

immgenT SOP

This SOP describes the steps for preparing the samples for T cell profiling with multimodal single-cell genomics (CITE-seq, RNA and TCR) in the immgenT OpenSource program.

The success of the project depends on the reproducible generation of cell preparations. ADT (DNA-tagged antibodies) staining is reliable but highly dependent on staining conditions, and it is thus essential that all preparations strictly follow the steps and limits described below.

Per Immgen standard, 6-8 week-old B6 mice imported from Jackson Lab should be used, in duplicates. Male and female mice should be used when possible.

Up to 10 samples can be profiled in parallel. Each sample will be tagged using Total-Seq C hashtag antibodies. Each replicate should be tagged with a different hashtag. In every experiment, plan to sort 10,000 splenocytes as standard across experiments (it will be used for batch correction).

After dissection of organs of interest, the first step is to obtain single cell suspensions using your preferred optimized protocol to enrich in immunocytes (i.e. enzymatic digestion, Percoll, etc).

Each sample is then stained with hashtags and fluorescent antibodies (e.g. CD45 and CD3e). Keep in mind that some digesting enzymes can trim epitopes (such as CD3). Negative selection strategies are not preferred in order to prevent negative selection of rare T cell population with unusual surface antigen expression (i.e. Ly6c, a classic myeloid marker, is also present in a subset of T cells).

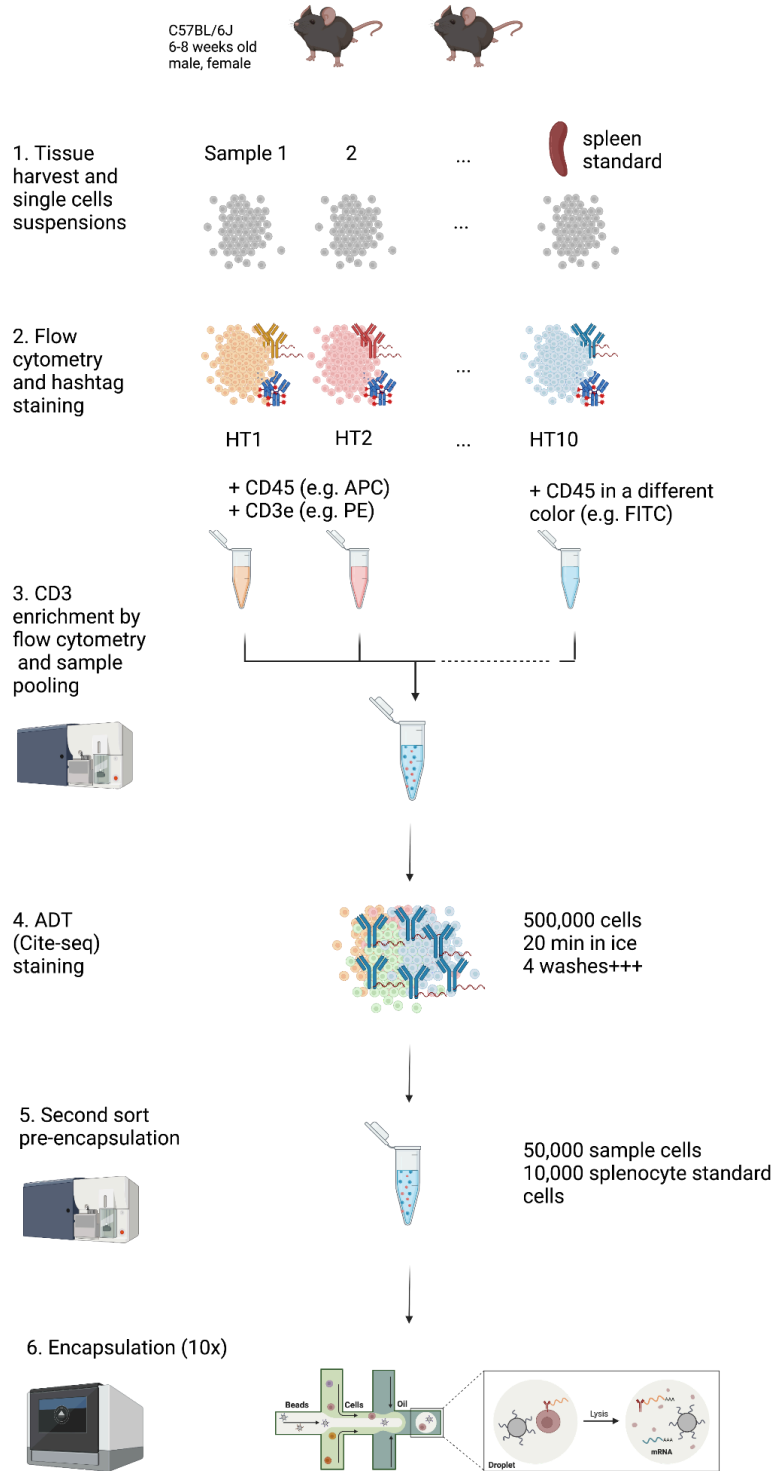
The spleen standard should be stained with a different CD45 antibody than the main samples (e.g. CD45-FITC for the standard and CD45-APC for the samples). With this strategy, we can sort a limited number of standard cells and make sure the sample cells are predominantly sequenced.

A first sort by flow cytometry is performed to enrich T cells (either total T cells with CD3e, or a more specific sort). Should aim for a total of 500,000 cells (across all samples in the same run) as this maximizes the expensive ADT reagents, ideally sorted and pooled into the same tube. 500,000 cells are necessary for optimal ADT staining. If fewer than 500,000 cells are sorted, filler cells (i.e. unstained splenocytes) should be used to compensate. Plan to sort ~50,000 CD45+ splenocytes as internal standard into the same collection tube.

After this first sort, ADT staining: staining the cells with DNA-tagged antibodies is the most sensitive step and has been optimized: i) antibody aggregates need to be removed during the ADT tube preparation ii) staining requires 500,000 cells in a volume of 50uL iii) background free antibodies can overwhelm bound ADT signal during sequencing so thorough washes (x4!) and sorting by flow cytometry are required.

The last sort controls how many cells will be encapsulated for single-cell sequencing. Typically, 50,000 sample cells are sorted (e.g. previously labeled with CD45-APC), 5,000-10,000 standard cells (e.g. previously labeled with CD45-FITC). Filler cells, unlabeled, aren't sorted.

As a rule of thumb, if one aims to obtain good sequencing profiles for $x=10,000$ cells, $2*x=20,000$ cells (~50% encapsulation efficiency) should be sorted before encapsulation (i.e. during the 2nd sort), $4-6*x=40,000-60,000$ cells should be sorted during the first sort (~30%-50% recovery efficiency).



1. Reagents, consumables, mice

1.1. Reagents

- Buffer (Magnesium, Chloride, and EDTA-free) for staining and collection tubes
 - E.g. Phenol-free DMEM + 2%FCS + 10mM HEPES
- ACK Lysing Buffer
- Fcblock
- Antibodies:
 - anti-CD3e
 - anti-CD45 (color 1 for sample)
 - anti-CD45 (color 2 for spleen standard)
 - TotalSeqC Anti-Mouse Hashtags 1-10 (Biolegend #155861-155879)
 - ADT (DNA-tagged antibodies) TotalseqC ImmGen T panel of 128 antibodies (Biolegend Part no. 900004815)

Single Cell supplies:

- 10x Genomics 5' v2 kit (PN 1000265); 4 rxns
- 10x Genomics Chip K Single Cell Kit (PN 1000287); 2 chips
- 50% Glycerol Solution

1.2. Consumables

- Eppendorf 1.5ml lo-bind tubes (Eppendorf # 022431021)

1.3. Mice

- For baseline studies, the Immgen standard mice (C57Bl/6J mice raised in SPF conditions at the Jackson Laboratory, Bar Harbor, shipped at 5 weeks of age, to be used at 6 weeks of age after 1 week of acclimation)
- Both male and female
- Euthanize all mice between 8:30 and 9:30 am to minimize circadian variation, by cervical dislocation (no CO₂)

2. Prepare solutions

ADT tube preparation (~45min)

Tip: start after the beginning of the first sort to make sure enough cells will be obtained for ADT staining

- Equilibrate for 5 min at RT
- Spin down vial at 10,000g for 30sec
- Add 27.5ul staining buffer (DMEM + 2% FCS + 10mM HEPES) to ADT tube and vortex (yielding ADT 2x mix)
- Incubate tubes for 30 min to ensure full resuspension
- Spin down at 14,000g for 10min at 4C. Transfer to a lobind tube and maintain at 4C (ice).

3. Tissue preps, flow cytometry-hashtag staining

Goal: obtain a single-cell suspension from tissues. Stain with fluorescent antibodies and hashtag for the first sort enriching for CD3+ T cells.

- Dissect organs and place in ice-cold medium as quickly as possible (no more than 15 min)
- When relevant, ensure that tissues are picked clean of attached lymph nodes

3.1. Primary tissues

- Variable protocols, but refer to ImmGen Cell Preparation and Sorting Protocol when applicable ([link](#))
- If digestion is required to release the T cells, limit digestion time at 37°C to 20 minutes or less, if possible, in glucose-containing medium.
If necessary, increase enzyme concentration to decrease digestion time.
- Final output should be a single-cell suspension with concentration $\geq 10\text{-}20\text{M}$ cells/mL (1-2M cells/100ul)
- Stain:
 - Stain 1-2M cells in 100uL buffer containing Hashtag antibody 1uL and the fluorescent antibodies required (at a minimum anti-CD3e)
 - Incubate in dark in ice for 20 min
 - Wash x2 and resuspend in 200uL for sorting.

Stain up to 5M cells with 1ul of HT

3.2. Spleen standard for the experiment

- Dissect the spleen(s) from a C57Bl/6 mouse (6 wo, JAX), and place it in ice-cold medium within 5 minutes of euthanasia.

- If applicable: digest the spleen using the same cocktail of enzymes used for the tissues. Adjust the time to allow digestion (but no more than 20 min).
- Homogenize passing through a 40um filter using 1mL syringe plunger into 50mL tube
- Spin at 500g for 5 min, at 4°C, then remove supernatant.
- Red blood cell lysis:
 - Resuspend cell pellet in 1 mL ice-cold ACK Lysing Buffer for 2 minutes in ice.
 - Top up tube with ice-cold staining medium, spin at 500g for 5 minutes at 4°C, then remove supernatant and resuspend in 1mL ice-cold staining medium
- Count cells with hemocytometer (trypan blue or other).
- Stain:
 - Stain 1 million splenocytes in 50uL buffer with anti-CD45 (conjugated to a fluorochrome different from that used for the main tissues) + Hashtag antibody 0.5 uL
 - Incubate in dark in ice for 20 min
 - Wash x2 and resuspend in 200uL for sorting

4. First sort (CD3+ enrichment)

Goal: ADT staining requires 500K cells. This step sorts T cells by flow cytometry to ensure enough T cells for ADT staining. According to the sample being prepared, sort may be for total T cells (anti-CD3) or specific subsets (e.g. anti-TCRb, anti-TCR or more refined populations)

Save pictures or download sorting plots, and store the FCS files from the sort

- Right before sorting: add viability dye, pass through a filter into a FACS tube → rinse filter with 200uL buffer
- Sort all different samples, each stained with a different hashtag, into a single collection 1.5mL LoBind tube containing 300ul staining medium
- Sort up to 450k live CD45+ CD3+ cells from target tissues
- Sort 50K-100k live, CD45+(FITC or other distinguishable from tissues) cells from spleen

5. ADT staining

Critical: Unbound ADT significantly (sometimes catastrophically) overwhelms the bound ADT signal during sequencing. Thorough washes (x4!) and sorting by flow cytometry are required.

New optimization starting Feb 2024 = IGT68+

Staining in 5% FCS instead of 2%

No LoBind tubes

Save supernatant to be safe

Wash x2 instead of x4

Use swinging buckets, not fixed rotors, to pellet the cells at the bottom.

Add filler cells after staining to visualize the pellet better and possibly improve pelleting.

- If less than 500k total cells are obtained after first sort, add unstained splenocytes (filler cells) to the collection tube to reach 500k cells
- Spin down at 500g for 5min at 4C and remove supernatant
- Resuspend in 20ul of staining medium + 5 ul Fcblock
- Add 25ul of ADT (2x) mix and gently pipette mix
- Incubate in dark in ice for 20min, gently resuspending every 5-10min
- Add 500K filler cells at the end of the wash (use as sink for unbound Ab and to identify the pellet)
- Wash 4x with 1mL staining medium, spinning at 500g for 5min at 4C
- Resuspend in 300ul of staining medium for sorting

6. Second sort (final sample)

Save pictures or download sorting plots, and store the FCS files from the sort

- Sort two populations into a single collection tube (1.5mL LoBind tube) containing 100ul staining medium
- Balance relative proportion of spleen standard vs. sample T cells using the different CD45 colors: for a single 10x lane:
 - Sort ~50k live T cells
 - Sort 5k live, CD45+ control splenocytes

7. Encapsulation using 10x Genomics 5'v2 NextGEM with Feature Barcoding

- Spin down at 500g for 5min at 4C
- Aspirate to 30uL and gently resuspend
- Using all 30uL, complete GEM Generation and Barcoding section of the 10x 5'v2 Protocol ([link](#)), 10x doc number CG000330• Rev A
- Freeze to -80 and ship on dry ice