**Antibody Titration Protocol**

This protocol is optimized for simultaneous titration of up to 10 surface Abs and 20 intracellular Abs. It is also designed to recapitulate the conditions present during the typical staining protocol, i.e. barcoding and subsequent staining in aggregate.

1. Prepare blood samples for lysis. If only titrating phenotypic markers, only Unstim samples will be required (unless phenotypic fluctuation is dependent on stimulation). If titrating functional markers, both Unstim and Stim will be required. The best stim for this purpose is a combined stimulation with IFNα + LPS, which will induce activation of all functional markers we typically measure. See step 2 below to determine how many samples will need to be prepared.
2. You will need to determine how many cells to prepare for the titration. This is calculated based on how the Abs will be used in a panel. For example, we typically run one half of a 1mL blood sample for clinical studies, so the blood samples to be used for titration should be arranged accordingly. In the example below, the assumption is that one half of a 1mL blood sample (a half-sample) will be used for each position on a barcode plate. Because we use 5 reactions’ worth of Ab cocktail to stain a barcode plate of 20 half-samples, we are staining the equivalent of 4 half-samples per reaction. Consequently, the same number of half-samples will be needed for the titration. Since there are 5 reactions (one for each concentration) per sample type (Unstim vs. Stim) in the titration below, this will require 5 reactions x 4 half-samples per reaction = 20 half-samples per sample type, i.e. 10 full Unstim and 10 full Stim samples. If only titrating surface Abs, only 10 full Unstim samples will be required.
3. Lyse the appropriate number of samples following the standard RBC lysis protocol. After lysis is complete, combine all of the leukocytes from the same sample type (Unstim or Stim) together and measure the volume of the combined suspension. Divide this number by 5, and transfer that amount into each of 5 cluster tubes, each labeled with sample type and one of the titration concentrations (0.5, 1, 2, 4, 8μg/mL). Do this for each sample type (Unstim and Stim).
4. Wash the samples with 1mL (total volume) CSM, PBS, then 0.02% saponin/PBS

**IMPORTANT NOTE:** Use a Pasteur pipette with a non-filtered 200μL pipette tip (green stacking box) to aspirate each cluster tube individually. **DO NOT** use the multi-channel aspirator, as it is not optimized for cluster tube racks.

1. Barcode the samples in the cluster tubes according to the standard protocol.
2. For every titration, a counterstain must be employed. Determining the Abs to use in the counterstain will depend on which cell subsets will need to be identified in order to determine the optimal concentration of each Ab being titrated. For instance, CD4 and CD8 will need to be used in order to identify a CD4- CD8- T-cell population for titrating an antibody targeting TCRγδ. Similarly, CD14 and CD16 will need to be used in order to identify classical monocytes for titrating intracellular Abs targeting TLR4 pathway effectors.
3. During the barcoding incubation, prepare the counterstain mix (Surface Master Mix) that will be used in conjunction with the surface Abs to be titrated. This mix will be used as the diluent for each serial dilution of the surface Abs being titrated. Shown below is an example:

Prepare Surface Master Mix (SMM):

5.5μL CD235ab-113

2.8μL CD61-113

5.5μL CD66-139

5.5μL CD7-141

5.5μL CD19-142

11μL CD4-145

5.5μL CD8a-146

5.5μL CD3-170

46.8μL + 683.2μL CSM 🡪 730µL SMM

1. The final volume of SMM is calculated based on how much is required to prepare the serial dilutions. This will depend on how many surface Abs are to be titrated. Below is an example of the serial dilutions to be prepared for titrating 10 surface Abs. The 730µL SMM in the example is calculated from what is required to make the serial dilutions below (171+165+154+132+88 = 710), with 20µL extra to account for pipetting error.

Prepare Surface Serial Dilutions:

17μL ea. CD45-115 / CD45RA-143 / CD11c-147 / CD123-148 / TCRγδ-152 / CD16-165 / CD25-169 / CCR2-173 / HLADR-174 / CD14-175 + 171μL SMM 🡪 341μL **8μg/mL**

165μL 8μg/mL + 165μL SMM 🡪 330μL **4μg/mL**

154μL 4μg/mL + 154μL SMM 🡪 308μL **2μg/mL**

132μL 2μg/mL + 132μL SMM 🡪 264μL **1μg/mL**

88μL 1μg/mL + 88μL SMM 🡪 176μL **0.5μg/mL**

NOTE: You will notice that in the above example, for the 8μg/mL reaction, the amount of SMM will be about half of what will be in each of the subsequent serial dilutions. This is not an issue for most of our Abs, which will stain well regardless. Consequently, the fewer surface Abs that are titrated, the less of an issue this will be, and is the reason why this protocol is optimized for titrating a maximum of 10 surface Abs, beyond which the counterstain for the 8μg/mL sample will be compromised.

1. In the above example, the 17μL of each Ab to be titrated is based on calculating how much of a 0.2mg/mL solution of each Ab is required for 5 – 100μL reactions (+10% overage for pipetting error), one at each concentration (0.5, 1, 2, 4, and 8μg/mL). If titrating both surface and intracellular Abs, you will require 2 sets of reactions (Unstim vs. Stim), so there will be 10 total reactions, and the calculation will be: (4.4 + 2.2 + 1.1 + 0.55 + 0.275μL) x 2 = 17.05μL each Ab. If titrating *only* intracellular Abs, surface serial dilutions are not necessary, and only the SMM will be required. The volume of SMM would therefore be calculated based on how much is needed for 10 – 100μL reactions (+ 10% extra for pipetting error).
2. After barcoding, wash the samples according to the standard protocol. Aspirate each sample until only 20μL remains in each cluster tube. Combine the samples **from each concentration** into each of 5 separate labeled FACS tubes (0.5, 1, 2, 4, 8μg/mL). Each FACS tube will contain ~40μL cells in CSM. See diagram below.

+

+

+

+

+

Unstim

Stim

0.5

1

2

4

8µg/mL

After barcoding, pool cells in FACS tubes according to concentration

0.5

1

2

4

8µg/mL

1. Add 2μL FcX to each FACS tube, and incubate with shaking at 600rpm at RT for 10 min.
2. Add 160μL of each serial surface Ab dilution to the appropriate FACS tube and incubate with shaking at 600rpm at RT for 30 min.
3. During the incubation, prepare the intracellular serial dilutions. These are calculated the same way as with the surface Abs above, except CSM is used as the diluent instead of SMM, unless you wish to counterstain with functional markers as well. The example below assumes no intracellular counterstain:

Prepare Intracellular Serial Dilutions:

17μL ea. p38-151 / pSTAT3-154 / pS6-155 / Tbet-160 / FoxP3-162 / IκB-164 / pNFκB-166 / pSTAT6-168 + 205μL CSM 🡪 341μL **8μg/mL**

165μL 8μg/mL + 165μL CSM 🡪 330μL **4μg/mL**

154μL 4μg/mL + 154μL CSM 🡪 308μL **2μg/mL**

132μL 2μg/mL + 132μL CSM 🡪 264μL **1μg/mL**

88μL 1μg/mL + 88μL CSM 🡪 176μL **0.5μg/mL**

1. After the incubation is complete, wash cells 2X with CSM.
2. Permeabilize cells in each FACS tube with 700μL 100% methanol at 4°C for 10 min.
3. Wash cells 2X with PBS, then 1X with CSM.

NOTE: when adding the CSM for the 3rd wash, make sure to watch out for cells adhering to the side of the FACS tube. If they do, dislodge them by pipetting up and down and scraping the pipette tip against the side where they have adhered. This generally occurs with low cell numbers.

1. Aspirate the supernatant from each FACS tube until ~40μL remains and vortex briefly to re-suspend the cells.
2. Add 160μL of each intracellular serial dilution to the appropriate FACS tube, and incubate with shaking at 600rpm at RT for 30 min.
3. Wash each FACS tube 2X with CSM.
4. Re-suspend and pool **all** samples in 2mL of intercalator cocktail into one of the FACS tubes.
5. Incubate O/N at 4°C and run the next day on CyTOF using an appropriate panel. Make sure to create a barcode deconvolution spreadsheet according to how you barcoded each reaction in step 5.