CITE-seq

Cell staining

- 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.* Miltenyi kit) is recommended.
- Resuspend ~1-2 million cells in 100 µl Staining buffer (2%BSA/0.01%Tween in 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 antibody-oligo pool to cells.
- Incubate for 30 minutes at 4˚C.
- Wash cells 3 times with 1 mL Staining buffer (2%BSA/0.01%Tween in PBS), 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]).
- Filter cells through 40 µm strainers (*e.g.* Flowmi cell strainer).
- Verify cell concentration, single cellularity and high cell viability on hemocytometer after filtration.

Run Drop-seq (Macosko *et al.*, 2015) or 10x Genomics single cell 3' (v2 or v3) assay as described until before cDNA amplification.

At cDNA amplification step:

Add "additive" primers to cDNA PCR to increase yield of ADT products:
ADT PCR additive primer (0.2 µM stock): 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-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!**
    - 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.
    - 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 <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.

by Marlon Stoeckius mstoeckius@nygenome.org

- 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 45 µl water.
- Pipette mix vigorously and incubate at room temperature for 5 minutes.
- Place tube on magnet and transfer clear supernatant into PCR tube.

- <u>Amplify ADT sequencing library</u>:
  - Prepare 100uL PCR reaction with purified ADTs:
    - 45 µl purified ADT 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 <u>P5-SMART-PCR hybrid</u> oligo.
      - For 10x use <u>SI PCR</u> 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		

- 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 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 PCR tube.
- ADT libraries are now ready to be sequenced.
  - Quantify libraries by standard methods (QuBit, BioAnalyzer, qPCR).
  - ADT libraries will be around 180 bp (Figure 1).

Sequencing CITE-seq libraries:

- ADT 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.

ADT library structure:

5' Read 1 Seq primer | Cell Barcode | UMI | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | Antibody Barcode | Read 2 Seq primer 3'

3' Read 1 Seq primer | Cell Barcode | UMI | AAAAAAAAAAAAAAAAAAAAAAAAAAAAA | Antibody Barcode | Read 2 Seq primer 5'

Read 1:

Cell Barcode | UMI | TTTTTTTTTTTTT ...

Read 2:

Antibody Barcode | B AAAAAAAAAAAAAAAAAAA ...

Figures

Figure 1.

a

b

Figure 1. 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. To further enrich for ADT specific product the purified ADT library can be reamplified for ~2-3 additional cycles with ADT specific primer sets or P5/P7 generic primers. (**b**) A clean ADT library will contain a predominant single peak at around 180 bp.

Oligonucleotide 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). See examples with a 12nt barcode.

5'CCTTGGCACCCGAGAATTCCACATGATTGGCTCBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A

Oligos required for ADT library amplification:

- Drop-seq P5-SMART-PCR hybrid primer (for Drop-seq only)
  5'AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C

- 10x Genomics SI-PCR primer (for 10x Genomics Single Cell 3P (v2 and v3) and 5P kits)
  5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC

- ADT cDNA PCR additive primer
  5'CCTTGGCACCCGAGAATTCC

- Illumina Small RNA RPI1 primer (for ADT amplification; i7 index 1, Oligonucleotide sequences © 2015 Illumina, Inc)
  5'CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

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

by Marlon Stoeckius mstoeckius@nygenome.org

Materials and Kits needed:

- Antibody-oligo conjugates for CITE-seq (*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)
- PBS (Common lab suppliers)
- Tween20 (Common lab suppliers)
- BSA (DNAse, RNAse and protease free, *e.g.* VWR #0332-25G)
- Live Cell Enrichment Kit (optional, *e.g.* Miltenyi)