MBLI Application Note

MHC Tetramer Guided Staining of peptide-specific CD8+ T cells in Peripheral Mononuclear Cells (PBMC) in a 96-well plate format, a practical note

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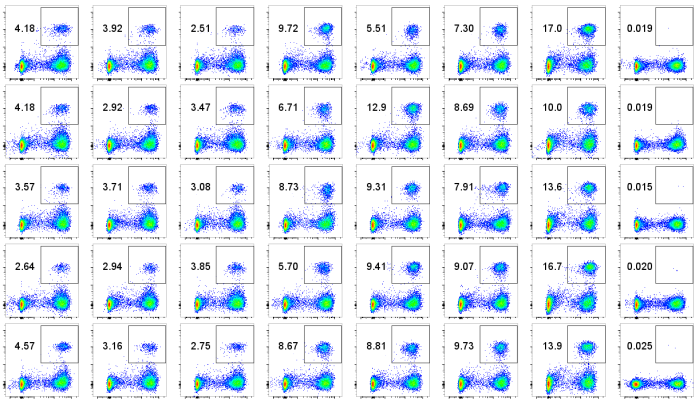
MBL tetramers are provided with a protocol analyzing T cells in a single tube. This protocol is in depth and provides great information for how to set up and run your experiments using MHC tetramers.

Here we describe a method of tetramer staining and flow analysis in a 96-well plate format, for use with an automated plate handler.

The collected plate-based flow data can be analyzed and visualized using appropriate flow analysis software.



Basic Tips for Tetramer Staining

There are a few fundamental considerations in the experimental design that will ensure robust detection of activated CD8 (Class I) and CD4 (Class II) T Cells

- MHC Tetramer is allele-specific to match the donor PBMC and the peptide of interest.
- A volume tetramer-titration curve has been previously performed for optimal staining.
- The sample and antibody cocktail, including MHC class I tetramer, is incubated in the dark at room temperature for at least 30 minutes. On the other hand, a 60-minute incubation for staining with MHC class II in 37 °C is recommended.
- A live/dead marker is used to select for live cells and ensure a more accurate reading.
- Perform the flow analysis soon after staining. You have the option to fix the tetramer stained cells should you need to delay flow cytometry analysis.

Below is an example staining protocol in a 96-well plate format and specific for MHC class I Tetramer.

The method has been verified in our lab and is a standard staining procedure for the use of MBLI Tetramers. In experiments where cells are stimulated then stained in the same plate, it is recommended that the wells on the outermost edge of the plate (Rows A & H, and Columns 1 & 12) are filled with at least 100 µl sterile water or PBS. This will maintain the humidity of the plate and reduce the risk of evaporation.

Safety

- Consult the corresponding safety forms for precautions associated with the reagents.
- Wear proper Personal Protective Equipment (PPE) when performing this SOP
- Any spilled material needs to be disposed according to your institution's EHS policy.

Materials

- Multi-channel pipettes 20-200 µl & 1-10 µl
- Single channel pipettes 1000, 200, 20 & 2 µl
- Reagent reservoirs
- 96 well U- or V-Bottom plates
- Benchtop Centrifuge with plate holder
- Waste containers for tips and liquid waste

Reagents

- FACS Wash buffer (0.5 mM EDTA in Phosphate Buffered Solution)
- FACS Incubation Buffer (PBS + 0.5% Fetal Bovine Serum or 0.5% Human AB Serum)
- Compensation beads (BD 552843)
- Basic Tetramer staining panel (MHC Class I example)

Fluorophore	Target	Final Dilution	Company	Product Code
FITC	CD3	1:100	MBL	FP10255010
PE	MHC Tetramer	2.5 µl per well	MBL	Various
7AAD	Live/Dead	5 µl per well	MBL	FP00020050
AOC	CD8	1:100	MBL	FP10588010

Procedure

- Remove cells from the incubator, and transfer to a U- or V-bottom plate. Alternatively, cells previously stimulated in a 96-well plate format can also be used.
- Pellet cells (300xg, 5 min), remove supernatant, add 60 ul of FACS Incubation buffer and resuspend cells by pipetting.
- Prepare a master 4X mix of the antibody staining in FACS Incubation buffer. For example, if the dilution of CD3 antibody is 1/100, prepare a master mix of 1/25.
- Add 20 µl of the master mix to each well and mix by pipetting.
- Add 2.5 µl of each Tetramer to each well and mix by pipetting.
- Cover and incubate at RT for 30 min in dark conditions such as a closed drawer.
- Add 100 µl of FACS Wash Buffer, pellet cells (300xg, 5 min), and remove supernatant.
- Repeat previous wash step with 150 µl FACS Wash Buffer, remove supernatant.
- Add 200 µl of FACS Wash buffer.
- Prior to flow analysis, add 5 ul 7-AAD.
- Flow analysis: collect at least 250,000 events per well to ensure acquisition of the low frequency Tetramer positive events.
  - FSC/SSC, Gate on Lymphocytes
  - Lymphocytes, Gate on Singlets
  - Singlets, Gate on Live CD3+
  - Tet versus CD8 for Tetramer Positive Gate