

## 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  $\mu$ l 0.5M NaCl.
  - Centrifuge full speed (~18,000g) 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  $\mu$ l) of 100% EtOH.
  - Incubate at -80°C for at least 30 minutes.
  - Centrifuge full speed (~18,000g) in table top centrifuge for 30 minutes at 4°C.
  - Wash pellet in 80% EtOH.
  - Centrifuge full speed (~18,000g) in table top centrifuge for 30 minutes at 4°C.
  - Wash pellet in 80% EtOH.
  - Centrifuge full speed (~18,000g) in table top centrifuge for 30 minutes at 4°C.
  - Air dry pellet.
- Resuspend oligo pellet in 20  $\mu$ l 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  $\mu$ l DMSO to make 10mM solution.
  - Add 10  $\mu$ l of Biotin-NHS to oligo (100 nmoles ~4 fold excess over oligo).
  - Incubate at RT for 5 minutes.
  - Repeat 4 more additions of 10  $\mu$ l Biotin-NHS to oligo and incubations for 5 minutes. Final volume now 70  $\mu$ l.
  - Purify oligo by Ethanol precipitation:
    - Increase volume to 400  $\mu$ l (add 330  $\mu$ l water) to reduce DMSO concentration to <10%.
    - Add 1:10 vol. 5M NaCl (40  $\mu$ l).
    - Add 3 vol. 100% Ethanol (1.2 mL).
    - Incubate for at least 30 minutes at -80°C.
    - Centrifuge full speed (~18,000g) in table top centrifuge for 30 minutes at 4°C.
    - Wash pellet in 500  $\mu$ l 80% EtOH.
    - Centrifuge full speed (~18,000g) in table top centrifuge for 30 minutes at 4°C.
  - Resuspend pelleted oligo in 20  $\mu$ l PBS pH 8.5.
  - Add 10  $\mu$ l 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  $\mu$ l Biotin-NHS to oligo and incubations for 5 minutes. Final volume now 70  $\mu$ l.
- Clean oligos vigorously to eliminate biotin carryover by two steps:
  1. Ethanol precipitation:
    - Increase volume to 400  $\mu$ l (add 330  $\mu$ l water) to reduce DMSO concentration to <10%.
    - Add 1:10 vol. 5M NaCl (40  $\mu$ l).
    - Add 3 vol. 100% Ethanol (1.2 mL).
    - Incubate for at least 30 minutes at -80°C.
    - Centrifuge full speed (~18,000g) in table top centrifuge for 30 minutes at 4°C.
    - Wash pellet in 500  $\mu$ l 80% EtOH.
    - Centrifuge full speed (~18,000g) in table top centrifuge for 30 minutes at 4°C.
    - Wash pellet in 500  $\mu$ l 80% EtOH.
    - Centrifuge full speed in (~18,000g) table top centrifuge for 30 minutes at 4°C.
    - Air dry pellet shortly.
    - Resuspend pellet in water at an estimated concentration of >100  $\mu$ M ~200  $\mu$ L.
  2. Size exclusion on Bio-Spin P6 desalting column according to manufacturer's protocol:
    - Spin oligos at 1000g for 4 minutes at room temperature.
    - Collect flow through containing biotinylated purified oligo.

- Quantify oligo, if needed adjust concentration to 100  $\mu$ M 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  $\mu$ g of antibody are needed for conjugation.*
- Clean 15  $\mu$ g 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  $\mu$ l PBS pH 8.5.
  - Combine 15  $\mu$ l antibody with 200  $\mu$ l PBS pH 8.5 and transfer to column.
  - Spin at room temperature 4 minutes 14,000g.
  - Discard flowthrough.
  - Add 400  $\mu$ l PBS pH 8.5 to column.
  - Spin at room temperature ~4 minutes at 14,000g until all liquid has drained to ~20  $\mu$ l mark on column.
  - Recover concentrated purified antibody by placing column upside down in new tube and spin for 2 minutes at 3,000g.
  - Adjust volume of recovered purified antibody to 30  $\mu$ l with PBS pH 8.5.
- Streptavidin label antibodies using a 10  $\mu$ g streptavidin kit (see materials) per manufacturer's protocol with the following modifications:
  - *Note: 10 $\mu$ g streptavidin kit conjugates ~ 2 streptavidin tetramers to each antibody on average when using 15  $\mu$ g of Antibody input.*
  - Add 3  $\mu$ l of modifier solution (from kit) to 30  $\mu$ l recovered purified antibody.
  - Add purified antibody solution containing modifier directly onto the lyophilized reactive 10 $\mu$ g streptavidin.
  - Mix by flicking the tube carefully.
  - Incubate for at least 3 hours (or overnight) at room temperature.
  - Quench reaction by adding 3  $\mu$ l quenching solution (from kit).
  - Add 4  $\mu$ l 5M NaCl to increase the NaCl concentration to ~0.5M.
  - Add 4  $\mu$ l Tween 20 (0.1% in H<sub>2</sub>O) 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. 10 $\mu$ g 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  $\mu$ l PBS.
  - Transfer to oligo-labelled antibody to 50 kDa cutoff column.
  - Spin at room temperature for 4 minutes at 14,000g.
  - Discard flow through.
  - Wash antibody-oligo-conjugate 7 times in 0.5M NaCl/PBS on column (Spin at RT ~4min ~14,000g, per cutoff column protocol).
  - Perform the final wash with 1x PBS.
  - Spin at room temperature ~4 minutes at 14,000g until all liquid has drained to ~20  $\mu$ l mark on column.
  - Recover concentrated purified antibody by placing column upside down in new tube and spin for 2 minutes at 3,000g.
  - Adjust volume of recovered purified antibody to 30  $\mu$ l with PBS.
- Validate oligo-conjugation by running ~0.7  $\mu$ g 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.
  - 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.

**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 14,000g.
  - Discard flow through.
  - Wash antibody-panel 2 times in 400 µl 0.5M NaCl/PBS on column (Spin at RT ~4min ~14,000g, per cutoff column protocol).
  - Perform the final wash with 400 µl 1x PBS.
  - Spin at RT ~4 minutes at 14,000g 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 3,000g.
  - Adjust volume to 100 µl with cell staining buffer (2%BSA/0.02%Tween, PBS)
- **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.
  - Make note of cell viability (>95%) and also include dead cells in the total cell count!
  - If you observe many dead cells live cell enrichment by FACS is recommended!
- Resuspend ~500,000 cells in 100 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 100 µl Antibody-oligo pool (containing ~1-2 µg of each Antibody or titrated amounts).
  - Final volume is now 200 µl.
- 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 450g 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]).
- **Verify cell concentration by counting on hemocytometer.**

**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 ~2 minutes until solution is clear.
  - Carefully remove and discard the supernatant.
  - Add 400  $\mu$ l 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  $\mu$ l water.
  - Perform another round of 2X SPRI purification by adding 100  $\mu$ l SPRI reagent directly onto resuspended beads.
  - Incubate 10 minutes at room temperature.
  - Place tube on magnet and wait ~2 minutes until solution is clear.
  - Carefully remove and discard the supernatant.
  - Add 200  $\mu$ l 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (1<sup>st</sup> Ethanol wash).
  - Carefully remove and discard the ethanol wash.
  - Add 200  $\mu$ l 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (2<sup>nd</sup> 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 2 minutes.
  - Resuspend beads in 45  $\mu$ l 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 100 $\mu$ l PCR reaction with purified ADTs:
    - 45  $\mu$ l purified ADTs.
    - 50  $\mu$ l 2x KAPA Hifi PCR Master Mix.
    - 2.5  $\mu$ l Truseq Small RNA RPIx primer (containing i7 index) 10 $\mu$ M.
    - 2.5  $\mu$ l P5 oligo at 10 $\mu$ M 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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 2 minutes.
  - Resuspend beads in 20  $\mu$ l water.
  - Pipette mix vigorously and incubate at room temperature for 5 minutes.
  - Place tube on magnet and transfer clear supernatant to PCR tube.

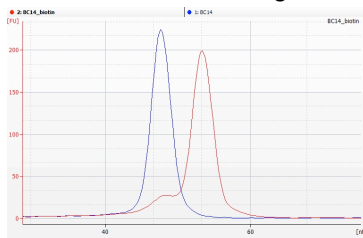
- ADT library is now ready to be sequenced.
  - Quantify library by standard methods (QuBit, BioAnalyzer, qPCR).
  - ADT library will be between 170-180 bp (Figure 2).

## 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).

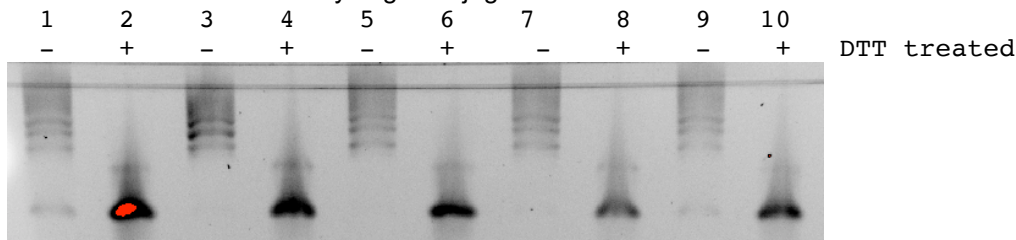
## Figures

**Figure 1.** Verification of oligo biotinylation.



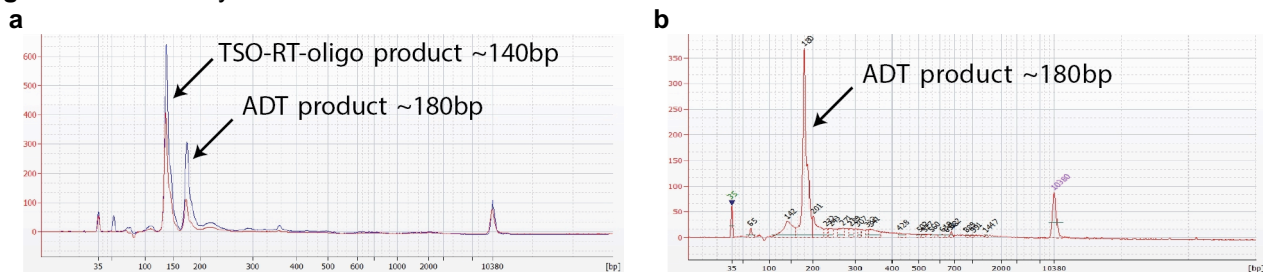
Run 100 fmoles of biotinylated (red trace) and unconjugated oligo (blue trace) on a Small RNA Bioanalyzer chip. You will expect to see a clear size shift between conjugated and unconjugated oligos and over 90% labelling.

**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 170-180 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/CCTTGGCACCCGAGAATTCCAATCAGCGBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC2: /5AmMC12/CCTTGGCACCCGAGAATTCCACGATGTBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC3: /5AmMC12/CCTTGGCACCCGAGAATTCCATTAGGCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC4: /5AmMC12/CCTTGGCACCCGAGAATTCCATGACCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC5: /5AmMC12/CCTTGGCACCCGAGAATTCCAACAGTBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC6: /5AmMC12/CCTTGGCACCCGAGAATTCCAGCCAATBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC7: /5AmMC12/CCTTGGCACCCGAGAATTCCAAGATCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC8: /5AmMC12/CCTTGGCACCCGAGAATTCCAAGCTGBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC9: /5AmMC12/CCTTGGCACCCGAGAATTCCAGATCAGBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC10: /5AmMC12/CCTTGGCACCCGAGAATTCCAGGCTACBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC11: /5AmMC12/CCTTGGCACCCGAGAATTCCAGGCTACBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC12: /5AmMC12/CCTTGGCACCCGAGAATTCCACTGTABAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC13: /5AmMC12/CCTTGGCACCCGAGAATTCCAAGTCAABAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
- v2\_BC14: /5AmMC12/CCTTGGCACCCGAGAATTCCAAGTTCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Oligos required for ADT library amplification:

- P5-SMART-PCR hybrid oligo (For Drop-seq)  
5' AATGATACGGCGACCACCGAGATCTACACGCTGTCCGCGAAGCAGTGGTATCAACGCAGAGT\*A\*C
- Illumina PE 1.0 P5 oligo (For 10x Single Cell Version 2, Oligonucleotide sequences © 2015 Illumina, Inc)  
5' AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGAT\*C\*T
- Illumina Small RNA RPI1 (i7 index 1, Oligonucleotide sequences © 2015 Illumina, Inc)  
CAAGCAGAAGACGGCATACGAGATCCTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

**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, USA)
- PCR Thermocycler (e.g. Bio-Rad, T100)
- Magnetic tube rack (e.g. Invitrogen, USA)
- Qubit (Invitrogen, USA)
- Hemocytometer (e.g. Fuchs Rosenthal)
- 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)