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Not for use in diagnostic procedures.**

MBL
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T-Select MHC Tetramer

I-A^b ESAT-6$_{1-20}$ Tetramer-PE (20 tests)

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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 CD4$^+$ antigen-specific T cells requires cognate recognition of the T cell receptor (TCR) by a class II MHC/peptide complex. This can be done using T-Select MHC Class II Tetramers which are composed of four MHC class II molecules each bound to the specific peptide[1,2] 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, Tetramer-stained population includes specific CD4$^+$ 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[3]. 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 mouse MHC class II I-A^b and an epitope peptide derived from ESAT-6 (Early Secreted Antigenic Target 6) and can detect I-A^b-restricted ESAT-6-specific CD4$^+$ T cells.

Mycobacterium tuberculosis (Mtb) is a gram-positive bacterium that is well known as the causative agent of tuberculosis, including pulmonary tuberculosis. ESAT-6 is a major small molecule protein secreted by Mtb, which has strong antigenicity. Since the *M. bovis* BCG strain used in the tuberculosis vaccine lacks the *esat-6* gene, this antigen-specific T cell response is used to diagnose tuberculosis infection in BCG-vaccinated individuals[6]. ESAT-6$_{1-20}$ is an I-A^b-restricted immunodominant epitope, which is widely used for monitoring the immune response *in vivo* and in the development of new vaccines. Transgenic mice with I-A^b/ESAT-6$_{1-20}$-specific TCR have also been created[7),8)], therefore, the I-A^b ESAT-6$_{1-20}$ Tetramer is expected to be an important tool in future experiments.

A Tetramer, which is constructed with the same allele (I-A^b) of interest and an irrelevant peptide, may be used as a negative control Tetramer. If it is difficult to use the negative control Tetramer, use of a cell population that does not contain CD4$^+$ T cells expressing the desired TCR is recommended as a control.

MHC restriction: I-A^b

Peptide Sequence

ESAT-6 (1-20 aa, MTEQQWNFAGIEAAASAIQG)

Mouse Strain I-A Haplotypes:

I-A allele	I-A^b	I-A^d	I-A^k	I-A^S
Mouse strains	C57BL/-BXSB/Mp 129/-	BALB/c DBA/2	C3H/He	SJL/J B10.S

Conjugates

TS-M707-1: Streptavidin-Phycoerythrin (SA-PE)
Excites at 486-580 nm
Emits at 586-590 nm

Storage Conditions

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

Reagents

200 μL liquid - 10 μL/test
T-Select MHC Class II Mouse Tetramer - 20 tests
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 0.09% NaN_3.

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).

References for ESAT-6_{1-20}

1) Brandt L, *et al. J. Immunol.* **157**: 3527-3533 (1996)
2) Winslow GM, *et al. J. Immunol.* **170**: 2046-2052 (2003)
3) Jung Y, *et al. J. Exp. Med.* **201**: 1915-1924 (2005)
4) Scott-Browne JP, *et al. J. Exp. Med.* **204**: 2159-2169 (2007)
5) Khader SA, *et al. Nat. Immunol.* **8**: 369-377 (2007)
6) Ganguly N, *et al. Tuberculosis* **88**: 510-517 (2008)
7) Reiley WW, *et al. PNAS* **105**: 10961-10966 (2008)
8) Gallegos A, *et al. J. Exp. Med.* **205**: 2359-2368 (2008)

Statement of Warnings

1. This reagent contains 0.09% sodium azide. Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be flushed with running water while being discarded. These precautions are recommended to avoid deposits in metal piping in which explosive conditions can develop. If skin or eye contact occurs, wash excessively with water.
2. Specimens, samples and material coming in contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions.
3. Never pipette by mouth and avoid contact of samples with skin and mucous membranes.
4. Minimize exposure of reagent to light during storage or incubation.
5. Avoid microbial contamination of reagent or erroneous results may occur.
6. 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
- mouse CD4-FITC (clone GK1.5), MBL, PN D341-4
- 7-AAD Viability Dye, Beckman Coulter, Inc., PN A07704
- Clear Back (Human FcR blocking reagent), MBL, PN MTG-001

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 x 10^7 cells/mL. 50 µL of sample is required for each T-Select MHC Tetramer determination.
2. Add 10 µL of Clear Back (human FcR blocking reagent, MBL, PN MTG-001) to each 12 x 75 mm test tube.
3. Add 50 µL of cell suspension into each test tube (e.g. 1 x 10^6 cells per tube).
4. Incubate for 5 minutes at room temperature.
5. Add 10 µL of T-Select MHC Tetramer and vortex gently.
6. Incubate for 30-60 minutes at 2-8°C or room temperature (15-25°C) protected from light.
7. Add any additional antibodies (e.g. anti-mouse CD4) and vortex gently.
8. Incubate for 30 minutes at 2-8°C protected from light. If red blood cell lysis is necessary, lyse red blood cells using commercially available reagents.
9. Add 3 mL of PBS or FCM buffer (2% FCS/0.05% NaN_3/PBS).
10. Centrifuge tubes at 400 x g for 5 minutes.
11. Aspirate or decant the supernatant.
12. Suspend the pellet in 500 µL of FCM buffer and analyze it immediately, or suspend it in 0.5% paraformaldehyde/PBS and store the sample in a dark room at 2-8°C. Be sure to analyze it within 24 hours.

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. Clear Back reagent (human FcR blocking reagent) may effectively block non-specific binding caused by macrophages or endocytosis, resulting in clear staining when cells are stained with MHC Tetramer and antibodies. Please refer to the data sheet (MBL, PN MTG-001) for details.
B. A Tetramer that is constructed with the same allele of interest and an irrelevant peptide may be used as a negative control.
C. The use of CD45 antibody and gating of the lymphocyte population are recommended in order to reduce contamination of unlysed or nucleated red blood cells in the gate.
D. Apoptotic, necrotic, and/or damaged cells are sources of interference in the analysis of viable cells by flow cytometry. Cell viability should be

determined by 7-aminoactinomycin D (7-AAD) staining; intact viable cells remain unstained (negative).

E. Cells do not require fixation prior to analysis if the stained cells are analyzed by flow cytometry within several hours.

References for T-Select MHC Tetramer

1. Altman JD, *et al*. *Science* **274**: 94-96 (1996)
2. McMichael AJ and O 'Callaghan CA, *J Exp Med* **187**: 1367-1371 (1998)
3. Nepom GT, *et al*. *Arthritis Rheum* **46**: 5-12 (2002)
4. Lyons AB and Doherty KV, *Current Protocols in Cytometry* **2**: 9.11.1-9.11.9 (1998)
5. Novak EJ, *et al*. *J Clin Ivest* **104**: R63-R67 (1999)

Example of Tetramer Staining

All data were kindly provided by Dr. Shunsuke Sakai and Dr. Masao Mitsuyama (Department of Microbiology, Kyoto University Graduate School of Medicine).

C57BL/6 mice were injected with the human tubercle bacillus H37Rv strain or BCG Pasteur strain in the tail vein (5 × 10^5 CFU /mouse). The spleen and lungs were excised to prepare cells four weeks after immunization, and staining for MHC class II Tetramer was performed.

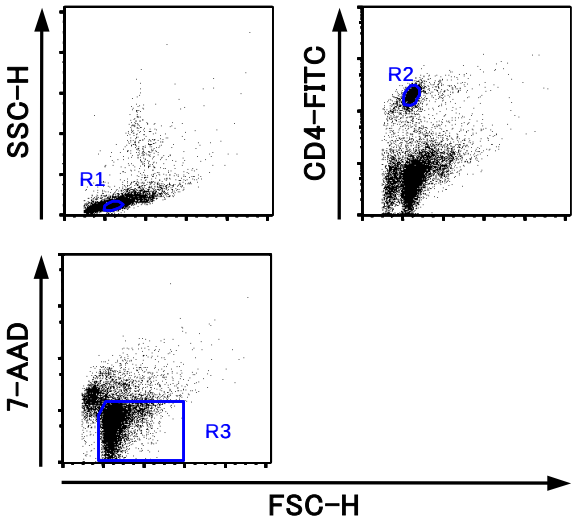
Procedure

1. Prepare immunized mice splenocytes (1 x 10^6 cells) hemolyzed with ACK lysis buffer, and wash in FCM buffer (2% FCS/0.05% NaN_3/PBS) in each test tube. Immunized mice lung cells (1 x 10^6 cells) are treated with Collagenase D/DNase I, followed by hemolysis.
2. Add 1 mL of FCM buffer, and centrifuge at 400 x g for 5 minutes.
3. Aspirate the supernatant carefully. Add 20 μL of Clear Back (MBL, PN MTG-001) and 20 μL of FCM buffer. Incubate for 5 minutes at room temperature.
4. Add 10 μL of I-A^b ESAT-6_{1-20} Tetramer-PE (MBL, PN TS-M707-1) or I-A^b MOG_{35-55} Tetramer-PE as a negative control for splenocyte staining (MBL, PN TS-M704-1) to each test tube and mix well. Incubate the cells for 60 minutes at room temperature.
5. Add 2 μL of mouse CD4-FITC (clone GK1.5) and 5 μL of 7-AAD to each test tube and mix well. Incubate for 20 minutes at 4°C.
6. Add 1 mL FCM buffer, and centrifuge at 400 x g for 5 minutes.
7. Aspirate the supernatant carefully. Suspend the cells in 300 μL of 20 μg/mL 7-AAD/1% paraformaldehyde/PBS and fix samples at 4°C in the dark for more than 30 minutes.
8. Analyze the prepared samples using flow cytometry.

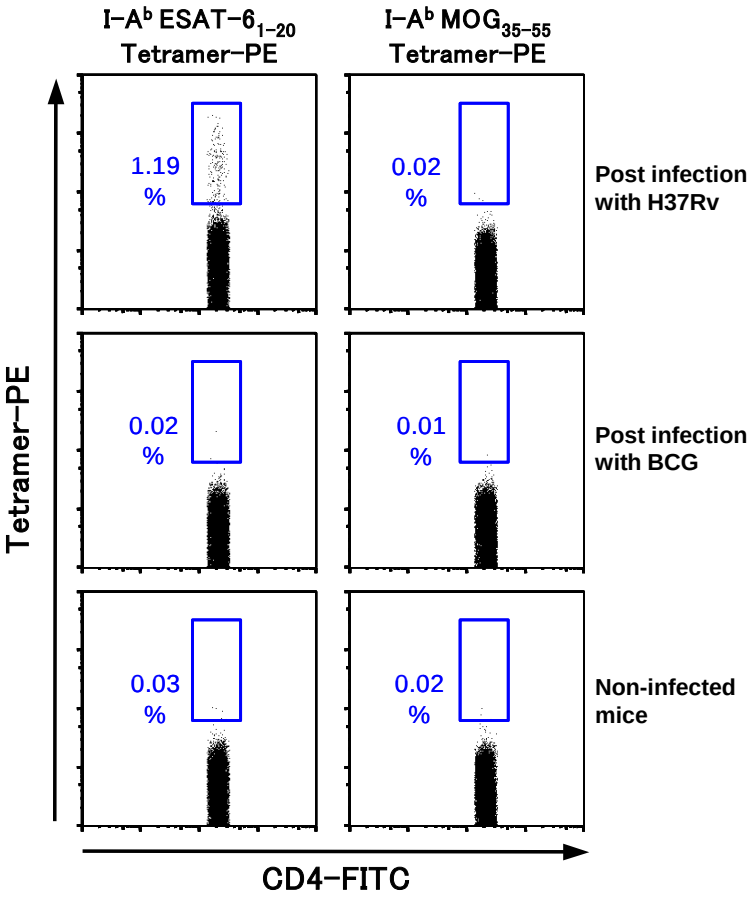
Results

The lymphocyte population was defined by an FSC/SSC gate (R1), $CD4^+$ T cell population was defined by FSC/CD4-FITC (R2), and the viable cell population was defined by an FSC/7-AAD (R3). Data were analyzed by triple gating (R1, R2 and R3).

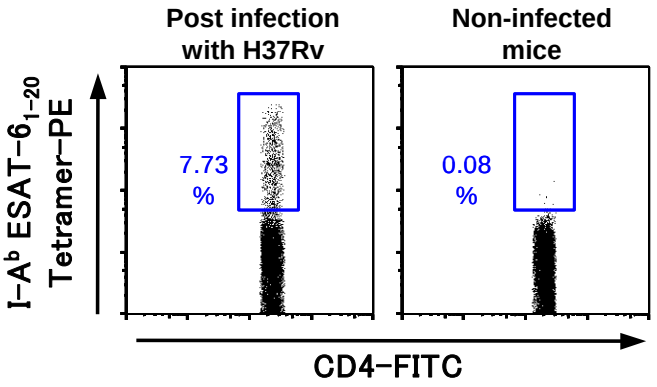
[Gating (splenocytes)]



[Tetramer staining (splenocytes)]



[Tetramer staining (lung cells)]



The I-A^b ESAT-6$_{1-20}$ Tetramer$^+$ CD4$^+$ T cells could be detected after infection with the human tubercle bacillus H37Rv strain. Tetramer$^+$ CD4$^+$ T cells were not detected in the negative control mice (non-infected or BCG infected) or by the negative control Tetramer I-A^b MOG$_{35-55}$ Tetramer-PE.