CITE-seq & Cell Hashing protocol

For experiments involving cell hashing, we recommend using the cost per cell calculator from the Satija Lab to plan experiments, determine number of hashes, number of cells to load, expected doublet rates (detected and undetected) and cost considerations.

Cell staining for Drop-seq or 10x Genomics

- Obtain all single cell suspensions from different samples/conditions that will be multiplexed in the run. Keep samples in separate tubes until after cell hashing and shortly before loading cells into the single cell RNA-seq instrument. When aiming to super-load the same sample into one run, divide the sample up into equal proportions before staining with distinct cell hashing antibodies. Keep cell suspensions on ice (unless otherwise stated) at all times.
- 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 (*e.g.* Dead Cell Removal kit) is recommended!
- Resuspend ~1-2 million cells in 100 µl Staining buffer (2%BSA/0.02%Tween, PBS).
- 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 and 1 µg of single cell hashing antibody (pool).
- Add antibody-oligo pool to cells.
- 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 400*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 by counting on hemocytometer after filtration.
- Pool all different samples/conditions at desired proportions and immediately proceed to next step.

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

At cDNA amplification step:

Add "additive" primers to cDNA PCR to increase yield of ADT and/or HTO products:
ADT PCR additive primer (2 µM): 1 µl (for 10x Genomics) or 0.4 µl (for Drop-seq)
HTO PCR additive primer (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 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 per manufacturer protocol:
    - Add 1.4X SPRI to supernatant to obtain a final SPRI volume of 2X SPRI.

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- 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.

- Amplify 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 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		

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- 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 2 minutes.
    - Resuspend beads in 20 µl water.
    - Pipette mix vigorously and incubate at room temperature for 5 minutes.
    - Place tube on magnet and transfer clear supernatant to PCR 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).

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Sequencing CITE-seq and Hashing libraries:

- We estimate that an average of 100 molecules per ADT or HTO per cell is sufficient to achieve useful information, we typically sequence our ADT / HTO libraries to obtain significantly more reads than this per cell. The number of reads required to obtain 100 molecules depends on the complexity of the sequencing library (*e.g.* duplication rate). ADT, HTO and cDNA sequencing libraries can be pooled at desired proportions. We typically sequence ADT at 10% and HTO libraries at 5% of a lane and cDNA library fraction at 85% of a lane (HiSeq2500 Rapid Run Mode Flowcell).

ADT or HTO library structure:

Read 1:

Read 2:

Figures

Figure 1.

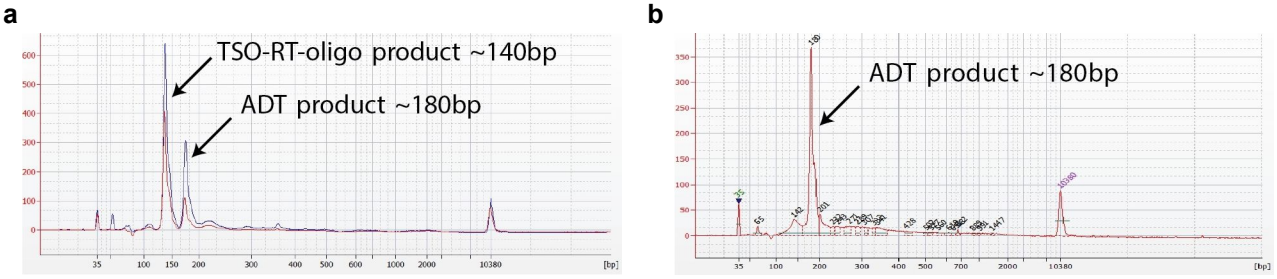

Figure 1. ADT (or Hashtag) library verification. (a) A TSO-RT-oligo product (~140 bp) can be amplified during the ADT PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. Sequential 2X SPRI purification of the ADT fraction after cDNA amplification 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.

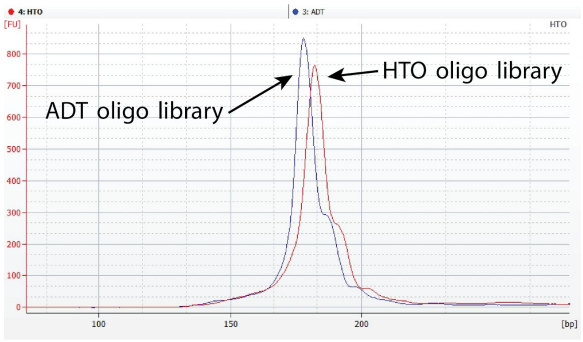

Figure 2. Verification of ADT and hashtag libraries. ADT and Hashtag libraries are very similar in size ~180bp but should appear as distinguishable products on a High Sensitivity Bioanalyzer, where the Hashtag library appears a few nucleotides larger compared to the ADT library.

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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'CCTTGGCACCCGAGAATTCCACATGATTGGCTCBAAAAAAAAAAAAAAAAAAAAAAAAAAA*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'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC*T*C`
- ADT cDNA PCR additive primer
  `5'CCTTGGCACCCGAGAATT*C*C`
- HTO cDNA PCR additive primer
  `5'GTGACTGGAGTTCAGACGTGTGC*T*C`
- Illumina Small RNA RPI1 primer (for ADT amplification; i7 index 1, Oligonucleotide sequences © 2015 Illumina, Inc)
  `5'CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A`
- Illumina TruSeq D701_s primer (for HTO amplification; i7 index 1, shorter than the original Illumina sequence)
  `5'CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGT*G*C`

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

Materials and Kits needed:

- CITE-seq and Cell Hashing Antibody-Oligo conjugates (*e.g.* BioLegend Totalseq)
- 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)
- E-gel 4% (Invitrogen, USA)
- 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 (*e.g.* Miltinyi)

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