL).
     - Add 3 vol. 100% Ethanol (1.2 mL).
     - Incubate for at least 30 minutes at -80˚C.
     - Centrifuge full speed (~18,000*g*) in table top centrifuge for 30 minutes at 4˚C.
     - Wash pellet in 500 µl 80% EtOH.
     - Centrifuge full speed (~18,000*g*) in table top centrifuge for 30 minutes at 4˚C.
     - Wash pellet in 500 µl 80% EtOH.
     - Centrifuge full speed in (~18,000*g*) table top centrifuge for 30 minutes at 4˚C.
     - Air dry pellet shortly.
     - Resuspend pellet in water at an estimated concentration of >100 µM ~200 µL.
  2. Size exclusion on Bio-Spin P6 desalting column according to manufacturer's protocol:
     - Spin oligos at 1000*g* for 4 minutes at room temperature.
     - Collect flow through containing biotinylated purified oligo.

- Quantify oligo, if needed adjust concentration to 100 µM with TE and store at -20˚C.
- Verify sufficient biotinylation by running control and biotinylated oligo on Agilent Bioanalyzer Small RNA Chip (see Figure 1).
    - If less than 90% of oligo is biotinylated, repeat biotinylation.

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.*
    - 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).*

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 purified oligo directly onto 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 0.5M NaCl/PBS on column (Spin at RT ~4min ~14,000*g*, per cutoff column protocol).
    - Perform the final wash with 1x 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 by running ~0.7 µg of Antibody on 4% Agarose E-gel.

- Release oligo by treating with 0.2M DTT for 10min at 90˚C and compare to untreated antibody.
- Run ~0.7 µg of antibody complex on 4% Agarose gel (E-gel) for 4 minutes.
- Cool gel before visualization on ice (see Figure 2).

○ 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.

CITE-seq run:

Prepare antibody panel shortly before CITE-seq run

- Make antibody panel by pooling all 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.
    - 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 0.5M NaCl/PBS.
  - Transfer biotin-blocked antibody panel to 50kDa cutoff column.
  - Spin at RT 4 minutes 14,000*g.*
  - Discard flow through.
  - Wash antibody-panel 2 times in 400 µl 0.5M NaCl/PBS on column (Spin at RT ~4min ~14,000*g*, per cutoff column protocol).
  - Perform the final wash with 400 µl 1x PBS.
  - Spin at RT ~4 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*.
- Use pool for cell labelling immediately. Do not store merged antibody-oligo pool.

Cell staining for Drop-seq or 10X

- Carefully count cells to ensure accurate quantitation.
- Resuspend ~500,000 cells in 200 µl Staining buffer (2%BSA/0.02%Tween, PBS).
- Add 5 µl Fc Blocking reagent (FcX, BioLegend).
- Incubate 10 minutes at 4˚C.
- Add cleaned Antibody-oligo pool (containing ~1-2 µg of each Antibody or titrated amounts).
- Incubate for 30 minutes at 4˚C.
- Wash cells 3 times with 1 mL Staining buffer (2%BSA/0.02%Tween, PBS), spin 5 minutes 450*g* at 4˚C.
- Resuspend cells in PBS at appropriate concentration for downstream application. **(*e.g.*** for **10x** [~500 cells/µl] or **Drop-seq** [~ 200 cells/µl]).

Run Drop-seq or 10x Genomics single cell 3' assay according to Macosko *et al.*, 2015 (Drop-seq) or manufacturer's instructions (10x Genomics) until after the cDNA amplification step.

After cDNA amplification: Separate ADTs (~180bp) and cDNAs (>300bp).

- Perform SPRI selection to separate ADTs and full length cDNAs.
- **DO NOT DISCARD SUPERNATANT FROM 0.6X SPRI. THIS CONTAINS THE ADTs!**
  - Add 0.6X SPRI.
  - Incubate 5 minutes and place on magnet.
  - Supernatant contains ADTs.
  - Beads contain full length cDNAs.

- **cDNA >300bp (beads fraction).**
  - Proceed with standard 10x or Drop-seq protocol for cDNA sequencing library preparation.

- **ADTs ~180bp (supernatant fraction).**
  - Purify ADTs using two 2XSPRI purifications per manufacturer protocol:
    - Add 1.4X SPRI to supernatant to obtain a final SPRI volume of 2XSPRI.
    - Incubate 10 minutes at room temperature.
    - Place tube on magnet and wait 1 minute until solution is clear.
    - Carefully remove and discard the supernatant.

- Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (only one Ethanol wash).
- Carefully remove and discard the ethanol wash.
- Centrifuge tube briefly and return it to magnet.
- Remove and discard any remaining ethanol.
- Resuspend in beads in 50 µl water.
- Perform another round of 2X SPRI purification by adding 100 µl SPRI reagent directly onto resuspended beads.
- Incubate 10 minutes at room temperature.
- Place tube on magnet and wait 1 minute until solution is clear.
- Carefully remove and discard the supernatant.
- Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (1st Ethanol wash).
- Carefully remove and discard the ethanol wash.
- Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (2nd Ethanol wash).
- Carefully remove and discard the ethanol wash.
- Centrifuge tube briefly and return it to magnet.
- Remove and discard any remaining ethanol and allow the beads to air dry for 1 minute.
- Resuspend beads in 45 µl water.
- Pipette mix vigorously and incubate at room temperature for 5 minutes.
- Place tube on magnet and transfer clear supernatant to PCR tube.

- Amplify ADT sequencing library:
  - Prepare 100uL PCR reaction with purified ADTs:
    - 45 µl purified ADTs.
    - 50 µl 2x KAPA Hifi PCR Master Mix.
    - 2.5 µl Truseq Small RNA RPIx primer (containing i7 index) 10uM.
    - 2.5 µl P5 oligo at 10uM depending on application:
      - For Dropseq use P5-SMART-PCR hybrid oligo.
      - For 10x use Illumina PE 1.0 P5 oligo.
    - Cycling conditions:

95˚C	3 min		
95˚C	20 sec	|	
60˚C	30 sec	|	8-12 cycles
72˚C	20 sec	|	
72˚C	5 min		

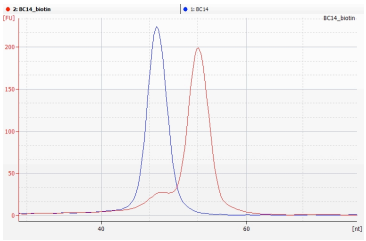
- Purify PCR product using 1.6X SPRI purification by adding 160 µl SPRI reagent.
  - Incubate 5 minutes at room temperature.
  - Place tube on magnet and wait 1 minute until solution is clear.
  - Carefully remove and discard the supernatant.
  - Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (first Ethanol wash).
  - Carefully remove and discard the ethanol wash.
  - Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (second Ethanol wash).
  - Carefully remove and discard the ethanol wash.
  - Centrifuge tube briefly and return it to magnet.
  - Remove and discard any remaining ethanol and allow the beads to air dry for 1 minute.
  - Resuspend beads in 45 µl water.
  - Pipette mix vigorously and incubate at room temperature for 5 minutes.
  - Place tube on magnet and transfer clear supernatant to PCR tube.
  - Wash beads twice in 80% Ethanol.
  - Elute in 20 µl water.
- ADT library is now ready to be sequenced.
  - Quantify library by standard methods (QuBit, BioAnalyzer, qPCR).

Sequencing CITE-seq libraries:

- We estimate that an average of 100 molecules per ADT per cell is sufficient to achieve useful information. The number of reads required to obtain 100 molecules depends on the complexity of the sequencing library (*e.g.* duplication rate). ADT and cDNA sequencing libraries can be pooled at desired proportions. To obtain sufficient read coverage for both libraries we typically sequence ADT libraries in 10% of a lane and cDNA library fraction at 90% of a lane (HiSeq Rapid Run Lane).

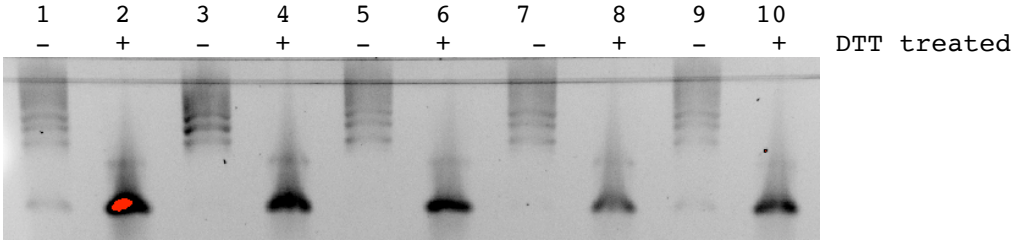
Figures

Figure 1. Verification of oligo biotinylation.



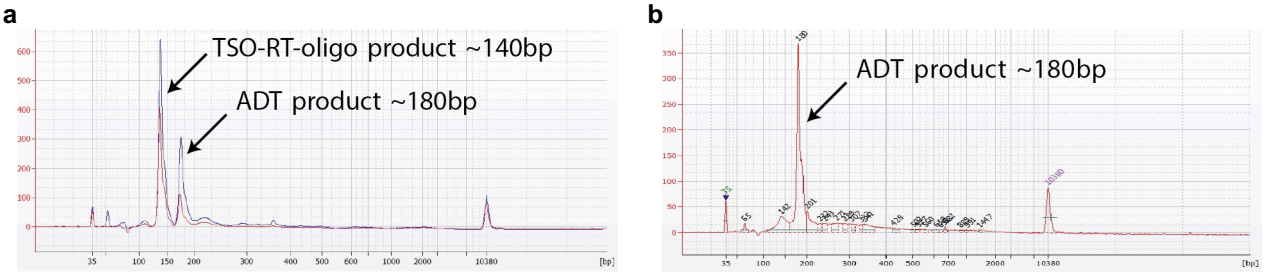
Run 100 fmoles of biotinylated (red trace) and unconjugated oligo (blue trace) on a Small RNA Bioanalyzer chip. You will expect to see a clear size shift between conjugated and unconjugated oligos and over 90% labelling.

Figure 2. Verification of antibody-oligo conjugation



Antibody-labelled-oligonucleotides with and without DTT treatment (oligo release) were run on 4% Agarose gel to verify both oligo labelling and removal of unbound oligo. Successfully conjugated and purified antibody-oligo complexes should appear as a high molecular weight smear with some laddering (lanes 1, 3, 5, 7 & 9) which entirely collapses to an oligo band upon reduction, as seen in lanes 2, 4, 6, 8 & 10. Lane 1 (and to a lesser extend lane 9) are examples of carryover of unconjugated oligo.

Figure 3. ADT library verification.



(**a**) A TSO-RT-oligo product (~140 bp) can be amplified during the ADT PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. Sequential 2X SPRI purification of the ADT fraction after cDNA amplification reduces carryover of primers from cDNA amplification, and minimizes the amplification of this product during ADT-library amplification. (**b**) A clean ADT library will contain a predominant single peak at around 180 bp.

Oligo sequences:

Oligos contain standard small TruSeq RNA read 2 sequences and can be amplified using standard Truseq Small RNA primer sets (RPIx - primers):

- v2_BC1: /5AmMC12/**CCTTGGCACCCGAGAATTCCAATCACGBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC2: /5AmMC12/**CCTTGGCACCCGAGAATTCCACGATGTBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC3: /5AmMC12/**CCTTGGCACCCGAGAATTCCATTAGGCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC4: /5AmMC12/**CCTTGGCACCCGAGAATTCCATGACCABAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC5: /5AmMC12/**CCTTGGCACCCGAGAATTCCAACAGTGBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC6: /5AmMC12/**CCTTGGCACCCGAGAATTCCAGCCAATBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC7: /5AmMC12/**CCTTGGCACCCGAGAATTCCACAGATCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC8: /5AmMC12/**CCTTGGCACCCGAGAATTCCAACTTGABAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC9: /5AmMC12/**CCTTGGCACCCGAGAATTCCAGATCAGBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC10: /5AmMC12/**CCTTGGCACCCGAGAATTCCATAGCTTBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC11: /5AmMC12/**CCTTGGCACCCGAGAATTCCAGGCTACBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC12: /5AmMC12/**CCTTGGCACCCGAGAATTCCACTTGTABAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC13: /5AmMC12/**CCTTGGCACCCGAGAATTCCAAGTCAABAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**
- v2_BC14: /5AmMC12/**CCTTGGCACCCGAGAATTCCAAGTTCCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**

Oligos required for ADT library amplification:

- P5-SMART-PCR hybrid oligo (Drop-seq)
  5'AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C

- Illumina PE 1.0 P5 oligo (10x Single Cell Version 2)
  5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGAT*C*T

- Illumina Small RNA RPI1 (i7 index 1)
  CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

Materials and Kits needed:

- Flow-cytometry grade monoclonal antibodies, unlabeled and purified
- Antibody streptavidin conjugation kit
  (*e.g.* Bio-Rad LYNX kit #LNK163STR, or, Innova Biosciences Lightning-Link kit # 708-0030)
- NHS Biotinylation reagent
  (*e.g.* Thermo, EZ-Link Sulfo-NHS-SS-Biotin, No weight format. #21328)
- Centrifugal Filters 50kDa cutoff:
  (*e.g.* Millipore Amicon Ultra – 0.5mL Centrifugal Filters #UFC505024)
- FC blocking reagent (*e.g.* BioLegend)
- Desalting columns.
  (*e.g.* Bio-Rad, Micro Bio-Spin 6 Columns. #732-6221)
- 8-strip PCR tubes, emulsion safe (!) (*e.g.* TempAssure PCR 8-strips, USA Scientific)
- Bioanalyzer chips and reagents (DNA High Sensitivity and small RNA kit, Agilent)
- SPRIselect reagent (GE Healthcare, B23317)
- E-gel 4% (Invitrogen)
- PCR Thermocycler (*e.g.* Bio-Rad, T100)
- Magnetic tube rack (e.g. Invitrogen, USA)
- Qubit (Invitrogen, USA)
- DMSO (Common lab suppliers).
- PBS (Common lab suppliers)
- Na-Azide (Common lab suppliers)
- NaCl (Common lab suppliers)
- Tween20 (Common lab suppliers)