Immunological Genome Project

ImmGen Cell preparation and Sorting

The success of the ImmGen project depends on the reproducible generation of cell populations in a consistent and reproducible manner, minimizing site-to-site differences. It is thus essential that all cell preparations intended for ImmGen profiling strictly follow the steps and limits described below. To achieve >99% sort purity needed for rigorous RNAseq profiling, preparations must involve two rounds of purification: either a magnetic selection (preferably negative) followed by a single flow cytometry sorting, or 2 back-to-back rounds of sorting. Also essential are a carefully selected dump channel, and dead-cell exclusion. Low-level contamination kills.
For chromatin analysis, where contamination is usually much less an issue but cell yields are limiting, a single sort by flow cytometry to yield >95% purity is acceptable in most cases

Reagents

Phenol-red free DMEM (Sigma-Aldrich# D1145)
ACK Lysing Buffer (Lonza BioWhittaker# 10-548E – order from Fischer Scientific)
Propidium Iodide (Sigma-Aldrich# P4170)
TCL buffer (Qiagen #1031576)
2-Mercaptoethanol, 14.3M (Sigma-Aldrich# M6250)

Staining Medium

Staining and cell prep medium: Phenol-red free DMEM, 0.1% azide, 10 mM HEPES, 2% FCS

Consumables

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

Time tables

1. Total cell preparation time, from euthanasia to final collection tubes, must be held to 3 hrs or better for “standard preparations” or 4-5 hrs for more intricate preparations.
2. Replicate sorts should be performed on separate days to smooth out variations across parallel samples.

Mice

3. Mice housed in SPF conditions[1]. In most instances, the mice used will be C57Bl/6J shipped from the Jackson Laboratory, Bar Harbor, and used 6 to 10 days from reception.
4. All mice used are males[2]. Positively verify sex of all animals, data from mixed origins are dangerously confounded.
5. Euthanize all mice between 8:30 and 9:30 am to minimize circadian variation, by cervical dislocation (no CO2), with sorter booked in consequence.

Harvesting tissues

1. Dissect organs and place in ice-cold medium within 5 minutes of euthanasia.
2. For preparation of cells from non-lymphoid tissues, perfuse tissues with cold PBS or collagenase-containing medium.

[1] Except when variant health status is being tested
[2] Except when gender influence is specifically tested

3. If harvesting cells from non-lymphoid tissues (and thymus), ensure that tissues are picked clean of attached lymph nodes.

Harvesting Peritoneal Cavity cells

1. Sacrifice mouse; pin feet to dissecting board, belly up
2. Cut and pull back skin to expose abdominal cavity (without cutting peritoneum):
3. Make a Y-shaped incision in skin with dissecting scissors
4. Peel back skin with forceps, exposing peritoneal membrane
5. Inject 10ml staining medium into peritoneal cavity using a 25g needle and a 10ml syringe
6. Remove syringe and pierce peritoneal membrane with a 9" glass disposable pasteur pipette
7. Remove as much liquid as possible and transfer to 15ml conical tube (avoid collecting any blood)
8. Centrifuge 1200rpm for 7 minutes
9. Resuspend pellet in 100 ul of staining medium

Harvesting Spleen and Lymphoid Organs

1. Dissect the spleen and place it in ice-cold medium within 5 minutes of euthanasia.
2. Homogenize by passing through a 100-µm filter using a 1 mL syringe plunger to press the tissue through the filter.

   for best yields of DC's, make sure to homogenize thoroughly, especially in the white connective tissues
3. Spin at 1500 rpm for 4 min, at 4C, then remove supernatant.
4. Lyse red blood cells in ice-cold ACK Lysing Buffer; use 1 ml per spleen for 3 minutes @ 4C. Top up tube with ice-cold staining medium, spin at 1500 rpm for 4 minutes @ 4C, then remove supernatant and resuspend in ice-cold staining medium, at staining volume.

Harvesting tissues that require enzymatic digestion

Limit digestion time at 37^{o}C to 20 minutes or less, in glucose-containing medium. If necessary, increase enzyme concentration to decrease digestion time.

Antibody staining

Except when preparing cells for the Standard ImmGen Sets (e.g. 14-cell set), pick the Ab/fluorochrome combination that works best in your sorter, and is compatible with PI as a cell viability die (more recent dies have not been vetted for RNAseq).

1. Resuspend cells in ice-cold staining medium at a concentration of 10^8 to 5.10^8 / ml
2. Stain cells with appropriate antibodies for 15 minutes:
   a. For 14 cell set, follow staining procedure as outlined in 14 cell set sorting SOP
   b. Add anti-FcR 2.4G2 to prevent non-specific FcR binding.
   c. Include a 'dump' channel containing a combination of antibodies to exclude cells that may contaminate the population of interest.
   d. Include anti-CD45 when dealing with tissues that contain predominantly non-hematopoietic cells.
3. If a second step staining is required (conjugated streptavidin, anti-Ig), top up tube with ice-cold staining medium, spin 5 minutes and resuspend for secondary staining.
4. Wash with medium, spin for 2 min (4C) at 2500 rcf, and then resuspend cells in medium. Be sure to filter cells before sorting.
5. Immediately prior to sorting, exclude dead cells by adding propidium iodide to a final concentration of 5 ng/ml (from working stock 1 µg/ml, stored 1 month in the dark at 4C, itself diluted down from 500 µg/ml stock)

FACS sorting

1. All cell populations sorted to achieve 99% purity.
2. Include a doublet exclusion gate to eliminate cell aggregates.

a. For ULI RNAseq (2 consecutive sorts required)

3. Collect at least 10^4 cells from first round sorts into tubes containing 200 µl staining medium (keep cold).
4. Add more propidium iodide before second sort.
5. Sort 1,000 cells into a LoBind tube containing 5 ul TCL buffer (Qiagen) supplemented to 1% v/v βMercaptoethanol.
6. After sort vortex, keep on ice for 5 minutes, give a quick spin.
7. Freeze on dry ice.

b. For ATACseq (one sort round sufficient)

7. Prepare Lo-bind tubes with 100 µL of BAMBANKER for cell collection. Keep on ice.
8. Collect 10,000 cells in the prepared collection tubes kept on ice.
9. Keep sorted cells on ice for at least 30 minutes and then immediately freeze in a freezing container with isopropyl alcohol, leaving it at -80˚C overnight
10. Keep the cells at -80˚C (ship to core lab if needed)