Arming Pulmonary Macrophages for Pathogen Defense
Program Project

SOP for Sample collection for RNAseq processing
Version 1.0

I. Objective of the SOP

The objective of this document is to provide detailed guidelines for collection of small numbers of highly purified cells for RNAseq, including experimental design considerations through cryopreservation of flow cytometrically sorted cells. Our typical workflow will use 100 highly purified cells as starting input for RNA amplification.

II. Version History

v1.0 Updated to include references to other sample preparation protocols. Current stable as of April 2015.

v0.1 Preliminary version with sparse information, for the purpose of preliminary sample collection. Prepared Jan-Feb 2015 by Charlie Kim.

III. Methods

A. Experimental Design

i. *Sample size*. The data we have been getting from RNAseq of 100 cells has been more variable than previous experiments with 1,000 cells analyzed on microarrays. We recommend preparing a bare minimum of 5 samples per group from independent mice, and preferably 6-8 samples per group.

ii. *Experimental design*. The most effective designs are comparative in nature, so consider what population will serve as a good baseline. Be sure to account for confounding factors in your design. This might, for example, include taking untreated samples in a kinetic course, as the baseline might change with time.

B. Preparation of single cell suspension

i. *Euthanasia and dissection*. Work as quickly as possible to avoid perturbation of the transcriptional profiles, which are particularly susceptible to cell death. We typically aim to have samples processed for flow cytometry within 2 hours of euthanasia.

ii. *Digestion*. Collagenase

C. Surface staining for flow cytometry

i. *Target population definitions*. See SOP documents for staining panels and gating strategies.

ii. *Staining protocol*. See SOP for cell preparation.

D. Sorting samples to high purity

i. *FACSAria setup*. To obtain the best data, setup and gating must be highly consistent between experiments. There are few specific requirements for this since there is a fair amount of variation between instruments; just be sure to be as consistent as possible on any given instrument.

Droplet targeting should be performed with high precision to ensure cells are directly sorted into the lysis buffer. After getting the sort stream roughly into position, typically when a capped tube, use a dry tube of the same type into which you'll be sorting to do a very quick test sort. Ensure that the droplets are landing at the very bottom of the tube; if some are hitting the sides, adjust your stream position until it is perfect.

ii. *Sort parameters*. Perform a Purity sort. It is preferable not to exceed 5,000 events per second, which achieves the lowest conflict rate (good for yield) and highest purity. However, this must be balanced with time – for very rare populations (<0.1%) in abundant samples, it may be desirable to sacrifice purity and yield by performing a very fast first sort, and subsequently performing two more rounds of sorting to obtain high purity.

iii. *Sort workflow.*

1. First sort: sort primary sample into 300µl of ice-cold PBS, ideally into a low retention tube. Typical yields on the second sort will be 10-50%, depending on your cell type, so aim for a minimum of 3,000 cells on the first sort if possible. Higher cell numbers are also useful because your cells will be more concentrated during the second sort, making the collection go faster and minimizing the risk of perturbing transcriptional profiles.
2. Pipette the volume of the first sort gently down the walls of the first sort capture tube to recover any cells on the walls, and transfer the entire volume to a fresh, pre-chilled FACS tube.
3. Perform a second sort directly into 100µl of ice-cold RNA lysis buffer (RNAqueous Micro kit, AM1931 Life Technologies). **DO NOT EXCEED 100 CELLS PER TUBE**. Such samples create problems downstream and will be discarded.
4. Immediately and aggressively flick mix the tube approximately 10-20 times, which will wash down the walls with lysis buffer and lyse the cells to inactivate RNases.
5. Snap freeze on dry ice. These samples can be stored at -80C for a few days, or longer term in liquid nitrogen.
6. Ideally, you should collect multiple aliquots of 100 cells, which will provide a buffer against losses due to problems during shipping, user errors during amplification, etc. without having to re-run the entire experiment.
7. Ship the samples on dry ice in batches to be amplified together.

E. Checking purity

i. This should be performed at least once or twice for every cell population being profiled. Some cell types are stickier than others, resulting in different amounts of contamination following a sort.

ii. Use the procedures above, but on the second sort, sort into <u>300µl of ice-cold PBS</u>.

iii. Re-load this sample on the FACSAria and run it a third time to assess the purity of your cells following the second sort. If desired, a third sort can be performed and checked.