

T-Select MHC class I mouse Tetramer

H-2K^b OVA Tetramer

-SIINFEKL (50 tests)

Background

T lymphocytes play a central role in immune system. Total T cell and T cell subset counts are measured by detection of various cell surface molecules. Enumeration of CD8⁺ antigen-specific T cells requires cognate recognition of the T cell receptor (TCR) by a class I MHC/peptide complex. This can be done using T-Select MHC class I Tetramers which are composed of four MHC class I molecules each bound to the specific peptide and conjugated with a fluorescent protein. Thus, T-Select MHC Tetramer assays allow quantitation of the total T cell population specific for a given peptide complexed in a particular MHC molecule. Furthermore, since binding does not depend on functional pathways, this population includes specific CD8⁺ T cells regardless of functional status. Measurements may be performed in whole blood or isolated lymphocyte/mononuclear cell preparations. In some cases where frequency is low, it may be necessary to perform an *in vitro* cell expansion. Specific cell staining is accomplished by incubating the sample with the T-Select MHC Tetramer reagent, then washing away excess Tetramer. The number of Tetramer positive lymphocytes is then determined by flow cytometry.

This Tetramer reagent comprises murine class I MHC H-2K^b and epitope peptide derived from ovalbumin (OVA), and it can detect an H-2K^b-restricted OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells. The CD8⁺ T cell epitope, OVA₂₅₇₋₂₆₄, of the OVA model antigen has been a useful tool in immunology. The OT-I transgenic mouse strain carries a TCR transgene specific for the OVA₂₅₇₋₂₆₄ peptide, and it is useful for studying T cell immunology.

A Tetramer, which is constructed with the same allele (H-2K^b) of interest and an irrelevant peptide, may be used as a negative control Tetramer. Alternatively, a cell population devoid of Tetramer-positive cells may be used as a negative control.

MHC Restriction: H-2K^b

Origin and Sequence of CTL Epitope:

OVA (257-264 aa, SIINFEKL)

Reagents

The Tetramer is dissolved in an aqueous buffer containing 0.5 mM EDTA, 0.2% BSA, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% ProClin. The concentration of class I MHC/peptide complexes (monomer) is adjusted as shown below.

TS-5001-1C: 25 µg/mL of monomer

TS-5001-2C: 100 µg/mL of monomer

Storage Conditions

Store at 2 to 8°C. Do not freeze. Minimize exposure to light. The expiration date is indicated on the vial label.

Conjugates

• TS-5001-1C

Streptavidin-Phycoerythrin (SA-PE)

Excites at 486-580 nm

Emits at 586-590 nm

• TS-5001-2C

Streptavidin-Allophycocyanin (SA-APC)

Excites at 633-635 nm

Emits at 660-680 nm

Evidence of Deterioration

Any change in the physical appearance of this reagent may indicate deterioration and the reagent should not be used. The normal appearance is a clear, colorless to pink (SA-PE), or light blue (SA-APC).

Usage

This reagent is for use with standard flow cytometry methodologies.

Reagent Preparation

No preparation is necessary. These T-Select MHC Tetramer reagents are used directly from the vial after a brief vortex on low setting. However, depending on murine cell type and assay conditions, it may be necessary to optimize Tetramer labeling of antigen-positive, CD8-positive T cells. Optimal labeling is determined by performing a checkerboard titration of both class I Tetramer and anti-murine CD8 antibody reagents.

Mouse Strain H-2K Haplotypes:

H-2K allele	H-2K ^b	H-2K ^d	H-2K ^k
Mouse strains	C57BL/-, BXSB/Mp, 129/-	BALB/c, DBA/2, NOD	C3H/He

References for Products

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- 25) Tsukamoto H, *et al. Cancer Immunology* **1**: 64-76 (2013)

References for T-Select MHC Tetramer

- Altman JD, *et al. Science* **274**: 94-96 (1996)
 McMichael AJ, *et al. J Exp Med* **187**: 1367-1371 (1998)
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Statement of Warnings

1. Specimens, samples and material coming in contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions.
2. Never pipette by mouth and avoid contact of samples with skin and mucous membranes.
3. Minimize exposure of reagent to light during storage or incubation.
4. Avoid microbial contamination of reagent or erroneous results may occur.
5. Use Good Laboratory Practices (GLP) when handling this reagent.

Materials Required But Not Supplied

- 12 x 75 mm polypropylene test tubes
- Transfer pipettes
- Pipettors and disposable pipette tips
- Vortex mixer
- Centrifuge capable of 150 x g or 400 x g
- Aspirator
- PBS
- Red blood cell lysis reagent
- 7-AAD Viability Dye, Beckman Coulter, Inc., PN A07704
- Clear Back (human FcR blocking reagent), MBL, PN MTG-001
- anti-mouse CD8-FITC (KT15), MBL, PN K0227-4
- anti-mouse CD8-Alexa Fluor® 647 (KT15), MBL, PN K0227-A64

Procedure for Whole Blood

1. Collect venous blood specimen according to established protocol into a blood collection tube using an appropriate anti-coagulant. If the mouse line that is being used is transgenic and the T cell receptor is specific for the peptide, 100 µL of whole blood should be adequate. If the blood specimen is not being derived from a transgenic line, you may require more than 100 µL in order to perform the rare event analysis.
2. To each 12x75 mm test tube add 10 µL of T-Select MHC Tetramer.
3. Add 100 µL of whole blood into each test tube.
4. Vortex gently.
5. Incubate for 30-60 minutes at 2-8°C protected from light.
6. Add any additional antibodies (e.g. anti-CD8) and vortex gently.
7. Incubate for 30 minutes at 2-8°C protected from light.
8. Lyse red blood cells using commercially available reagents.
9. Prepare samples according to description of the package insert.
10. Store prepared samples at 2-8°C protected from light for a minimum of 1 hour (maximum 24 hours) prior to analysis by flow cytometry.

Procedure for Cell Preparations and Cell Suspensions

1. Collect lymph node, spleen or thymus and prepare a single-cell suspension according to an established protocol. Cells should be re-suspended at a concentration of 2×10^7 cells/mL. 50 μ L of sample is required for each T-Select MHC Tetramer determination.
2. To each 12x75 mm test tube add 10 μ L of Clear Back (human FcR blocking reagent, MBL, PN MTG-001).
3. Add 50 μ L cell suspension into each test tube (e.g. 1×10^6 cells per tube).
4. Incubate for 5 minutes at room temperature.
5. Add 10 μ L of T-Select MHC Tetramer and vortex gently.
6. Incubate for 30-60 minutes at 2-8°C protected from light.
7. Add any additional antibodies (e.g. anti-CD8) and vortex gently.
8. Incubate for 30 minutes at 2-8°C protected from light.
If red blood cell lysis is necessary, proceed to step 8-16 in the **Procedure for Whole Blood** section. If red blood cell lysis is not necessary, continue to step 9 below.
9. Add 3 mL of PBS or FCM buffer (2% FCS/0.09% NaN₃/PBS).
10. Centrifuge tubes at 400 x g for 5 minutes.
11. Aspirate or decant the supernatant.
12. Resuspend the pellet in 500 μ L of PBS with 0.5% paraformaldehyde or formalin.
13. Store at 4°C protected from light for a minimum of 1 hour (maximum 24 hours) prior to analysis by flow cytometry.

Limitations

1. For optimal results with whole blood, retain specimens in blood collection tubes at room temperature, while rocking, prior to staining and analyzing. Refrigerated specimens may give aberrant results.
2. Recommended cell viability for venous blood specimens is > 90%.
3. Prolonged exposure of cells to lytic reagents may cause white blood cell destruction and loss of cells in the population of interest.
4. All red blood cells may not lyse under the following conditions: nucleated red blood cells, abnormal protein concentration or hemoglobinopathies. This may cause falsely decreased results due to unlysed red blood cells being counted as leukocytes.

Technical Hints

- A. In an experiment where cells are stained with T-Select MHC Tetramer and antibodies, Clear Back (human FcR blocking reagent) may effectively block non-specific binding caused by macrophages or endocytosis, resulting in clear staining. Please refer to the data sheet (MBL, PN MTG-001) for details.

- B. A Tetramer, which is constructed with the same allele of interest and an irrelevant peptide, may also be used as a negative control.
- C. We recommend the use of the CD8 antibody clone KT15, because some clones inhibit or enhance Tetramer-specific binding to TCR.
- D. In the case of OT-I TCR transgenic mice, it is necessary to perform a cross-titration experiment with the Tetramer and the CD8 antibody (clone KT15) to determine the optimal concentration of both reagents.
- E. To reduce contamination of unlysed or nucleated red blood cells in the gate, we recommend the use of CD45 antibody and gating of the lymphocyte population.
- F. Apoptotic, necrotic, and/or damaged cells are sources of interference in the analysis of viable cells by flow cytometry. Non-viable cells should be evaluated and discriminated following 7-AAD-positive labeling when viable cells remain unstained (negative).
- G. The cells do not need to be fixed before analysis if stained cells are analyzed by flow cytometry within several hours.

Related Products

Please check our web site (<https://www.mbl-chinawide.cn/>) for up-to-date information on products and custom MHC Tetramers.

Experimental data 1: OVA₂₅₇₋₂₆₄ peptide immunized mice

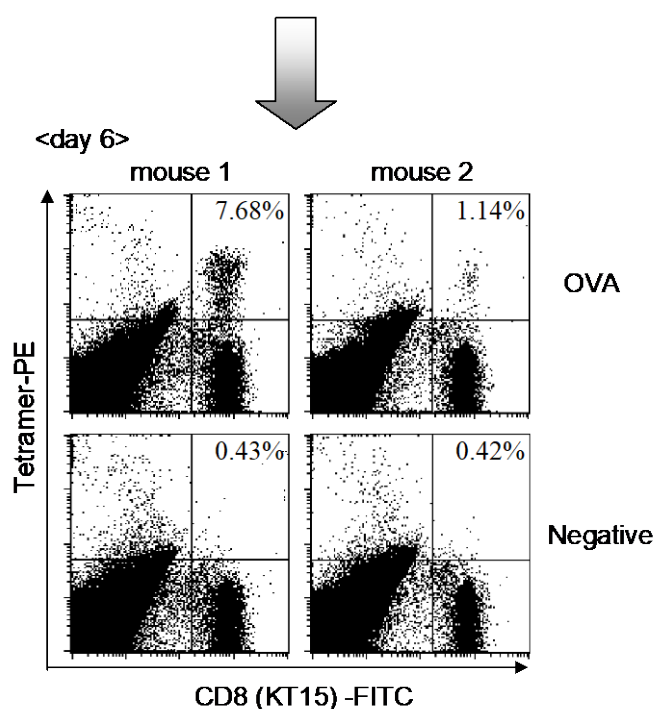
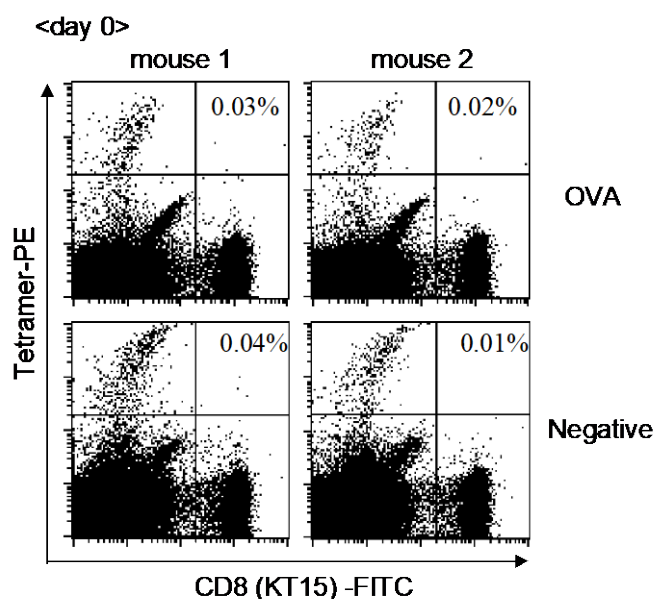
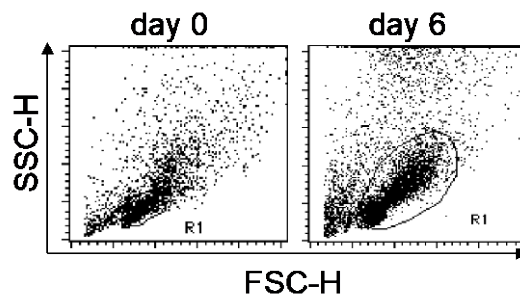
C57BL/6 mice were intraperitoneally immunized with 100 nmol of OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) and OVA₃₂₃₋₃₃₉ helper peptide (ISQAVHAAHAEINEAGR) in complete Freund's adjuvant. Splenocytes were prepared from the immunized mice 10 days after immunization. Splenocytes were stained with MHC class I Tetramer on day 0. An aliquot of the splenocytes was stimulated with OVA₂₅₇₋₂₆₄ peptide-pulsed splenocytes isolated from non-immunized mice. Staining for MHC class I Tetramer was performed on day 6.

Procedure:

1. Prepare immunized mice splenocytes or peptide stimulated cells for 6 days (1×10^6 cells) hemolyzed with ACK lysis buffer, and wash in FCM buffer (2% FCS/0.05% NaN₃/PBS) in each test tube.
2. Add 1 mL of FCM buffer, and centrifuge at 400 x g for 5 minutes.
3. Aspirate the supernatant carefully. Add 10 μ L of Clear back (MBL PN MTG-001) and 30 μ L of FCM buffer. Incubate for 5 minutes at room temperature.
4. Add 10 μ L of H-2K^b OVA Tetramer-PE (MBL PN TS-5001-1C) or H-2K^b Negative Tetramer-PE (MBL PN TS-M008-1) to each test tube and mix well. Incubate the cells for 30 minutes at 4°C.
5. Add 10 μ L of mouse CD8-FITC (clone KT15) to each test tube and mix well. Incubate for 20 minutes at 4°C.
6. Add 1 mL of FCM buffer, and centrifuge at 400 x g for 5 minutes.
7. Aspirate the supernatant carefully. Suspend the cells with 400 μ L of FCM buffer.
8. Analyze prepared samples by flow cytometry.

Results:

The lymphocyte population was defined by an FSC/SSC gate (R1). Data were analyzed by gating on the lymphocyte cell population (R1). The frequency of MHC Tetramer⁺ and CD8⁺ T cells is shown as a percentage of total CD8⁺ T cells.



Limited staining was observed in freshly isolated splenocytes (day 0). However, the H-2K^b OVA Tetramer-positive CD8⁺ T cells could be detected after *in vitro* stimulation (mouse 1 and 2). Tetramer-positive CD8⁺ T cells were not detected in the negative control (H-2K^b Negative Tetramer-PE).

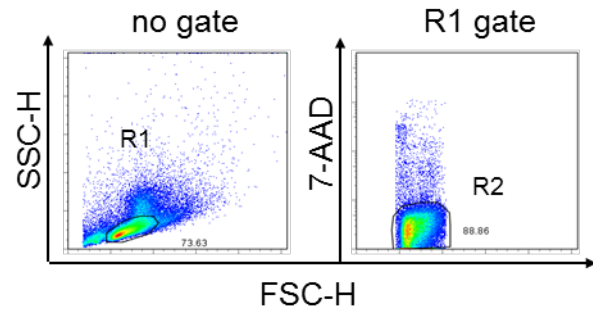
*The percentages of tetramer-positive CD8 T cells were different in each mouse though these mice were treated equally. In order to avoid such individual differences, at least two mice should be immunized.

*The nonspecific staining was detected in the results because 7-AAD was not used in this Procedure. Please refer to Technical Hints F.

*CD8 antibody clone 53.6.7 and H-2K^b Negative Tetramer-PE should not be used together. In the case of clone 53.6.7, H-2K^b β -galactosidase Tetramer-PE (MBL PN TS-M501-1) should be used as a negative control tetramer.

Results:

The lymphocyte population was defined by an FSC/SSC gate (R1), and the viable cell population was defined by an FSC/7-AAD (R2). Data were analyzed by double gating on the lymphocyte and viable cell population (R1 and R2).



Experimental data 2: Mouse CD8 antibody comparison (KT15 vs 53.6.7)

The OT-I transgenic mouse strain has a TCR transgene specific for the OVA₂₅₇₋₂₆₄ peptide, and it is bred and maintained in the C57BL/6 genetic background. OT-I splenocytes were stained with two different mouse CD8 antibody clones (KT15 or 53.6.7) and H-2K^b OVA Tetramer-PE.

Procedure:

1. Prepare OT-I splenocytes (1×10^6 cells/sample) hemolyzed with ACK lysis buffer, and wash in FCM buffer (2% FCS/0.05% NaN₃/PBS) in each test tube.
2. Add 1 mL of FCM buffer, and centrifuge at 400 x g for 5 minutes.
3. Aspirate the supernatant carefully. Add 10 μ L of Clear back (MBL PN MTG-001) and 30 μ L of FCM buffer. Incubate for 5 minutes at room temperature.
4. Add 10 μ L of H-2K^b OVA Tetramer-PE (MBL PN TS-5001-1C) or H-2K^b β -galactosidase Tetramer-PE (MBL PN TS-M501-1) to each test tube and mix well. Incubate the cells for 20 minutes at 4°C.
5. Add serially diluted mouse CD8 antibody (clone KT15 or 53.6.7) to each test tube in a final assay volume of 100 μ L.
6. Mix well and incubate for 20 minutes at 4°C.
7. Add 1 mL of FCM buffer, and centrifuge at 400 x g for 5 minutes.
8. Aspirate the supernatant carefully. Suspend the cells with 400 μ L of FCM buffer.
9. Add 5 μ L of 7-AAD for the exclusion of nonviable cells in flow cytometric assays.
10. Analyze prepared samples by flow cytometry. All measurements were performed under the same compensation conditions.

In the case of 53.6.7, CD8⁺ T cells in OT-I splenocytes were stained with H-2K^b β-galactosidase Tetramer-PE. When OT-I splenocytes were stained with KT15 and H-2K^b β-galactosidase Tetramer-PE, Tetramer-positive cells were not observed. These indicate that the CD8 antibody, 53.6.7, changes the fine specificity of MHC Tetramer binding to its specific TCR. The CD8 coreceptor helps to increase the degree of TCR occupancy at the T cell surface and stabilize the interaction between the TCR and MHC Tetramer. 53.6.7 is thought to have the enhancing effect on this interaction.

On the other hand, higher concentration of KT15 inhibited the staining of CD8⁺ T cells in OT-I splenocytes. Titration of KT15 revealed an optimal signal to noise ratio at 0.63 μ L/sample for OT-I splenocytes. These results indicate that KT15 has weak inhibitory effect on the interaction between the TCR and MHC Tetramer. MBL strongly recommends to use of appropriately titrated KT15 when OT-I splenocytes are stained with MHC Tetramer. As shown above Experimental data 1, no particular problem was observed except a case of OT-I splenocytes.

*The number in parenthesis indicates the ratio of tested to the recommended amount of antibody according to the data sheet.

