MHC Dextramer® Staining Protocol

Intended use	Staining of antigen-specific T cells using one or more fluorochrome-labelled MHC Dextramer® reagents in one sample.
Optimized for	Cat No. Wxxxxxx/Fxxxxxx/XDxxxxx MHC I Dextramer®, MHC II Dextramer®, and CD1d Dextramer®
Materials Provided	MHC Dextramer® PE, APC and/or FITC
Materials Required (not provided)	4 mL Falcon disposable 12 x 75-mm test tubes or equivalent LoBind® Eppendorf tubes or equivalent Stain and wash buffer: PBS, 1-5% FCS, pH 7.4 100 µM d-Biotin (e.g. Avidity, cat# BIO200) diluted in PBS, pH 7.4 10x PBS, pH 7.4 Antibodies identifying relevant surface markers (e.g., CD3, CD4, CD8 and optionally other desired antibodies and live-dead dye[A]). See the FAQ on immudex.com regarding recommended antibody clones. The optimal choice of fluorochromes depends on the flow cytometer and experimental setup.

Procedure

1. Thaw and prepare PBMCs[B] and resuspend 1-3 x 10^6 PBMCs (for clonal cells, use 2-5 x 10^4 instead) in 50 µL stain and wash buffer.
2. To prepare a pool of multiple MHC Dextramer® reagents (*calculation example can be found in Appendix 1)*, mix the following reagents in an empty 1.5 mL LoBind® Eppendorf tube[C]:
    a. Add 0.2 µL of 100 µM d-Biotin[D] per MHC Dextramer® reagent.
    b. Add 10 µL of each MHC Dextramer® reagent.
    c. Add 0.6 µL of 10x PBS[D] per MHC Dextramer® reagent.

NB: When staining with a single MHC Dextramer® reagent, a and c can be omitted.

3. Vortex the MHC Dextramer® pool briefly. The MHC Dextramer® pool must be used directly after preparation and cannot be stored.
4. Centrifuge the pool at 10.000 x g for 1 min. to avoid transferring any potential precipitate.
5. Add the MHC Dextramer® pool to the cell sample and vortex briefly.
6. Incubate in the dark at room temperature:
    a. MHC I Dextramer® pool: 10 min. incubation[E].
    b. MHC II Dextramer® pool: 30 min. incubation[E].
    c. MHC I and II Dextramer® pool: 30 min. incubation[E].
7. Add relevant antibodies in the volume/concentration according to manufacturer's instructions:
    a. If staining with MHC I Dextramer® reagents, use anti-CD8, anti-CD3, and optionally other phenotype markers.
    b. If staining with MHC II Dextramer® reagents, use anti-CD4, anti-CD3, and optionally other phenotype markers.
8. Incubate at room temperature in the dark for 20 min.

9. Wash cells by adding 2 mL stain and wash buffer. Centrifuge at 300 x g for 5 min. and remove the supernatant. Repeat washing for a total of 2 washes[F].
10. Resuspend the pellet in desired volume of stain and wash buffer suitable for your flow cytometer.
11. Proceed to analyze the samples on a flow cytometer or store at 2-8 °C in the dark. For optimal results, do not store the samples longer than 2 hours before acquisition. Alternatively, fixed cells[G] can be stored at 2-8C in dark for up to 24 hours.

Procedural notes

A. Live-dead staining can be performed at the beginning or end of staining procedure according to manufacturer's instructions.
B. MHC Dextramer® staining can be performed on any cell suspensions, cell lines, TILs, or whole blood, if the cells are non-fixed. For whole-blood samples, stain with MHC Dextramer® before Red Blood Cell (RBC) lysis or use non-fixable RBC lysing solution.
C. Always keep MHC Dextramer® reagents stored at 2-8 °C in the dark – the plastic vial only partially protects the reagents against light.
D. d-biotin is required to avoid artefacts in the staining. 10x PBS will balance the salt concentration of the pool.
E. Incubation time may be increased when using a high number of reagents in pool staining and requires optimization.
F. Staining can be performed using 96-well microtiter plates. In that case after antibody incubation make 4 sequential washes using 200 µL stain and wash buffer per well. Centrifuge at 300 x g for 5 min. between each wash and remove supernatant.
G. MHC Dextramer® stained cells can be fixed using 2% Methanol free formalin in PBS. Fixed samples may be washed and resuspended in stain and wash buffer prior to acquisition on a flow cytometer.

Technical support

For additional Tips & Tricks, FAQs and protocols, please visit https://www.immudex.com/resources/ or contact our support team at customer@immudex.com
Telephone: +45 3110 9292 (Denmark)

Analysis Guidelines

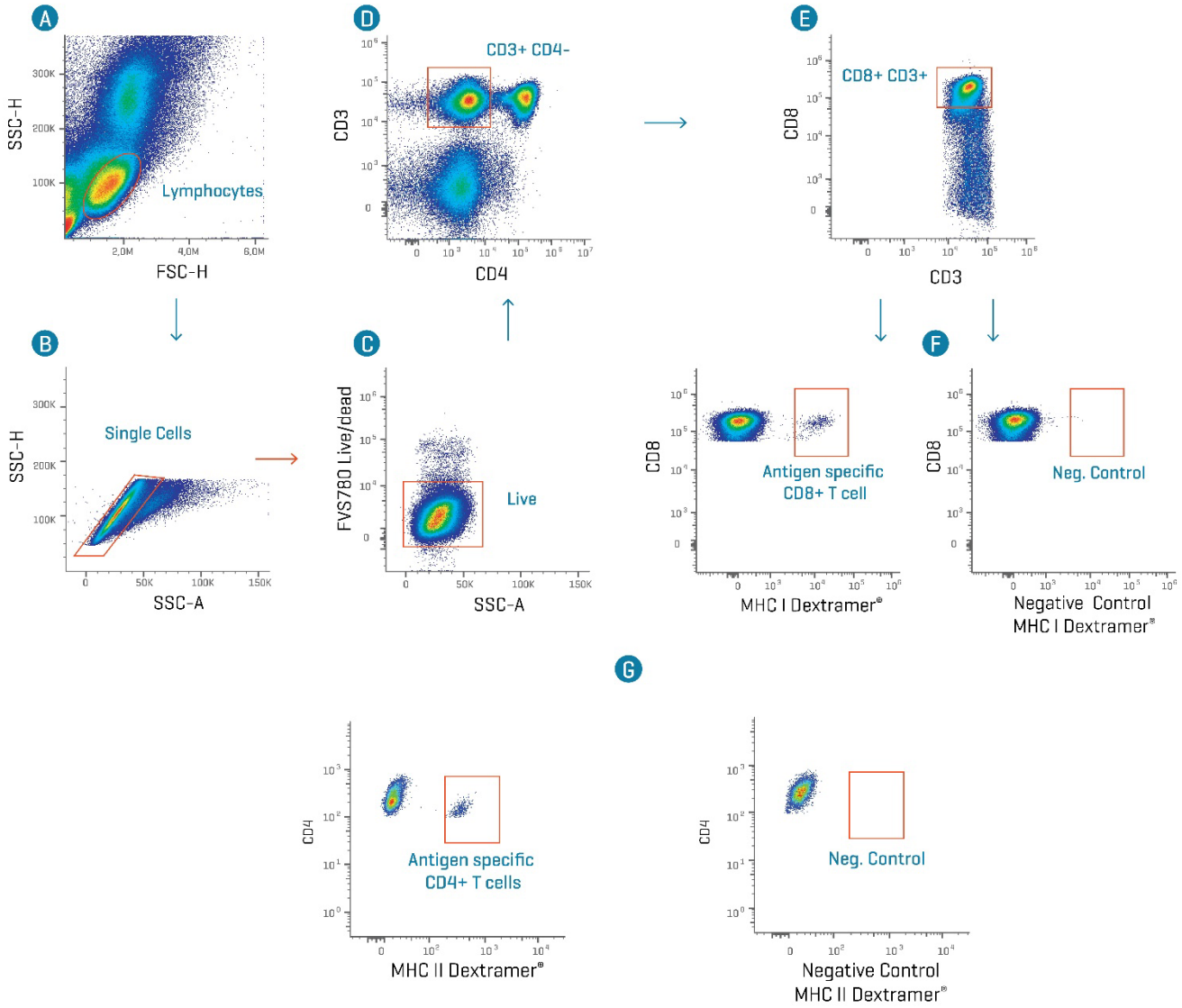

Fig. 1: Flow cytometry gating strategy using MHC I Dextramer® to identify antigen specific T-cells from samples of thawed hPBMCs. (A-F) gating of CD8+ antigen specific T cells. (**A**) Lymphocytes were identified based on the forward (FSC) - and side scatter (SSC) profiles. (**B**) Next, doublets were excluded by gating the single cells in a side scatter height (SSC-H) & side scatter area (SSC-A) profile plot. (**C**) Dead cells were excluded according to the live-dead stain (FVS780), and the live cells were gated for further characterization. (**D**) To exclude $CD4^+$ T cells and Natural killer cells (NK) (positive for CD8 but not CD3), the $CD3^+$/CD4- population was gated. (**E**) The $CD3^+$/$CD8^+$ T cells were then gated, and (**F**) subsequently, the antigen-specific population of cells were determined by comparing the results of gating the MHC I Dextramer® labeled or MHC I Dextramer® Negative Control labeled cells. (**G**) Flow cytometry plots showing CD4+ T helper cells labeled with MHC II Dextramer® or Negative Control MHC II Dextramer®.

Appendix 1
Calculation Examples

Preparation of pools of MHC Dextramer® reagents for staining 1 sample:

Examples	100 µM d-Biotin	Total MHC Dextramer® Reagents	10x PBS	Total Volume
Per each MHC Dextramer®	0.2 µL	10 µL per MHC Dextramer®	0.6 µL	10.8 µL
2 MHC Dextramer® reagents	0.4 µL	20 µL MHC Dextramer®	1.2 µL	21.6 µL
3 MHC Dextramer® reagents	0.6 µL	30 µL MHC Dextramer®	1.8 µL	32.4 µL
10 MHC Dextramer® reagents	2 µL	100 µL MHC Dextramer®	6 µL	108 µL

Preparation of pools of MHC Dextramer® reagents for staining 2 samples:

Note: When preparing a pool for more than 1 sample, we recommend preparing 20% overage of the pool, which is included in the examples below.

Examples	100 µM d-Biotin	Total MHC Dextramer® Reagents	10x PBS	Total Volume
Per each MHC Dextramer®	0.2 µL	12 µL per MHC Dextramer®	0.7 µL	12.9 µL
2 MHC Dextramer® reagents	0.5 µL	24 µL MHC Dextramer®	1.4 µL	25.9 µL
3 MHC Dextramer® reagents	0.7 µL	36 µL MHC Dextramer®	2.2 µL	38.9 µL
10 MHC Dextramer® reagents	2.4 µL	120 µL MHC Dextramer®	7.2 µL	129.6 µL