# FINAL IMMGEN SORTING SOP

*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, preparations will involve either a round of negative magnetic selection followed by a single flow sorting step, or 2 rounds of sorting.*

## Reagents

Phenol-red free DMEM (Sigma-Aldrich D1145)
ACK Lysing Buffer (Lonza BioWhittaker 10-548E – order from Fischer Scientific)
Propidium Iodide (Sigma-Aldrich P4170)
Trizol (Invitrogen 15596-026)

## Staining Medium

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

## Time tables

1. Total cell preparation time, from euthanasia to Trizol, must be held around 4 hours for "short preparations" or 6-7 hours 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, Maine, and used 6 to 10 days from reception.
4. All mice used are males[2]. Positively verify gender of all animals.
5. Tissues should be pooled from 3 (or more, but not less) mice for each batch[3].
6. Euthanize all mice between 8:30 and 9:30 am by cervical dislocation (no CO2), 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.
3. If harvesting cells from non-lymphoid tissues (and thymus), ensure that tissues are picked clean of attached lymph nodes.

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[1] Except when variant health status is being tested
[2] Except when gender influence is specifically tested
[3] Except when individual genetic variants or transgenics are tested

## Processing lymphoid or similar organs

1. Disrupt tissues by gentle teasing between frosted glass slides.
2. Lyse red blood cells in ice-cold ACK lysing buffer, 1ml per $10^8$ cells for <3 minutes @ 4C.
3. Top up tube with ice-cold staining medium, spin at <500g for 5 minutes @ 4C and resuspend in ice-cold staining medium.

## Processing tissues that require enzymatic digestion

Limit digestion time at 37C to 15 minutes, in glucose-containing medium. If necessary, increase enzyme concentration to decrease digestion time.

## Magnetic pre-purification (Dynal beads)

Negative depletion will be used to pre-purify cells when needed (i.e. no direct engagement of surface receptors on the selected cells). Use Dynal beads and keep total time, from incubation with beads to magnetic separation, to <20 minutes.

## Antibody staining

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. Add anti-FcR 2.4G2 to prevent non-specific FcR binding.
    b. Include a 'dump' channel containing a combination of antibodies to exclude cells that may contaminate the population of interest.
    c. 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. 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.
3. Collect first round sort into tubes containing 200 μl staining medium (keep cold). Add more propidium iodide and perform second sort immediately without centrifugation.
4. Collect $10^4$ to $5.10^4$ cells from the second round sort directly into 500 μl Trizol (co-sorted sheath fluid volume must be <100 μl).
5. When possible (i.e. not for very rare populations), check final sort purity by sorting another sample under the same conditions into 100 μl staining medium. Analyze and record.