Quick-Spin the tetramer tubes before opening, due to the change in altitude and the low volume.

Staining ex vivo samples with IA^{g7} Ins10-23 RE#3 Tetramers, from Kappler/Marrack Laboratory

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Before using the tetramers in a staining experiment, practice on control lymph nodes or spleen, with the counterstaining antibodies, data collection on the flow cytometer and gating strategies, until you are satisfied with the antibody dilutions, flow rate, amount of data acquired, etc.

Reagents Provided

1. Diluent[a] for tetramers, containing HAM57-597 (anti-mouse TCR Cβ) at 1ug/mL, 1mL
2. Diluent[a] for counter-staining antibodies, 1mL
3. 1879 IA^{g7} HEL tetramer, sufficient for 20 stains
4. 1946 IA^{g7} Ins10-23 RE #3 p8E tetramer (Epitope B), sufficient for 10 stains
5. 5168 IA^{g7} Ins10-23 RE #3 p8G tetramer (Epitope A), sufficient for 10 stains

Other Staining Reagents Required (The colors we use work well in the Cyan Flow Cytometer)

1. Anti-B220 (B cells) (We use RA3-6B2, AF488 or FITC)
2. F4/80 (macrophages) (We use AF488) These two ab's are the "dump" stain abs, detected in the same channel.
3. Anti-CD4 (We use GK1.5, PECy7)
4. Anti-CD8 (We use 53-6.7 APCefluor780)
5. Anti-CD44 (We use IM7, PerCPCy5.5)

It is imperative to use antibodies to all these markers, for appropriate dumping and gating strategies, to minimize background tetramer staining.

Cell requirements for ex vivo tissue: Staining is done in 96-well round bottom plates

1. Spleen, treated with ammonium chloride, $1.2x10^7$ cells per stain
2. Pancreatic or Peripheral Lymph Node, $1.2x10^7$ cells per stain (if possible)
3. Pancreatic Islet Cells: Following pancreatic islet isolation, the islets are incubated in CTM overnight at 37°C in 10% CO_2, to allow the T cells to migrate out of the tissue. Next day, the cells are passed through a 100um cell strainer and counted with a hemacytometer to minimize cell loss.
   (e.g. from four 8-wk NOD females, one can derive sufficient cells to stain two samples at about $1\text{-}3x10^5$ cells per stain)
4. Whole pancreas, digested with collagenase: Chop the pancreas into 1-2mm bits with scissors. For each two pancreases, place the bits in pre-warmed (37°C) 50mL digestion buffer (5% FBS in Balanced Salt Solution (BSS), containing 0.5mL 0.5M $CaCl_2$ and 0.5mL 10mg/mL collagenase). Incubate at 37°C for 15 minutes, mixing the tube every 5 minutes. Centrifuge the digested bits and wash once with 25mL 10% FBS in BSS. Re-suspend the cells in 10% FBS in BSS and pass through a 100um cell strainer. Spin and wash the cells multiple times in 10% FBS in BSS until the supernatant is clear. $2\text{-}3x10^7$ cells per stain (if possible)

Tetramer Dilution and Staining Protocol

NOTE: Quick-spin the tubes before opening them.

NOTE: Dilute only enough tetramer for that day's experiment. Do not store diluted tetramer.

1. Dilute the tetramers in the diluent that contains HAM57-597 (anti-mouse TCR Cβ) to 0.02mg/mL. Each stain uses 25uL of diluted tetramer.
2. For single stain controls, use the peripheral lymph node or spleen, since there is always an abundance of cells from these two tissues. Use $1/10^{th}$ the amount of cells for the single stain control wells as for a sample well. You will also need a single stain control well for the HEL tetramer alone .
3. Add the cells to the round bottom 96-well plate and spin. (We leave alternate wells in a row empty, and alternate rows empty, to avoid any sample mixing. 24 samples will fit in one plate.)
4. Toss off the supernatant, and re-suspend each well in 25uL of the appropriate tetramer. Mix the cells with the pipet tip. Use a fresh tip for each well.
5. Wrap the plate in plastic wrap, and place in a humidified, 10% CO_2, 37°C incubator for two hours. Every thirty minutes, gently agitate the plate to re-mix the well contents.
6. Prepare the counter-staining antibody dilutions in diluent without HAM57-597 during the two hour incubation. Make the antibodies 3.5X concentrated, because you will be adding 10uL to each well. For every antibody, prepare one single dilution, for the single stain compensation controls, and enough of the mixture of all antibodies to add to all the tetramer wells. (See Appendix A for a sample Excel spreadsheet with antibody and tetramer dilution scheme)
7. Add 10uL of the counterstaining antibodies to each well, with a fresh pipet tip each time, and mix the cells.
8. Incubate at room temperature in the dark for 20 minutes.
9. Wash the cells twice with 2% FBS in BSS containing 5mM sodium azide (BSSWB) .
10. If you are examining the cells on the flow cytometer the same day, re-suspend single stain samples in 300uL BSSWB and all other samples in 400uL BSSWB.
11. If you will examine the cells the next day, you may fix the cells with 1% paraformaldehyde (100uL per well) for 20 minutes at room temperature, then spin and wash once. After cells are fixed, a higher g-force and spin-time are required for complete recovery. Store at 4°C overnight in the dark.
12. Run the single stain controls for each color first, adjusting the voltages and compensations as necessary to get the positive and negative cells in the appropriate quadrants. Save the data from each single color sample after you are satisfied with the voltages and compensations. On the Cyan, you have to get the voltages correct at the time of acquisition, as they cannot be altered later. The compensated single-stain control samples can be used later, if there is a need to adjust compensation.

13. Run the samples using the settings established in step 12. On the Cyan, acquisition of 10-20,000 events per second is optimum for high cell density samples (spleen, lymph node or pancreas) but much lower speed is better for the pancreatic islet samples. For all the samples except pancreatic islets, acquire $3x10^6$ events if possible (about 3-4 minutes per sample on the Cyan). Obviously, if you cannot attain these many events, acquire until the tube is empty. In the islets, the percentage of tetramer positive cells allows collection of many fewer events.

Dumping and Gating Strategies in Flow Jo (See attached Appendix B) Use polygons, not quadrants or rectangles, to draw gates for steps 1 - 5.

1. Select the "live population" (low Side Scatter) on a FS/SS dot plot.
2. Select the Dump-negative, CD4+ population on a dot plot.
3. Select the Dump-negative, CD4+, CD8- gate on a dot plot.
4. Set up a dot plot of Live, Dump-, CD4+, CD8- cells, for CD44 vs Tetramer. Tetramer positive cells are almost always CD44 High.
5. Draw a polygon for the CD44Hi, Tetramer+ population, or:
6. Draw a rectangle around all the CD44 High cells, and send to a histogram.
7. Overlay the Insulin Tetramer over the HEL Tetramer histogram to see the tetramer positive population in an alternate view.

[a] Diluent Composition

1. 3 parts Complete Tumor Medium, CTM (ref)
2. 2 parts 24G2 (antiFcR), Normal Mouse Serum and Sodium Azide: 750uL 50% normal Mouse serum in BSS, 2.25mL 24G2 hybridoma supernatant, 75uL 0.5M sodium azide. (this formulation can be made in a larger quantity, sterile-filtered and aliquoted for storage at -20°C, for convenience).

Appendix A: Antibody and Tetramer Dilution Sample Spreadsheet, Kappler, et al.

Sample Spreadsheet for antibody and tetramer dilutions for NOD Pancreas and LN staining
Diluent for Tetramer is 1ug/mL HAM597 in 2:3 24G2/NMS/NaN3:CTM
Diluent for Antibodies is 2:3 24G2/NMS/NaN3:CTM

Antibody	Specificity	Fluor	Dilution	3.5XDil	Single	Mix
RA3-6B2	B220	FITC	400	114	0.5uL+	1.5uL+
F4/80	MacroPhg	AF488	200	57	1uL+55.5uL	3uL+
IM7	CD44	PerCPCy5.5	400	114	0.5uL+56.5uL	1.5uL+
GK1.5	CD4TCell	PECy7	800	228	0.5uL+113.5uL	0.75uL+
53-6.7	CD8TCell	APCefluor780	400	114	0.5uL+56.5uL	1.5uL+
						162.75uL

Single Stains for Tetramers	mg/mL	Dilution Scheme	ug/mL
PE-5168 RE3 p8E>G 040611	1.14	0.7uL+39uL	20

=171uL

Tetramer Mixes for Samples	mg/mL	Dilution	ug/mL
PE-1879 IAg7 HEL 020411	1.1	2.2uL + 20uL	20
PE-5168 RE3 p8E>G 040611	1.14	2.1uL + 120uL	20

Tetramers add 25uL per Uwell
Controls add 25uL HAM597 diluent per well
Abs add 10uL to the 25uL in the Uwell

Tetramers 37deg 10% CO2 shake @30'
Add 10uL antibodies, 20' RT
Wash 2X BSSWB
Add 100uL 1%PFA, 20' RT
Spin at 1500rpm (524g) 5' and wash 1X BSSWB
Final volumes 300uL SS, 400uL Tests

Appendix B: Gating Strategy for Pancreas and Pancreatic LN Stained with

IAg7-Ins10-23 Tetramer Mixture, Kappler, et al.