RPCI Tetramer/Dextramer Staining Procedure

Materials

CPT Tubes

BD BioSciences BD vacutainer CPT tube with sodium citrate
(BD BioSciences, 362760)

FCM buffer:

Phosphate buffered saline
Bovine serum albumin (Sigma A4503)
Sodium azide (Fisher 5227)
Na_2EDTA (Sigma)

- Dissolve in 1 liter of 1X PBS, 10 g BSA, 0.04 g Na_2EDTA and 1 g sodium azide. Stored at 4 - 8˚C, this solution is stable for 6 months.

UTI-Lyse (Dako, S3350)

Accucount Beads

Spherotech AccuCount Ultra(Cat # ACURFP-50-10)
or
Dako CytoCount (Cat # S2366)

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*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

MAbs

CD3 PC5: (Dako, clone UCHT1, Cat # C7067)
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).

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

MAb/Dextramer combinations per patient **using inclusion gate,** LD is optional.

1. LD / CD8 FITC / Neg PE Dextramer / CD3 PE-Cy5
2. LD / CD8 FITC / CMV APC Dextramer / CD3 PE-Cy5

Dextramer Staining Method

1. Harvest mononuclear cells (MNC) from CPT tube

2. Wash in one time with FCM buffer (centrifuge 1400 rpm, 5 minutes), resuspend in 0.5 ml of FCM buffer

3. Perform MNC cell count on using AcT 10 hematology analyzer

   Calculation for the final volume cells should be resuspended in, in step 4:
   Cell count (cells/μl) X 500 μl (volume from step 2) / 1 X 10^7

4. Resuspend MNC at a final concentration of 0.5 - 1 10^7 MNC/ml

5. Place 100 μl.(0.5 - 1 x 10^6 MNC/ tube) in a 12x75 flow tube (or Epependorf tube).

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

8. Incubate at RT for 10 minutes.

9a. Inclusion gating, add anti-CD8 FITC, anti-CD3 PC5, and Live Dead (LD) reagent at predetermined titers to each tube. Refer to Live Dead procedure for details on this method.

10. Incubate for 20 min at 4^{o}C in the dark.

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

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

13. Centrifuge 1400 rpm for 5 minutes, pour off supernatant and resuspend in PBS

14 Centrifuge 1400 rpm for 5 minutes, pour off supernatant and resuspend in 500 μl of 2% Formaldehyde.

[PW1]

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

CD8 count Method

1. Add 100 ul 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, and Live Dead (LD) reagent at predetermined titers to each tube. Refer to Live Dead procedure for details on this method.

3. Incubate for 20 min at 4°C 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 AccuCount or CytoCount to each tube

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