RPCI Tetramer Staining Procedure

Materials Required

Sodium Heparinzed Green Top tube (BD, 366480)

PBS buffer:
Phosphate buffered saline

iTAg™ MHC Tetramer Lyse Reagent:
Beckman Coulter, PN T08002

iTAg™ MHC Tetramer Fixative Reagent:
Beckman Coulter, PN T08003

Flow-Count Beads
FLOW-COUNT™ Fluorospheres (Beckman Coulter, PN 7547053)

Methanol Free Formalin
10% EM grade buffered Formalin (Polysciences 08379)
Dilute to 0.1% in PBS, store at room temperature indefinitely.
Alternatively, dilute a 2% solution 1:20 in PBS for a 0.1% final.

CMV Tetramers all PE conjugated
MHC Tetramer-(Phycoerythrin)
Excites at 486-580 nm
Emits at 586-590 nm

MHC (peptide sequence)	**Catalog #**
HLA-A*0101 (VTEHDTLLY)	PN T01045
HLA-A*0201 (NLVPMVATV)	PN T01009
HLA-B*0702 (TPRVTGGGAM)	PN T01046
HLA-B*0801 (ELRRKMMYM)	PN T01047
HLA-B*3501 (IPSINVHHY)	PN T01048
HLA-A*0201 (negative peptide)	PN T01044

MAbs
CD3 PC5: (Beckman Coulter, PN T20342)
CD4 PE : (Beckman Coulter, PN T20343)
CD8 FITC: (Beckman Coulter, PN T20222)

Specimen Collection:

For optimal results, whole blood should be collected into a tube containing an appropriate anti-coagulant (Na Heparin, EDTA, and Na Citrate are all exceptable). Samples should be processed within 24 hours of collection, and samples should remain at room temperature.

Compensation Controls

C1. Non stained
C2. CD8 FITC
C3. CD3 PE
C4. CD8 PECy5

MAb/Tetramer combinations per patient using inclusion gate

1. CD8 FITC / Neg PE Tetramer / CD3 PE-Cy5
2. CD8 FITC / CMV PE Tetramer / CD3 PE-Cy5

Instrumentation

BD FACSCanto A™ II

Procedure

1. Appropriately label tubes for each patient sample being tested.
2. Add 10 µL of specific iTAg MHC Class I Tetramer or Negative Tetramer and 10 µL each of anti-CD3 and anti-CD8 monoclonal antibody reagents into each tube.
3. Add 200 µL of whole blood into each tube. Specimens with low leukocyte counts (<3.0 x 103/µL) or low lymphocyte counts (<0.5 x 103/µL) may require whole blood volumes up to 400 µL. Under these circumstances, up to 4 mL of Lyse/Fixative solution is required (all other reagent volumes remain as described).
4. Vortex gently.
5. Incubate at room temperature (18–25°C) for 20–30 minutes, protected from light.
6. Prepare the Lyse/Fixative solution by combining 1 mL of lyse to 25 µL of Fixative. Each tube requires a minimum of 2 mL.
7. Add 2 mL of Lyse/Fixative solution to each tube and vortex immediately for one second after each addition.
8. Incubate at room temperature for at least 10 minutes, protected from light.
9. Centrifuge tubes at 150 x *g* for 5 minutes.
10. Aspirate or decant the supernatant.
11. Add 3 mL of PBS.
12. Centrifuge tubes at 150 x *g* for 5 minutes.
13. Aspirate or decant the supernatant.
14. Resuspend the cell pellet in 500 µL of PBS with 0.1% formaldehyde.
14. Vortex each tube for 5 seconds.
15. Store prepared samples at 4°C protected from light for a minimum of 1 hour (maximum 24 hours) until analysis by flow cytometry.

CD8 count Method

1. Add 100 µL of anti-coagulated (EDTA, Na Heparin, or ACD) whole blood to the bottom of a 12 x 75 mm polystyrene tube.

2. Add anti-CD8 FITC, anti-CD4 PE, and, anti-CD3 PC5 at predetermined titers to each tube.

3. Incubate for 30 min at 4^{o}C in the dark.

4. Prepare the Lyse/Fixative solution by combining 1 mL of lyse to 25 µL of Fixative. Each tube requires a minimum of 2 mL.

5. Add 1 mL of Lyse/Fixative solution to each tube and vortex immediately for one second after each addition.

6. Add 100 µL Flow-Count beads to each tube.

6. Store samples at 2 - 8^{o}C in the dark until analysis. (Samples can be run up to 24 hours after lysis).

Data Acquisition and Analysis

1. Analyze on a BD FACSCanto™ A flow cytometer.

2. Acquire data with BD FACSDiva™ v6 software.

3. Collect 20,000-30,000 CD3+CD8+ events.

4. Acquire all data using medium flow rate.

Instrument Set-up

PE Tetramers Setup on Canto A

09/12/2008

CD8 Cell Count:

On Tetramers:

Parameter	Type	Log	Voltage
FSC	H	off	100
SSC	A	off	412
FITC	A	On	476
PE	A	On	509
PE-Cy5	A	On	500
PE-Cy7	A	On	590
APC	A	On	541
APC-Cy7	A	On	500

Threshhold:
Parameter: PE-Cy5 Value : 850

Acquisition Setup:
Stopping Gate: P3(Beads) Events To Record: 10,000
Storage Gate: All Events. Events To Display: 1000
Flow Rate: Medium

Tetramers Samples:

Parameter	Type	Log	Voltage
FSC	H	off	60
SSC	A	off	412
FITC	A	On	476
PE	A	On	509
PE-Cy5	A	On	500
PE-Cy7	A	On	590
APC	A	On	541
APC-Cy7	A	On	500

Threshhold:

Parameter: FSC, Value : 47,000

Acquisition Setup:

Stopping Gate: P5(CD3+ & CD8+) Events To Record: 20,000-30,000
Storage Gate: All Events. Events To Display: 1000
Flow Rate: Medium