

**T-Select MHC Tetramer**

# HLA-A\*24:02 modified WT1 Tetramer -CYTWNQMNL (50 tests)

For Research Use Only. Not for use in diagnostic procedures.  
These T-Select MHC Tetramers use patented technology (Japanese patent No. P3728439).

## 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 human class I HLA-A\*24:02 and the modified 9-mer WT1 peptide, and it can detect HLA-A\*24:02-restricted WT1-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 HLA-A\*24:02-restricted modified 9-mer WT1 peptide (CYTWNQMNL) was initially identified by Dr. Haruo Sugiyama and his colleagues at Osaka University Medical School. A single amino acid substitution is introduced into the first anchor motif at position 2 of the natural WT1 peptide (CMTWNQMNL). The modified 9-mer WT1 peptide was shown to induce much stronger CTL activity than the natural peptide against WT1-expressing tumor cells, and is

used for clinical studies of WT1 peptide-based cancer immunotherapy for HLA-A\*24:02-positive patients.

A Tetramer, which is constructed with the same allele (HLA-A\*24:02) of interest and an irrelevant peptide, may be used as a negative control Tetramer.

**HLA Restriction:** HLA-A\*24:02

**Origin and Sequence of CTL Epitope**  
modified WT1 (235-243 aa, CYTWNQMNL)

## Reagents

500 µL liquid - 10 µL/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-M014-1

Streptavidin-Phycoerythrin (SA-PE)

Excites at 486-580 nm

Emits at 586-590 nm

TS-M014-2

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.

## 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.

## References for Products

- 1) Ohminami H, *et al. Blood* **95**: 286-293 (2000)
- 2) Oka Y, *et al. J Immunol* **164**: 1873-1880 (2000)
- 3) Tsuboi A, *et al. Cancer Immunol Immun* **51**: 614-620 (2002)
- 4) Oka Y, *et al. J Immunol* **164**: 1873-1880 (2000)
- 5) Oka Y, *et al. Int J Hematol* **78**: 56-61 (2003)
- 6) Azuma T, *et al. Clin Cancer Res* **10**: 7402-7412 (2004)
- 7) Oka Y, *et al. Curr Med Chem* **13**: 2345-2352 (2006)
- 8) Tsuboi A, *et al. Int J Hematol* **86**: 414-417 (2007)
- 9) Chiba Y, *et al. Jpn J Clin Oncol* **40**: 395-403 (2010)
- 10) Narita M, *et al. Int J Med Sci* **7**: 72-81 (2010)
- 11) Saitoh A, *et al. Med Oncol* **28**: 219-230 (2011)
- 12) Tamanaka T, *et al. Anticancer Res* **32**: 5201-5209 (2012)
- 13) Watanabe K, *et al. Biomedical Res* **34**: 41-50 (2013)
- 14) Kobayashi M, *et al. J Ovarian Res* **7**: 48 (2014)
- 15) Koido S, *et al. Clin Cancer Res* **20**: 4228-4239 (2014)

## High Specificity

The T cell surface CD8 enhances T cell antigen recognition by binding to HLA class I molecules. Therefore, MBL produced T-Select MHC class I human Tetramers with one point mutation at the HLA  $\alpha 3$  domain known to alter the interaction with CD8. These mutated Tetramers showed a greatly diminished nonspecific binding but retained specific binding. Alterations of CD8 binding by mutation of the MHC greatly improved the specificity of MHC-peptide multimers, thus providing efficient tools to sort specific human T cells for immunotherapy.

(French application Number; FR9911133)

## 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-CD8-FITC (T8), Beckman Coulter, Inc., PN 6603861
- Anti-CD8-PC5 (T8), Beckman Coulter, Inc., PN 6607011
- 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 blood by venipuncture into a blood collection tube containing an appropriate anti-coagulant.
2. Add 10  $\mu$ L of T-Select MHC Tetramer to each 12 x 75 mm test tube.
3. Add 200  $\mu$ 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 Peripheral Blood Mononuclear Cells

1. Prepare peripheral blood mononuclear cells (PBMC) according to established procedures. 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 PBMC 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.

9. Add 3 mL of PBS or FCM buffer (2% FCS/0.09% Na<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% formaldehyde.
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 PBMC culture 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 anti-CD8 antibody, clone SFC121Thy2D3 (T8, Beckman Coulter, Inc.), 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.

#### Related Products

##### WT1 Tetramers and Peptide

TS-M140-1	HLA-A*02:01 WT1 <sub>37-45</sub> Tetramer-VLDFAPPGA-PE
TS-M140-P	HLA-A*02:01 WT1 <sub>37-45</sub> peptide
TS-M016-1	HLA-A*02:01 WT1 <sub>126-134</sub> Tetramer-RMFPNAPYL-PE
TS-M014-1	HLA-A*24:02 modified WT1 Tetramer-CYTWNQMNL-PE
TS-M504-1	H-2D <sup>b</sup> WT1 <sub>126-134</sub> Tetramer-RMFPNAPYL-PE

##### Human Tetramers

###### Cancer

TS-M141-1	HLA-A*24:02 ACC-1 Tetramer-DYLQYVLIQI-PE
TS-M137-1	HLA-A*01:01 AIM-2 Tetramer-RSDSGQQARY-PE
TS-M112-1	HLA-A*24:02 CA9 <sub>219-227</sub> Tetramer-EYRALQLHL-PE
TS-M103-1	HLA-A*02:01 CEA Tetramer-YLSGANLNL-PE
TS-M080-1	HLA-A*02:01 CEA (N6D) Tetramer-YLSGADLNL-PE
TS-M101-1	HLA-A*02:01 CD33 Tetramer-AIISGDSPV-PE
TS-M102-1	HLA-A*02:01 CD33 A65Y Tetramer-YIISGDSPV-PE
TS-M084-1	HLA-A*02:01 EphA2 Tetramer-TLADFDPRV-PE
TS-0014-1C	HLA-A*02:01 gp100 (wild) Tetramer-ITDQVPFVSV-PE
TS-0013-1C	HLA-A*02:01 gp100 (mutant) Tetramer-ITDQVPFVSV-PE
TS-0035-1C	HLA-A*02:01 gp100 <sub>154-162</sub> Tetramer-KTWGQYVQV-PE
TS-M082-1	HLA-A*02:01 gp100 Tetramer-YLEPGPVTA-PE
TS-M089-1	HLA-A*24:02 gp100-intron 4 Tetramer-VYFFLPDHL-PE
TS-0016-1	HLA-A*02:01 Her-2/neu Tetramer-RLLQETELV-PE
TS-0015-1C	HLA-A*02:01 Her-2/neu E75 Tetramer-KIFGSLAFL-PE
TS-M083-1	HLA-A*02:01 HM1.24 Tetramer-KLQDASAEV-PE
TS-M010-1	HLA-A*24:02 hTERT Tetramer-VYGFVFRACL-PE
TS-M115-1	HLA-A*02:01 hTERT Tetramer-ILAKFLHWL-PE
TS-M086-1	HLA-A*02:01 IDO Tetramer-ALLEIASCL-PE
TS-M070-1	HLA-A*02:01 MAGE-A1 Tetramer-KVLEYVIKV-PE
TS-M071-1	HLA-B*07:02 MAGE-A1 Tetramer-RVRFVPSL-PE
TS-M072-1	HLA-A*02:01 MAGE-A2 Tetramer-YLQLVFGIEV-PE
TS-M073-1	HLA-A*24:02 MAGE-A2 Tetramer-EYLQLVFGI-PE
TS-M075-1	HLA-A*02:01 MAGE-A3 <sub>112-120</sub> Tetramer-KVAELVHFL-PE
TS-M076-1	HLA-A*02:01 MAGE-A3 <sub>271-279</sub> Tetramer-FLWGPRLV-PE
TS-M077-1	HLA-A*24:02 MAGE-A3 Tetramer-IMPKAGLLI-PE
TS-M078-1	HLA-A*02:01 MAGE-A10 Tetramer-GLYDGMHL-PE
TS-M138-1	HLA-A*02:01 MAGE-C1 Tetramer-ILFGISLREV-PE
TS-0009-1C	HLA-A*02:01 Mart-1 Tetramer-ELAGIGILTV-PE
TS-M091-1	HLA-A*24:02 MCPyV large T Ag Tetramer-EWWRSGGFSF-PE
TS-M088-1	HLA-A*02:01 MUC1 Tetramer-LLLLTVLTV-PE
TS-M011-1	HLA-A*02:01 NY-ESO-1 Tetramer-SLLMVTQC-PE
TS-M105-1	HLA-A*02:01 NY-ESO-1 C9V Tetramer-SLLMVTQV-PE
TS-M109-1	HLA-B*07:02 P2X5 Tetramer-TPNQRQNV-PE
TS-M081-1	HLA-A*02:01 p53 Tetramer-LLGRNSFV-PE
TS-M107-1	HLA-A*02:01 PAP <sub>299-307</sub> Tetramer-ALDVYNGLL-PE
TS-M136-1	HLA-A*24:02 PBF A24.2 Tetramer-AYRPVSRNI-PE
TS-M117-1	HLA-A*02:01 PRAME <sub>100-108</sub> Tetramer-VLDGLDVL-PE
TS-M119-1	HLA-A*02:01 PRAME <sub>142-151</sub> Tetramer-SLYSFPEPEA-PE
TS-M116-1	HLA-A*02:01 PRAME <sub>300-309</sub> Tetramer-ALYVDSLFFL-PE
TS-M118-1	HLA-A*02:01 PRAME <sub>425-433</sub> Tetramer-SLLQHLIGL-PE
TS-M120-1	HLA-A*02:01 PSA <sub>141-150</sub> Tetramer-FLTPKKLQCV-PE
TS-0017-1	HLA-A*02:01 PR-1 Tetramer-VLQELNVTV-PE
TS-M087-1	HLA-A*02:01 PSA Tetramer-KLQCVDLHV-PE
TS-M104-1	HLA-A*02:01 RHAMM Tetramer-ILSLELMKL-PE
TS-M095-1	HLA-A*02:01 PP2A Tetramer-SLLPAIVEL-PE
TS-M079-1	HLA-A*02:01 SSX-2 Tetramer-KASEKIFYV-PE
TS-M025-1	HLA-A*24:02 survivin-2B Tetramer-AYACNTSTL-PE
TS-M085-1	HLA-A*02:01 Survivin (T2M) Tetramer-LMLGEFLKL-PE
TS-0019-1C	HLA-A*02:01 Tyrosinase Tetramer-YMDGTMSQV-PE
TS-M090-1	HLA-A*24:02 Tyrosinase Tetramer-AFLPWHRLF-PE

###### Kits

4844	IMMUNOCYTO CD107a Detection Kit
8223	IMMUNOCYTO IFN-γ ELISPOT Kit
AM-1005	IMMUNOCYTO Cytotoxicity Detection Kit

Please check our web site (<http://ruo.mbl.co.jp>) for up-to-date information on products and custom MHC Tetramers.

### Experimental Data

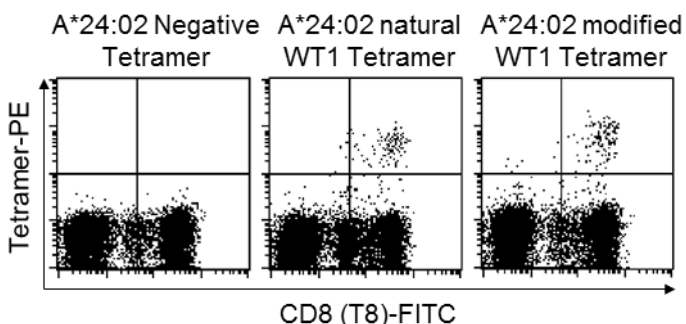
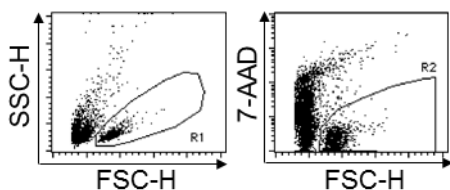
PBMCs isolated from an HLA-A24-positive healthy donor were subjected to mixed lymphocyte peptide culture under limiting dilution conditions (LD/MLPC). PBMCs were suspended in RPMI1640 supplemented with 5% (v/v) autologous plasma and mixed with modified WT1 peptide (10 µg/mL of CYTWNQMNL, MBL, PN TS-M014-P). Peptide-pulsed PBMC were seeded into round-bottom 96-well plates at the concentration of  $2-3 \times 10^5$  cells/100 µL/well. After 48 h, an equal volume of medium containing 100 U/ml interleukin-2 (IL-2) was added to each well, and every 2 to 3 days thereafter half of the medium was replaced with fresh medium containing IL-2 (50 U/ml).

On day 10 to 14, these cultured cells were stained with specific Tetramer and CD8 antibody as follows: First, an aliquot of cells from each of 8 vertical wells of a 96-well plate were collected to make 12 pooled samples. These samples were stained separately with MHC Tetramer. Second, to identify the MHC-Tetramer positive well within positive pools, cells in the individual wells within positive pools were stained with MHC Tetramer.

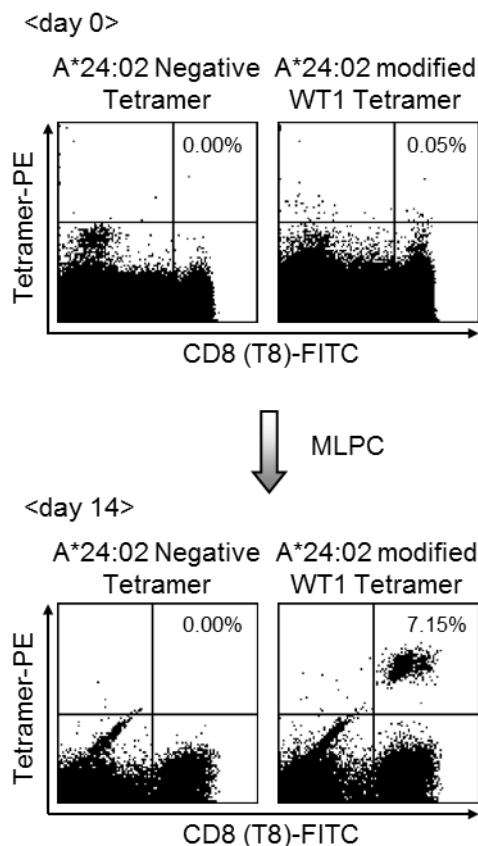
The lymphocyte population was defined by an FSC/SSC gate (R1), and the viable cell population was defined by an FSC/7-AAD (R2). Data were analyzed by double gating on the lymphocyte and viable cell population (R1 and R2).

Numbers in the top right quadrants represent the percentage of MHC Tetramer-positive cells in the total CD8<sup>+</sup> cells.

### Results 1: Staining comparison of modified and natural WT1 Tetramers



### Results 2: Tetramer staining of peptide-stimulated PBMCs for 14 days



Flow cytometric enumeration of MHC Tetramer-positive CD8<sup>+</sup> T lymphocytes in healthy individuals reveals that cancer antigen-specific CTLs were very rare compared the frequency of virus-specific CTLs. However, HLA-A\*24:02-modified WT1-specific CTLs were detectable in one or two wells of the 96-well plate in the peptide stimulated PBMCs of healthy donors.