CITE-seq & Cell Hashing Protocol

For experiments involving cell hashing, use cost per cell calculator to plan experiments, determine number of hashes, number of cells to load, expected doublet rates (detected and undetected) and cost considerations:

Obtain cells

- Carefully count all 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 is recommended!
  - Keep cell suspensions on ice (unless otherwise stated) at all times.

Multiplexing different samples

- Resuspend ~1-2 million cells of each sample in 100 µl Staining buffer (2%BSA/0.01%Tween, PBS) in 1.5 ml low bind tubes.
- Add 10 µl Fc Blocking reagent (FcX, BioLegend).
- Incubate for 10 minutes at 4˚C.
- While cells are incubating in Fc Block, prepare antibody-pool using ~0.5 - 1 µg (or titrated amounts) of each **CITE-seq antibody**.
- Add **CITE-seq antibody pool** to each tube.
- Add 0.5ug of a unique **Cell Hashing antibody** to each tube.
- Incubate for 30 minutes at 4˚C.
- Wash cells 3 times with 1 ml Staining buffer (2%BSA/0.01%Tween, PBS), spin at 4˚C for 5 minutes at 350*g*.
- **Keep samples separated until shortly before the run.**
- Merge all samples at desired proportions in 1 ml Staining buffer, spin 5 minutes 350*g* at 4˚C.
- Resuspend cells in PBS at appropriate concentration for downstream application.
  (*e.g.* for 10x ~500 cells/µl; for **Drop-seq** [~ 200 cells/µl]; for **super-loading** ~ 1,500 cells/µl or higher).
- Filter cells through 40 µm strainers (*e.g.* Flowmi cell strainer).
- Verify cell concentration, single cellularity and high cell viability on hemocytometer after filtration.
- **Immediately proceed to single cell run**.

Super-loading of one sample

- Resuspend ~1-2 million cells in 100 µl Staining buffer (2%BSA/0.01%Tween, PBS) in 1.5 ml low bind tube.
- Add 10 µl Fc Blocking reagent (FcX, BioLegend).
- Incubate for 10 minutes at 4˚C.
- While cells are incubating in Fc Block, prepare antibody-pool using ~0.5 - 1 µg (or titrated amounts) of each **CITE-seq antibody**.
- Add **CITE-seq antibody pool** to cells.
- Incubate for 30 minutes at 4˚C.
- Divide sample into 10 equal proportions into 1.5 ml low bind tubes (when using 10 Hashtags).
- Adjust volume to 50 µl with Staining buffer (2%BSA/0.01%Tween, PBS)
- Add 0.5ug of unique **Cell Hashing antibody** to each tube.
- Incubate for 20 minutes at 4˚C.
- Wash cells 3 times with 1 ml Staining buffer (2%BSA/0.01%Tween, PBS), spin at 4˚C for 5 minutes at 350*g*.
- **Keep samples separated until shortly before the run.**
- Merge cells from all tubes at equal ratios in 1 ml Staining buffer, spin 5 minutes 350*g* at 4˚C.
- Resuspend cells in PBS at appropriate concentration for desired super-loading yield.
  (~ 1,500 cells/µl or higher, use cost per cell calculator).
- Filter cells through 40 µm strainers (*e.g.* Flowmi cell strainer).
- Verify cell concentration, single cellularity and high cell viability on hemocytometer after filtration.
- **Immediately proceed to single cell run**.

Run Drop-seq (Macosko *et al.*, 2015) or 10x Genomics single cell 3' v2 assay as described until before cDNA amplification.

When super-loading we recommend adding 0.5U/µl RNAse inhibitor to the single cell RT master mix.

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At cDNA amplification step:

Add "additive" primers to cDNA PCR to increase yield of ADT and/or HTO products:
ADT PCR additive primer (0.2 µM): 1 µl (for 10x Genomics) or 0.4 µl (for Drop-seq)
HTO PCR additive primer (0.1 µM): 1 µl (for 10x Genomics) or 0.4 µl (for Drop-seq)

Subtract the total volume of additive primer from the water added to the PCR reaction.

After cDNA amplification: Separate ADT / HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp).

- Perform SPRI selection to separate mRNA-derived and antibody-oligo-derived cDNAs.
- **DO NOT DISCARD SUPERNATANT FROM 0.6X SPRI. THIS CONTAINS THE ADTs and hashtags!**
  - Add 0.6X SPRI to cDNA reaction as described in 10x Genomics or Drop-seq protocol.
  - Incubate 5 minutes and place on magnet.
  - Supernatant contains ADTs and Cell Hashtags.
  - Beads contain full length mRNA-derived cDNAs.

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

- **ADTs and Hashtags <180bp (supernatant fraction).**
  - Purify ADTs using two 2X SPRI purifications:
    - Add 1.4X SPRI to supernatant to obtain a final SPRI volume of 2X SPRI.
      For example: For 10x Genomics, the cDNA PCR reaction volume is 100 µl, 60uL (0.6X SPRI was added for capturing full length cDNAs, therefore add another 140 µl of SPRI to the supernatant. The final volume will be 300 µl containing 2X SPRI.
    - Transfer entire volume into a low-bind 1.5mL tube.
    - 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 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.
    - Mix by pipetting and 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 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 2 minutes (do not over dry beads).
    - Resuspend beads in 90 µl water.
    - Pipette mix vigorously and incubate at room temperature for 5 minutes.
    - Place tube on magnet and transfer clear supernatant into two PCR tubes.

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- Amplify CITE-seq ADT sequencing library:
  - Prepare 100uL PCR reaction with purified ADTs:
    - 45 µl purified ADT/Hashtag fraction
    - 50 µl 2x KAPA Hifi PCR Master Mix.
    - 2.5 µl TruSeq Small RNA RPIx primer (containing i7 index) 10 µM.
    - 2.5 µl P5 oligo at 10 µM depending on application:
      - For Drop-seq use P5-SMART-PCR hybrid oligo.
      - For 10x use SI PCR oligo.
    - Cycling conditions:

95˚C	3 min		
95˚C	20 sec	|	
60˚C	30 sec	|	~ 6-10 cycles
72˚C	20 sec	|	
72˚C	5 min		

- Amplify Cell Hashing HTO sequencing library:
  - Prepare 100uL PCR reaction with purified small fraction:
    - 45 µl purified ADT/Hashtag fraction
    - 50 µl 2x KAPA Hifi PCR Master Mix.
    - 2.5 µl TruSeq DNA D7xx_s primer (containing i7 index) 10 µM.
    - 2.5 µl P5 oligo at 10 µM depending on application:
      - For Drop-seq use P5-SMART-PCR hybrid oligo.
      - For 10x use SI PCR oligo.
    - Cycling conditions:

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

- Purify PCR products 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 2 minutes.
  - Resuspend beads in 30 µl water.
  - Pipette mix vigorously and incubate at room temperature for 5 minutes.
  - Place tube on magnet and transfer clear supernatant to low-bind tube.
- ADT and Hashtag libraries are now ready to be sequenced.
  - Quantify libraries by standard methods (QuBit, BioAnalyzer, qPCR).
  - ADT and Hashtag libraries will be around 180 bp (Figure 1 and 2).

Sequencing CITE-seq and Cell Hashing libraries:

- ADT, HTO and cDNA sequencing libraries can be pooled at desired proportions and sequenced in parallel. We typically allocate ~50K reads per cell for the cDNA library as recommended by 10x Genomics. We typically sequence the ADT library at ~2-5K reads per cell depending on the size of the antibody panel, and ~2K reads per cell for the HTO library.

ADT or HTO library structure:

Read 1:

Read 2:

Figures

Figure 1.

a **b**

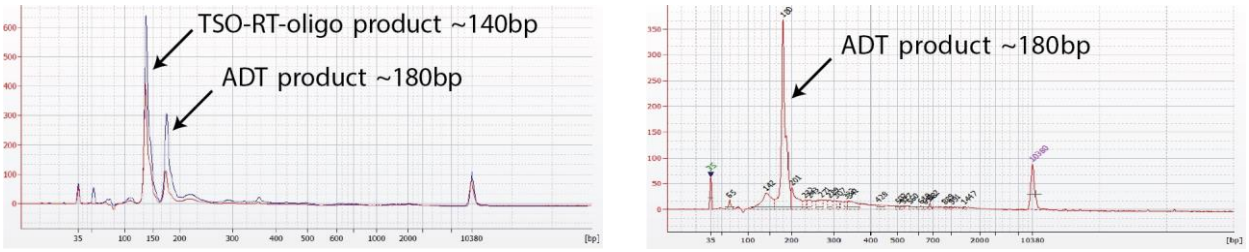

Figure 1. ADT (or Hashtag) library verification. (a) A TSO-RT-oligo product (~140 bp) can be amplified during the ADT PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. Sequential 2X SPRI purification of the ADT fraction after cDNA amplification reduces carryover of primers from cDNA amplification, and minimizes the amplification of this product during ADT-library amplification. To further enrich for ADT (or HTO) specific product the purified ADT library can be reamplified for ~3 additional cycles with ADT specific primer sets or P5/P7 generic primers. (**b**) A clean ADT (or HTO) library will contain a predominant single peak at around 180 bp.

Figure 2.

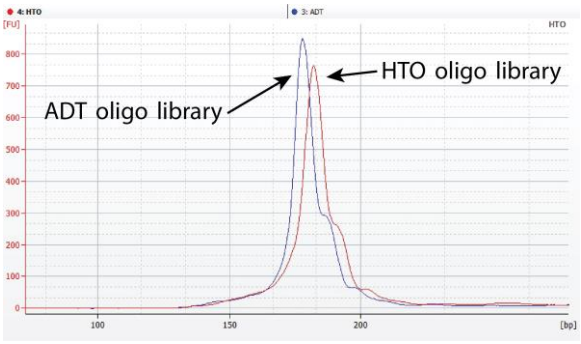

Figure 2. Verification of ADT and Hashtag libraries. ADT and Hashtag libraries are very similar in size ~180bp but should appear as distinguishable products on a High Sensitivity Bioanalyzer, where the Hashtag library appears a few nucleotides larger compared to the ADT library.

Oligonucleotide sequences:

CITE-seq antibody-oligos (ADTs):

CITE-seq antibody-oligos 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). See example below with a 12nt barcode:

5′CCTTGGCACCCGAGAATTCCACATGATTGGCTCBAAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A

Hashtag barcoding antibody-oligos (HTOs):

Cell Hashing antibody-oligos 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). See example below with a 12nt barcode:

5′GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGACCATCCAABAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A

Oligos required for ADT and HTO library amplification:

- Drop-seq P5-SMART-PCR hybrid primer (for Drop-seq only)
  5′AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C
- 10x Genomics SI-PCR primer (for 10x Single Cell Version 2 only)
  5′AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC
- ADT CITE-seq cDNA PCR additive primer
  5′CCTTGGCACCCGAGAATTCC
- HTO Cell Hashing cDNA PCR additive primer
  5′GTGACTGGAGTTCAGACGTGTGCTC
- Illumina Small RNA RPI1 primer (for ADT amplification; i7 index 1, Oligonucleotide sequences © 2015 Illumina, Inc)
  5′CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
- Illumina TruSeq D701_s primer (for HTO amplification; i7 index 1, shorter than the original D701 Illumina sequence)
  5′CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGC

  * Phosphorothioate bond
  B C or G or T; not A nucleotide

Materials and Kits needed:

- Antibody-oligo conjugates for CITE-seq and/or Cell Hashing (*e.g.* BioLegend Totalseq A)
- FC blocking reagent (*e.g.* BioLegend FcX)
- 8-strip PCR tubes**, emulsion safe (!)** (*e.g.* TempAssure PCR 8-strips, USA Scientific)
- Bioanalyzer chips and reagents (DNA High Sensitivity kit, Agilent)
- SPRIselect reagent (GE Healthcare, B23317)
- Low-bind 1.5 mL tubes (Common lab suppliers)
- 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)
- Tween20 (Common lab suppliers)
- TE pH 8.0 (Common lab suppliers)
- BSA (DNAse, RNAse and protease free, *e.g.* VWR #0332-25G)
- Dead Cell Removal Kit (optional, *e.g.* Miltenyi)