RPCI Dextramer Staining Procedure

A. Materials

Phosphate Buffered Saline

UTI-Lyse (Dako, Cat #: S3350)

Accucount Beads: Spherotech AccuCount Ultra(Cat # ACURFP-50-10)

Methanol Free Formalin
10% EM grade buffered Formalin (Polysciences 08379)
Dilute to 2% in PBS, store at room temperature indefinitely.

CMV Dextramers all PE conjugated

MHC (peptide sequence)	Epitope Origin	Catalog #
HLA-A*0101 (VTEHDTLLY)	HCMV pp50	WA101
HLA-A*0201 (NLVPMVATV)	HCMV pp65	WB106
HLA-A*0301 (KLGGALQAK)	HCMV pp65	
HLA-A*2402 (QYDPVAALF)	HCMV pp65	WF101
HLA-A*2402 (VYALPLKML)	HCMV pp65	WF102
HLA-B*0702 (RPHERNGFTVL)	HCMV pp65	WH103
HLA-B*0702 (TPRVTGGGAM)	HCMV pp65	WH104
HLA-B*0801 (ELRRKMMYM)	UL123	WI101
HLA-B*3501 (IPSINVHHY)	HCMV pp65	WK101
HLA-A*0201 (negative peptide)		WB100
HLA-A*0801 (negative peptide)		WB2666-RPE

MAbs
CD3 PcP: (BD, clone SK7, Cat # 340663)
CD4 PE (Dako, clone MT310, Cat # R0805)
CD8 FITC (Dako, clone DK25, Cat # F0765)

Optional: LIVE/DEAD® Fixable Violet Dead Cell Stain Kit for flow cytometry (Invitrogen Molecular Probes cat # L34955).

B. Compensation

C1. Non stained
C2. CD45 FITC
C3. CD4 PE
C4. CD8 PcP

C. MAb/Dextramer combinations per patient (using inclusion gating)

1. LD / CD8 FITC / Neg PE Dextramer / CD3 PcP
2. LD / CD8 FITC / CMV PE Dextramer / CD3 PcP

D. Dextramer Staining Method

1. Perform whole blood cell count using AcT 10 hematology analyzer.

2. Place 200 µL of anti-coagulated (EDTA, Na heparin, or ACD) whole blood in a 12x75 flow tube.

3. Add appropriate control or test Dextramer (10 µL: titrated first to determine the optimal concentration) to designated tube.

4. Incubate at RT for 10 minutes in the dark.

5. Inclusion gating, add anti-CD8 FITC, anti-CD3 PCP, and Live Dead (LD) reagent at predetermined titers to each tube. Refer to Live Dead procedure for details on this method.

6. Incubate for 30 min on ice in the dark.

7. Add 200 µL of UTI-Lyse reagent A to each tube, incubate 10 minutes at room temperature in the dark.

8. Add 2 mL of UTI-Lyse reagent B to each tube, incubate 10 minutes at room temperature in the dark.

9. Centrifuge 1400 rpm (400 x *g*) for 5 minutes, pour off supernatant and resuspend in PBS

10 Centrifuge 1400 rpm (400 x *g*) for 5 minutes, pour off supernatant and resuspend in 300-400 μl of 2% Formaldehyde.

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

12. Count 25,000 CD3 positive and CD8 events positive or until it times out at 5 minutes.

E 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 PCP, and Live Dead (LD) reagent at predetermined titers to each tube. Refer to Live Dead procedure for details on this method.

3. Incubate for 30 min on ice in the dark.

4. Add 100 µL of UTI-Lyse reagent A to each tube, incubate 10 minutes at room temperature in the dark.

5. Add 1 mL of UTI-Lyse reagent B to each tube, incubate 10 minutes at room temperature in the dark.

6. Add 100 µL of Spherotech AccuCount beads to each tube

7. Store samples at 2 - 8°C in the dark until analysis. (Samples can be run up to 24 hours after lysis).

8. Count 10,000 Bead events with no stopping time (i.e. set to 0 seconds) using a threshold on CD3.

PE Dextramers Setup on Deuce Canto

Dextramer parameters setup on Deuce

Dextramers & Tetramers Samples:

On Dextramers:

Parameter	Type	Log	Voltage
FSC	A	Off	208
SSC	A	Off	430
FITC	A	On	411
PE	A	On	464
PL3	A	On	513
PE-Cy7	A	On	456
APC	A	On	470
AAX750	A	On	495
PB	A	On	382
Qdot605	A	On	465

Threshhold:
Parameter: FSC Value : 47,000
Acquisition Setup:
Stopping Gate: P5(CD3+ & CD8+) Events To Record: 25,000
Storage Gate: All Events. Events To Display: 1000
Flow Rate: Medium

CD8 Cell Count:

On Dextramers:

Parameter	Type	Log	Voltage
FSC	A	off	208
SSC	A	off	386
FITC	A	On	411
PE	A	On	464
PL3	A	On	513
PE-Cy7	A	On	456
APC	A	On	470
AAX750	A	On	495
PB	A	On	382
Qdot605	A	On	465

Threshhold:
Parameter: FL3, Value : 750
Acquisition Setup:
Stopping Gate: P3(Beads) Events To Record: 10,000
Storage Gate: All Events. Events To Display: 1000
Flow Rate: Medium