

## 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  $\mu$ l Staining buffer (2%BSA/0.01%Tween in PBS).
- Add 10  $\mu$ l 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  $\mu$ g (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 350g at 4°C.
- Resuspend cells in PBS at appropriate concentration for downstream application.
  - (e.g. for **10x** ~500 cells/ $\mu$ l; for **Drop-seq** [~200 cells/ $\mu$ l]).
- Filter cells through 40  $\mu$ m strainers (e.g. Flowmi cell strainer).
- **Verify cell concentration and high cell viability on hemocytometer after filtration.**

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 products:**

**ADT PCR additive primer (0.2  $\mu$ M stock): 1  $\mu$ l (for 10x Genomics) or 0.4  $\mu$ l (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  $\mu$ l, 60 $\mu$ l (0.6X SPRI was added for capturing full length cDNAs, therefore add another 140  $\mu$ l of SPRI to the supernatant. The final volume will be 300  $\mu$ l 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  $\mu$ 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.
- 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  $\mu$ 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 (do not over dry beads).
- 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 into PCR tube.
- Amplify ADT sequencing library:
  - Prepare 100 $\mu$ l PCR reaction with purified ADTs:
    - 45  $\mu$ l purified ADT fraction
    - 50  $\mu$ l 2x KAPA Hifi PCR Master Mix.
    - 2.5  $\mu$ l Truseq Small RNA RPLx primer (containing i7 index) 10  $\mu$ M.
    - 2.5  $\mu$ l P5 oligo at 10  $\mu$ M 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		
- 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 any remaining ethanol and allow the beads to air dry for 2 minutes.
  - Resuspend beads in 30  $\mu$ 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 libraries are now ready to be sequenced.
  - Quantify libraries by standard methods (QuBit, BioAnalyzer, qPCR).
  - ADT libraries will be around 180 bp (Figure 1).



**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)
- SPRlselect 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)