

T-Select MHC Tetramer

# H-2D<sup>b</sup> WT1<sub>126-134</sub> Tetramer -RMFPNAPYL (50 tests)

For Research Use Only. Not for use in diagnostic procedures.  
These T-Select MHC Tetramers use patented technology (Japanese patent No. P4422903).

## 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<sup>+</sup> 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 with a particular MHC molecule. Furthermore, since binding does not depend on functional pathways, this population includes specific CD8<sup>+</sup> 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-2D<sup>b</sup> and WT1-derived epitope peptide, WT1<sub>126-134</sub>, and it can detect H-2D<sup>b</sup>-restricted WT1<sub>126-134</sub>-specific CD8<sup>+</sup> T cells.

Wilms' tumor gene 1 (WT1) is a zinc finger transcription factor with limited expression in normal adult tissues, but is overexpressed in the majority of leukemias and various types of solid tumors. In 2009, WT1 was ranked first in a list of 75 representative cancer antigens in a National Cancer Institute prioritization project. Many clinical trials of cancer immunotherapy targeting the WT1 have been carried out around the world.

The RMFPNAPYL peptide from WT1 is presented by both HLA-A\*02:01 and HLA-A\*02:06 in humans, as well as by H-2D<sup>b</sup> in C57BL/6 mice, and has been shown to be the target of WT1-specific responses in both species.

A Tetramer, which is constructed with the same allele (H-2D<sup>b</sup>) of interest and an irrelevant peptide, may be used as a negative control Tetramer.

**MHC Restriction:** H-2D<sup>b</sup>

## Origin and Sequence of CTL Epitope

WT1 (126-134 aa, RMFPNAPYL)

## Reagents

500  $\mu$ L liquid - 10  $\mu$ L/test

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<sub>3</sub>.

## Conjugates

TS-M504-1

Streptavidin-Phycoerythrin (SA-PE)

Excites at 486-580 nm

Emits at 586-590 nm

TS-M504-2

Streptavidin-Allophycocyanin (SA-APC)

Excites at 633-635 nm

Emits at 660-680 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.

## 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.

## 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).

## References for Products

- 1) Oka Y, *et al. J Immunol* **164**: 1873-1880 (2000)
- 2) Kohrt HE, *et al. Blood* **118**: 5319-5329 (2011)

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## Mouse Strain H-2D Haplotypes

H-2D allele	H-2D <sup>b</sup>	H-2D <sup>d</sup>	H-2D <sup>k</sup>
Mouse strains	C57BL/-, BXSB/Mp, 129/-,NOD	BALB/c, DBA/2	C3H/He, AKR/J

## 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)  
Bodinier M, *et al. Nat Med* **6**: 707-710 (2000)

## 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
- anti-mouse CD8-FITC (KT15), MBL, PN D271-4

- anti-mouse CD8-Alexa Fluor<sup>®</sup> 647 (KT15), MBL, PN D271-A64
- 7-AAD Viability Dye, Beckman Coulter, Inc., PN A07704
- Clear Back (human FcR blocking reagent), MBL, PN MTG-001

## 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  $\mu$ L of whole blood should be adequate. If the blood specimen is not being derived from a transgenic line, you may require more than 100  $\mu$ L in order to perform the rare event analysis.
2. To each 12 x 75 mm test tube add 10  $\mu$ L of T-Select MHC Tetramer.
3. Add 100  $\mu$ L of whole blood into each test tube.
4. Vortex gently.
5. Incubate for 30-60 minutes at 2-8°C or room temperature (15-25°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. Analyze prepared samples by flow cytometry. If necessary, store the samples at 2-8°C protected from light for a maximum of 24 hours prior to analysis.

## 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  $2 \times 10^7$  cells/mL. 50  $\mu$ L of sample is required for each T-Select MHC Tetramer determination.
2. Add 10  $\mu$ L of Clear Back (human FcR blocking reagent, MBL, PN MTG-001) to each 12 x 75 mm test tube.
3. Add 50  $\mu$ L of cell suspension into each test tube (e.g.  $1 \times 10^6$  cells per tube).
4. Incubate for 5 minutes at room temperature.
5. Add 10  $\mu$ L 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-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 8-9 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% NaN<sub>3</sub>/PBS).
10. Centrifuge tubes at 400 x g for 5 minutes.
11. Aspirate or decant the supernatant.
12. Resuspend the pellet in 500 µL of PBS with 0.5% paraformaldehyde or formalin.
13. Analyze prepared samples by flow cytometry. If necessary, store the samples at 2-8°C protected from light for a maximum of 24 hours prior to analysis.

### 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. If cell cultivation is needed, we recommend the use of heparin as an anti-coagulant.
- B. 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.
- C. A Tetramer that is constructed with the same allele of interest and an irrelevant peptide may be used as a negative control.
- D. We recommend the use of the CD8 antibody clone KT15 (MBL, PN D271-4), because some anti-CD8 antibodies inhibit Tetramer-specific binding to TCR.
- E. 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.
- F. 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).
- G. Cells do not require fixation prior to analysis if the stained cells are analyzed by flow cytometry within several hours.

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### Experimental Data: Peptide immunized mice

C57BL/6 mice were intraperitoneally immunized twice with 100 nmol of the WT1<sub>126-134</sub> peptide (RMFPNAPYL) and 85 nmol of HBc helper peptide (TPPAYRPPNAPIL, MBL, PN TS-M701-P) 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 the WT1<sub>126-134</sub> peptide (0.1 µg/mL) during the first seven days. Cultured cells were expanded with the WT1<sub>126-134</sub> peptide-pulsed syngeneic splenocytes for the next 7 days. Staining for MHC class I Tetramer was performed on day 7 and 14.

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

### Procedure:

1. Prepare immunized mice splenocytes or peptide stimulated cells (1 x 10<sup>6</sup> cells) hemolyzed with ACK lysis buffer, and wash in FCM buffer (2% FCS/0.05% NaN<sub>3</sub>/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 µL of Clear Back (MBL, PN MTG-001) and 30 µL of FCM buffer. Incubate for 5 minutes at room temperature.
4. Add 10 µL of H-2D<sup>b</sup> WT1<sub>126-134</sub> Tetramer-PE (MBL, PN TS-M504-1) or H-2D<sup>b</sup> Influenza NP Tetramer-PE as a negative control tetramer (MBL, PN TS-M502-1) to each test tube and mix well. Incubate the cells for 20 minutes at 4°C.
5. Add 10 µL of mouse CD8-FITC (clone KT15, MBL, PN D271-4) 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 µL of FCM buffer.
8. Add 5 µL of 7-AAD (MBL, PN A07704) for the exclusion of nonviable cells in flow cytometric assays.
9. Analyze prepared samples by flow cytometry.

### 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).

The frequency of MHC Tetramer<sup>+</sup> and CD8<sup>+</sup> T cells is

shown as a percentage of total CD8<sup>+</sup> T cells.

The H-2D<sup>b</sup> WT1<sub>126-134</sub> Tetramer-positive CD8<sup>+</sup> T cells could be detected in peptide stimulated splenocytes. Tetramer-positive CD8<sup>+</sup> T cells were not detected in the negative control (H-2D<sup>b</sup> Influenza NP Tetramer-PE).

