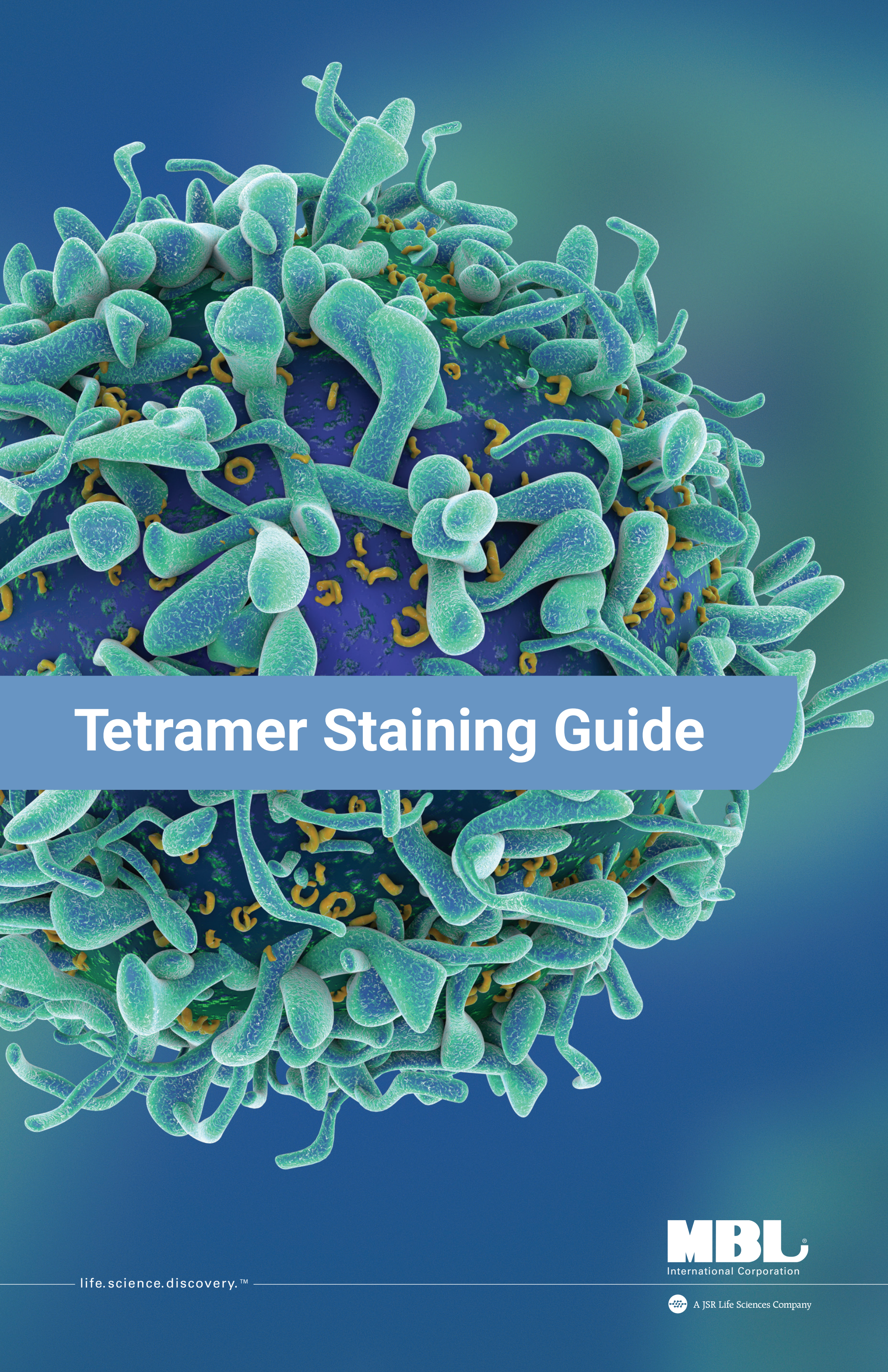

Tetramer Staining Guide

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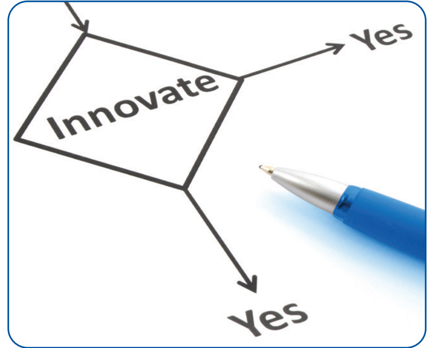

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Collaboration at every touch-point and uncompromising support to ensure you get the product and assistance when you need it

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Our products are used widely in academic research and medical institutions, pharmaceutical and biotechnology companies, government agencies as well as hospital and reference laboratories. We are focused on developing high quality products and have high standards for quality control in our manufacturing process. Our products include MHC monomers and multimers, antibodies, recombinant proteins, fluorescent proteins, and ELISA kits. Our products are highly cited and support numerous research fields relating to oncology, allergy, apoptosis, autophagy, epigenetics and neuroscience.

MBLI is known for its line of immune monitoring reagents, including MHC Tetramers and Monomers, as well as our line of QuickSwitch™ products for screening peptides and neoantigens. We help researchers to acquire reliable and reproducible immune monitoring results for the areas of immuno-oncology, autoimmune, vaccine development and more.

MBLI is committed to increasing the efficiency and effectiveness of its customers' science by delivering quality products with uncompromising support through our scientific sales, customer service, and technical support teams. Our goal is to ensure that our customers get the products and assistance when they need it.

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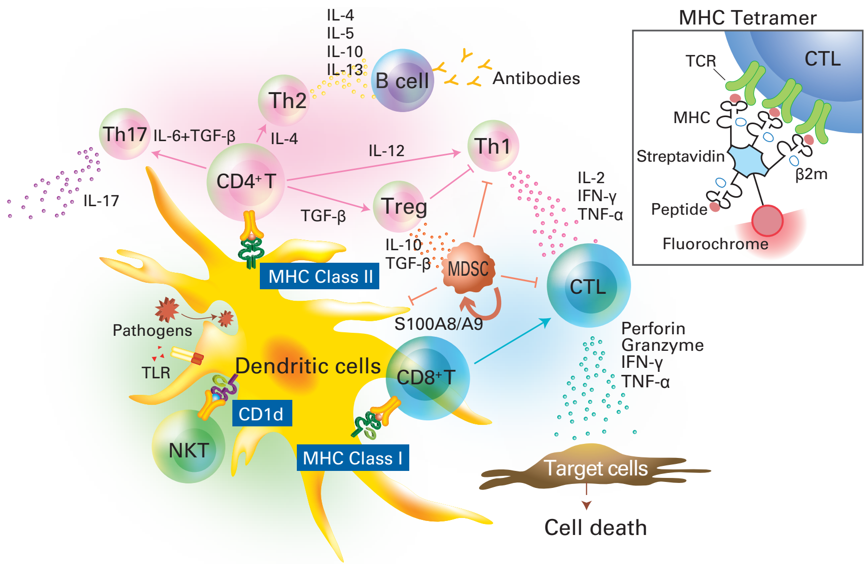
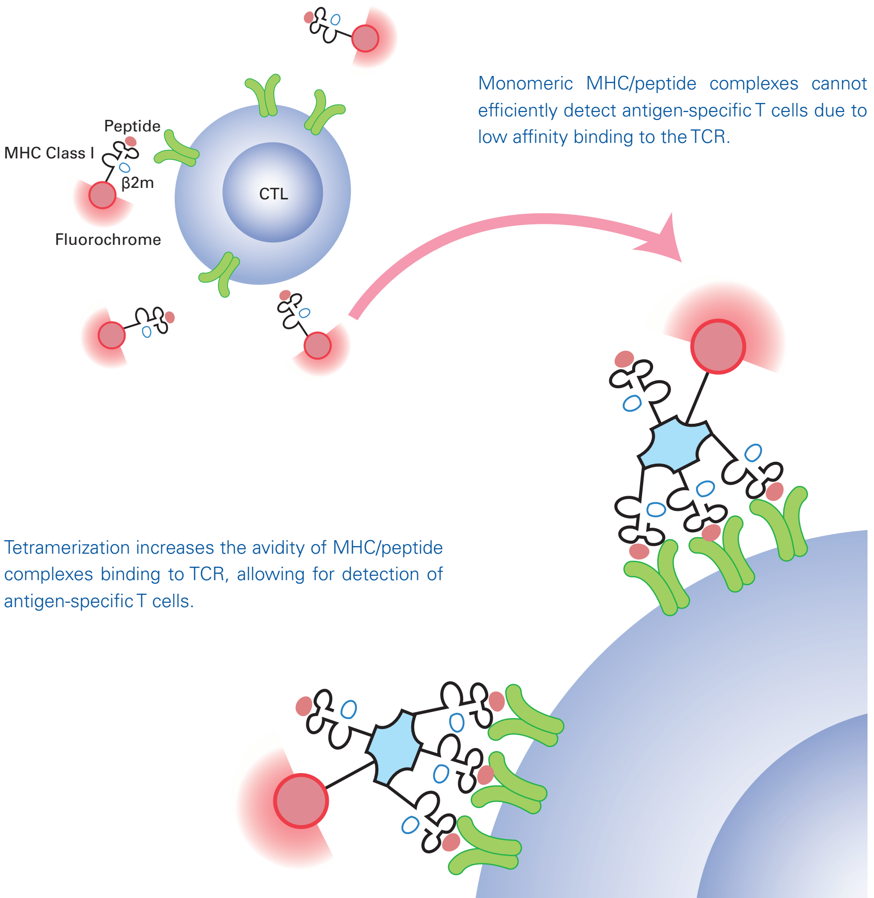
Reagents for antigen-specific T cell detection



T cell receptors (TCRs) expressed on T cells specifically recognize and bind to complexes of major histocompatibility complex (MHC) molecules and peptide fragments. CD8-positive T cells, also called cytotoxic T lymphocytes (CTL), recognize complexes of MHC class I molecules and peptide. CTL directly kill target cells including virus-infected and cancer cells. CD4-positive T cells recognize complexes of MHC class II molecules and peptide fragments. CD4-positive T cells are mainly helper T (Th) cells and are classified into Th1 and Th2 cells. Th1 cells affect CTL and macrophages, which are involved in cell-mediated immunity. Th2 cells act on dendritic cells and B cells to activate humoral immunity. In 1996, Altman et al., successfully detected antigen-specific T cells at a single cell level by flow cytometry using MHC Tetramer reagents (Science 1996, 274: 94). MHC Tetramers enable direct detection of antigen-specific T cells, which previously had been analyzed indirectly based on cytokine production or cytotoxic activity. MHC Tetramer reagents enable detailed analysis of function and phenotype of antigen-specific T cells. Since their discovery, MHC Tetramers have become accepted as essential tools for measuring T cell responses in basic research, clinical development, and immune monitoring in the fields of infectious disease, cancer vaccine therapy, cellular immunotherapy, transplantation tolerance, and autoimmune disease.

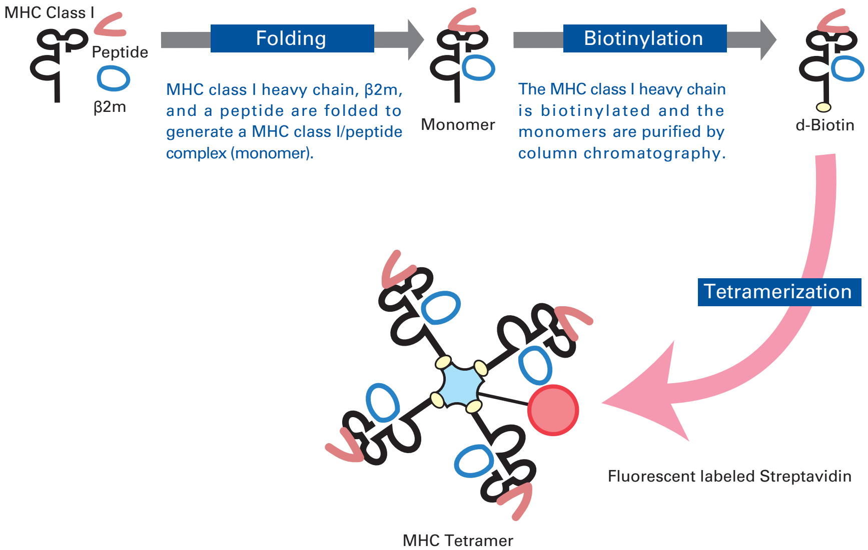
Detection of antigen-specific T cells by MHC Tetramers



TCRs recognize and bind to complexes composed of MHC molecules and specific peptides expressed on the surface of antigen-presenting cells. While it was believed that antigen-specific T cells could be detected using soluble MHC/peptide complexes, monomeric MHC/peptide complexes proved to be impractical due to their instability and low affinity to the TCR. To overcome this difficulty, MHC/peptide monomers are biotinylated and tetramerized with streptavidin to maintain stable binding to multiple TCR, enabling MHC/peptide Tetramers to be used as detection tools. MHC Tetramers are labeled with fluorescent molecules including phycoerythin (PE), allophycocyanin (APC) or Brilliant Violet™ 421 (BV421) and thus allow detection of antigen-specific T cells by flow cytometry or fluorescence microscopy.

Preparation of MHC class I Tetramer reagents

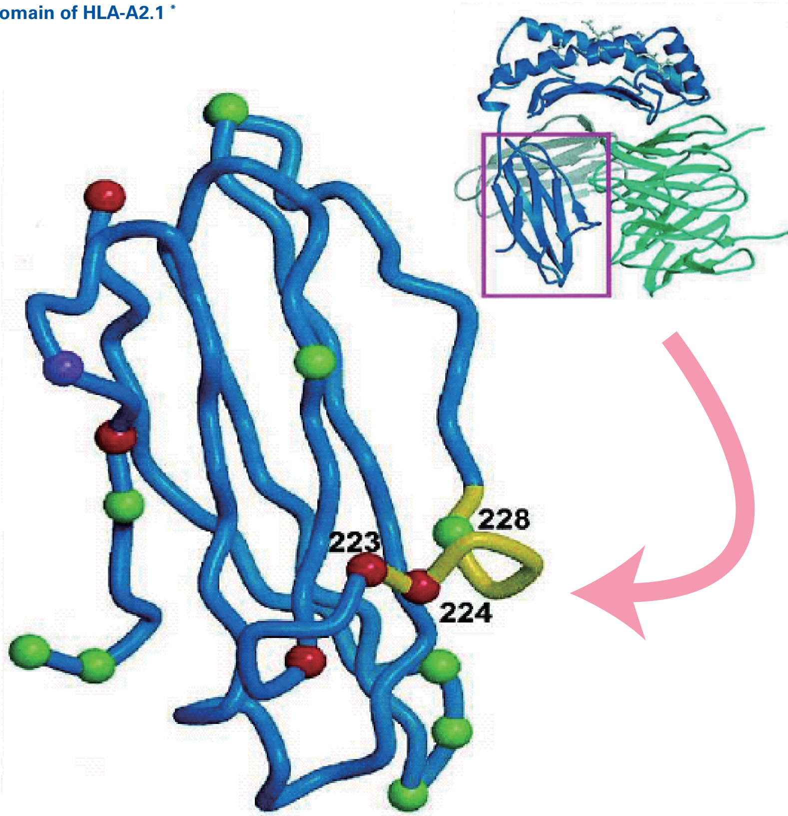
MHC class I Tetramer reagents are generated primarily using the method developed by Altman et al., who demonstrated that Tetramers of MHC class I/ peptide complexes could be used as probes for detection and quantitation of antigen-specific CTL (Science 1996, 274: 94–96).



MHC class I heavy chain, β2m, and peptide are folded to generate a MHC class I/ peptide complex (monomer), a process that is monitored daily for several days, depending on the particular peptide/MHC combination. The lysine residue in the C-terminus of MHC class I heavy chain is biotinylated using the BirA enzyme, and the monomers are purified by column purification. *E. coli*-expressed recombinant MHC class I heavy chain and β2-microglobulin (β2m) are folded in the presence of a peptide antigen to generate a soluble monomeric MHC class I/peptide complex (monomer). The monomer is then biotinylated by the biotin ligase enzyme BirA enzyme at a lysine residue present in the biotinylation sequence that has been added to the C-terminus of MHC class I heavy chain of the complex. The biotinylated monomer is purified by column chromatography. The purified biotinylated monomers are tetramerized by mixing with a fluorescently-labeled streptavidin to make MHC Tetramer reagents.

Greater specificity: The patented α3 mutation

α3 domain of HLA-A2.1 *



The human leukocyte antigen (HLA) system is the name of the major histocompatibility complex in humans. CD8 molecules are known to assist binding of HLA to CTL *in vivo* and thus HLA molecules have binding sites for CD8 molecules. Bodinier et al. reported that introducing a mutation (A245V) in the HLA class I heavy chain α3 domain minimized unwanted binding to CD8 molecules and dramatically improved specificity (Nat. Med. 2000, 6: 707). **MBL International has incorporated the patented mutation of the α3 domain in their HLA class I Tetramers*.**

*Only human and macaque HLA Class I Tetramers contain the alpha 3 mutation.

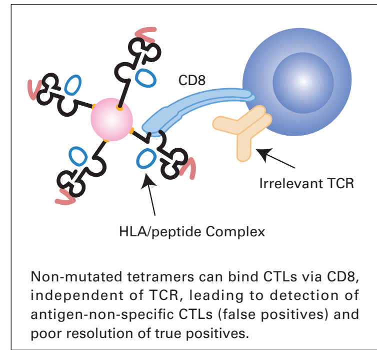
French Application Number; FR9911133
*1) Gao GF, et al., Nature 387: 630–634 (1997)

HLA-A*02:01 CMV pp65 Tetramer

Peripheral blood of HLA-A*02:01 donors positive or negative for CMV was stained with HLA-A*02:01 CMV pp65 Tetramer reagent. A dramatic improvement in specificity was observed in samples stained with the MHC Tetramer containing the α3 mutation (Figure 1b) compared with those stained with the conventional Tetramer lacking the mutation (Figure 1a).

Figure 1a

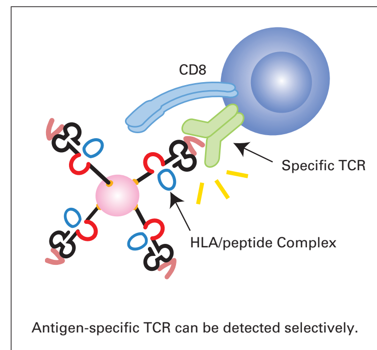
Wild type MHC/peptide complex



Non-mutated tetramers can bind CTLs via CD8, independent of TCR, leading to detection of antigen-non-specific CTLs (false positives) and poor resolution of true positives.

Figure 1b

iTAg HLA class I Tetramer



Antigen-specific TCR can be detected selectively.

HLA-A*24:02 EBV BRLF1 Tetramer

PBMC were separated from peripheral blood of HLA-A*24:02 positive healthy subjects (Figure 2). EBV BRLF1-specific CTL were induced by the MLPC method (see page 14) and stained with PE-labeled HLA-A*24:02 Negative (MBLI code no. TB-M007-1), or EBV BRLF1 Tetramers with (MBLI code no. TS-M002-1) or without the α3 mutation. Non-specific staining of CD8 positive cells was observed in the sample stained with the conventional, but not the α3-mutated, Tetramer.

Differences in Tetramer Quality Between Suppliers

MBL International tetramers have a clear advantage over academic tetramers and other commercial MHC multimer products, not only due to the reliability and high quality for every lot produced, but also due to the proprietary alpha-3 mutation. This mutation, engineered into the heavy chain of all of our human and macaque

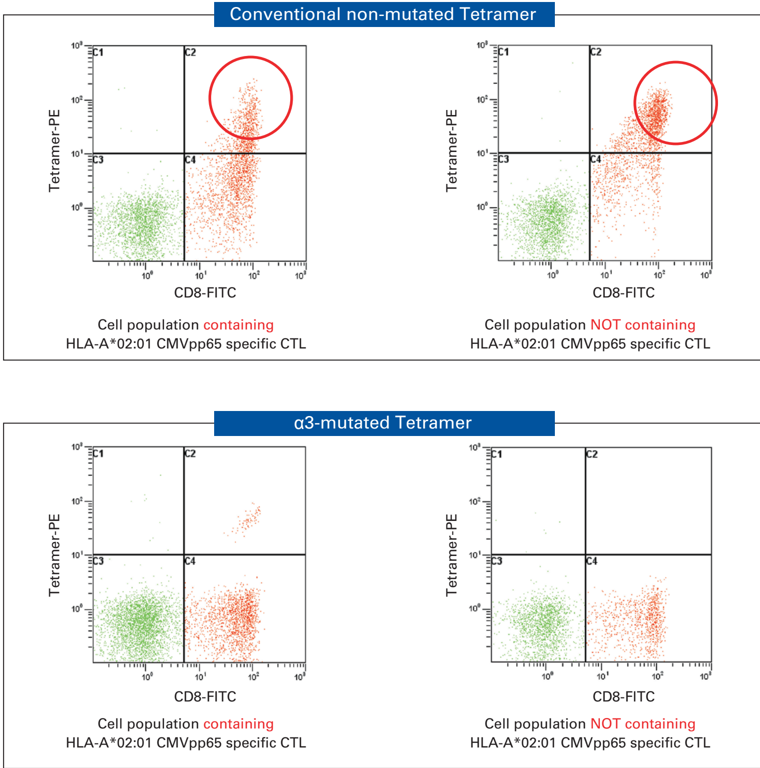
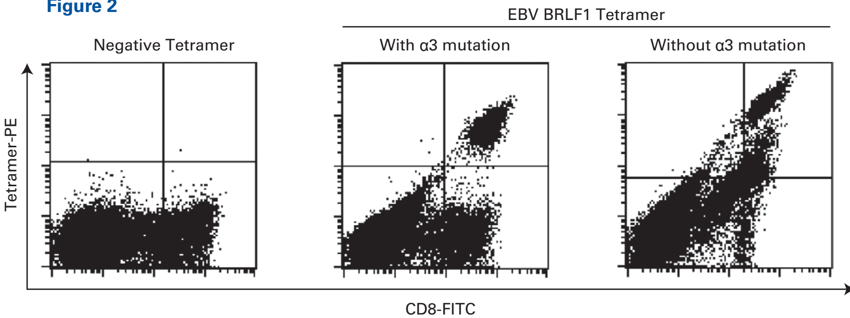


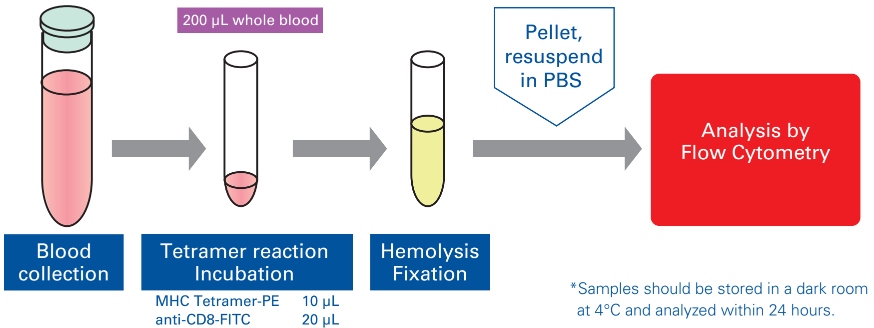
Figure 2



This data was analyzed within the lymphocyte gate and 7-AAD negative gate.

class I tetramers, helps decrease non-specific binding and leads to enhanced specificity. MBL International tetramers also save time and reduce errors due to the simple one-step staining procedure for Class I tetramers, where antibodies and tetramers are stained simultaneously for 30 minutes at room temperature. Other multimers require a two-step sequential staining protocol.

MHC Tetramer staining method



■ Procedure for whole blood samples

1) Collect blood by venipuncture into a blood collection tube containing an appropriate anti-coagulant.

2) To each 12x75 mm test tube add 10 µL of MHC Tetramer and any additional antibodies (e.g. anti-CD8).

3) Add 200 µL of whole blood into each test tube.

4) Vortex gently.

5) Incubate for 30 minutes at room temperature protected from light.

6) Lyse red blood cells using 2 mL of Lyse Reagent* supplemented with 50 µL Fixative Reagent** per tube.

7) Vortex for 5 seconds immediately after the addition of the Lyse/Fixative solution.

8) Incubate for a minimum of 10 minutes at room temperature protected from light.

9) Centrifuge tubes at 150 x g for 5 minutes.

10) Aspirate or decant the supernatant.

11) Add 3 mL of PBS.

12) Centrifuge tubes at 150 x g for 5 minutes.

13) Aspirate or decant the supernatant.

14) Resuspend the pellet in 500 µL of PBS with 0.1% formaldehyde. (12.5 µL Fixative Reagent**/1 mL PBS).

15) Store prepared samples at 4°C protected from light for a minimum of 1 hour (maximum 24 hours) prior to analysis by flow cytometry.

If hemolysis is incomplete, a non-specific staining pattern caused by diffuse reflection from erythrocytes may be observed. Inclusion of a CD45 antibody can help identify the true lymphocyte population.

Procedure for Peripheral Blood Mononuclear Cells (PBMC) samples

1) Prepare peripheral blood mononuclear cells (PBMC) according to established procedures. Cells should be resuspended at a concentration of 5×10^6 cells/mL. 200 μL of sample is recommended for each Tetramer determination.
2) To each 12x75 mm test tube add 10 μL of MHC Tetramer and any additional antibodies (e.g. anti-CD8).
3) Add 200 μL PBMC into each test tube (e.g. 1×10^6 cells per tube).
4) Vortex gently.
5) Incubate for 30 minutes at room temperature protected from light.
6) Add 3 mL of PBS.
7) Centrifuge tubes at 400 x g for 5 minutes.
8) Aspirate or decant the supernatant.
9) Resuspend the pellet in 500 μL of PBS with 0.5% formaldehyde. (62.5 μL Fixative Reagent**/1 mL PBS).
10) Store prepared samples at 4°C protected from light for a minimum of 1 hour (maximum 24 hours) prior to analysis by flow cytometry.

Procedure for Mouse spleen samples

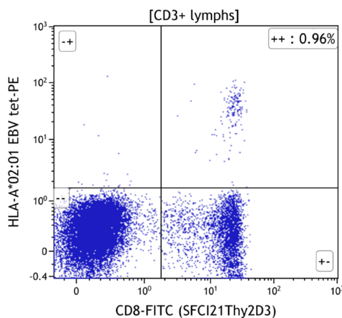
1) Collect lymph node, spleen or thymus and prepare a single-cell suspension according to an established protocol. Cells should be resuspended at a concentration of 5×10^6 cells/mL. 200 μL of sample is recommended for each Tetramer determination.
2) To each 12x75 mm test tube add 10 μL of MHC Tetramer and any additional antibodies (e.g. anti-CD8).
3) Add 200 μL cell suspension into each test tube (e.g. 1×10^6 cells per tube).
4) Vortex gently.
5) Incubate for 30 minutes at room temperature protected from light. If red blood cell lysis is necessary, proceed to step 6-15 in the PROCEDURE FOR WHOLE BLOOD section. If red blood cell lysis is not necessary, continue to step 6 below.
6) Add 3 mL of PBS.
7) Centrifuge tubes at 400 x g for 5 minutes.
8) Aspirate or decant the supernatant.
9) Resuspend the pellet in 500 μL of PBS with 0.5% paraformaldehyde or formalin.
10) Store at 4°C protected from light for a minimum of 1 hour (maximum 24 hours) prior to analysis by flow cytometry.

*VersaLyse™, Beckman Coulter A09777 **IO Test®3 10X Concentrate, Beckman Coulter A07800

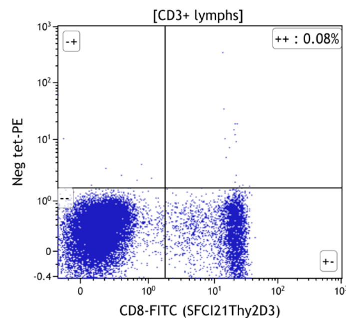
Negative Controls in Tetramer Assays

Controls help set up an experiment to get a clear answer for the experimental hypothesis and can also be used for troubleshooting when data does not look appropriate. In flow cytometry, controls are critical to help determine "real" events from artifacts.

Test



Negative Control



Negative controls are important in helping set the region or gate around antigen non-specific T cells to find the positive (i.e. antigen-specific) T cells. For example, in a dot plot displaying CD8 (x-axis) and class I tetramer (y-axis), a quadrant gate would be drawn based on the sample stained with a negative tetramer. The upper right quadrant (CD8+ Tetramer) is where the antigen-specific T cells events will appear when stained with the same panel containing the antigen-specific tetramer. An appropriate negative control for a particular tetramer will have five basic elements:

- Same fluorochrome
- Same MHC allele
- Peptide known to have no reactivity in the sample or in the system studied
- In the context of the exact flow antibody panel, with the identical staining conditions and acquisition parameters/settings
- A negative control (for human studies) that investigators have used is also HCV specific tetramers. Reference (Marc Davis' group): Evan W. Newell, Lawrence O. Klein, Wong Yu, and Mark M. Davis. Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. Nat Methods. 2009 Jul; 6(7): 497–499.

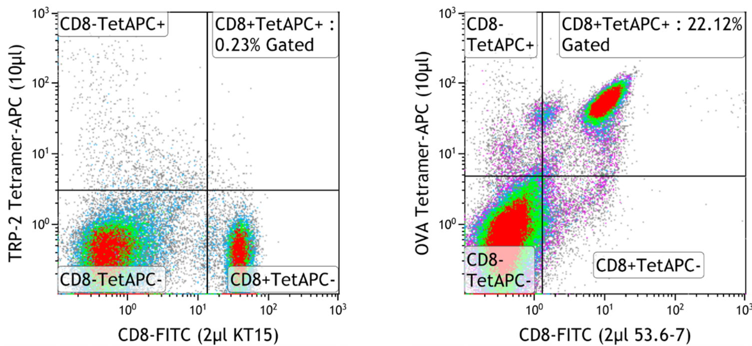
Often stocked tetramer products or customs with specificities unrelated to the experiment may be appropriate, but ultimately, it is up to the investigator to select the control most appropriate for his or her particular model or experiment.

Option for negative controls for human tetramer experiments:

For HLA-A*02:01 alleles, MBL International offers a "Negative Tetramer" in both PE (TB-0029-1) and APC (TB-0029-2). This tetramer is prepared with the HLA-A*02:01 allele and a proprietary peptide whose sequence does not occur in nature. For Class II alleles, tetramers made using the human CLIP peptide or a non-specific peptide can serve as negative controls

Options for negative controls for mouse tetramer experiments:

In mouse tetramer experiments, there are generally two options for selecting a negative control that will allow proper region/gate placement:



1. A tetramer of the same allele and fluorochrome as the specific tetramer, built with a non-specific peptide or a specificity known to be unreactive in the mouse model of interest may be appropriate. For example, H-2Kb tetramers specific for either β-gal or TRP2 have been used as negative controls in experiments using splenocytes from OT-I mice transgenic for the T cell receptor that binds specifically to H-2Kb OVA (SIINFEKL). For proper gate placement, the full panel of antibodies used for the experimental tetramer stain must be included in the control tetramer stain to account for fluorescent spillover contributed by fluorochromes from other channels of the flow cytometer.

2. In experiments involving immunized or treated mice, often cells from a naïve mouse, stained with the same tetramer/antibody panel as the experimental mouse cells, is an appropriate negative control.

Positive Control: Ways to Confirm your MHC Tetramer is Binding

A positive control for a tetramer is a sample that contains cells expressing the specific T cell receptor of interest, i.e. has the exact specificity of the tetramer. However, in many cases, you will not have access to a positive control, unless one of your experimental samples happens to show a positive result. In an experiment where no positive events are seen and no positive control was used, you cannot necessarily conclude that the donor/patient/mouse is negative for that T cell specificity. A tetramer experiment with no positive control and no positives in the experimentals is, therefore, uninterpretable.

Possible ways to obtain or create a positive control are listed below:

1. Positive control cells for certain specificities are available. A notable example is the OT-I mouse. Splenocytes, lymph node cells, or blood from this mouse are suitable positive controls for C57BL/6 mice that have been treated or immunized some way to generate a T cell response specific for H-2Kb OVA (SIINFEKL), as the OT-I mouse is transgenic for the T cell receptor that binds specifically to H-2KbOVA (SIINFEKL).

2. Positive control cells can be generated *in vitro*. The Mixed Lymphocyte-Peptide Culture (MLPC) method uses peptide-stimulation and cytokine culture to induce a specific T cell response.

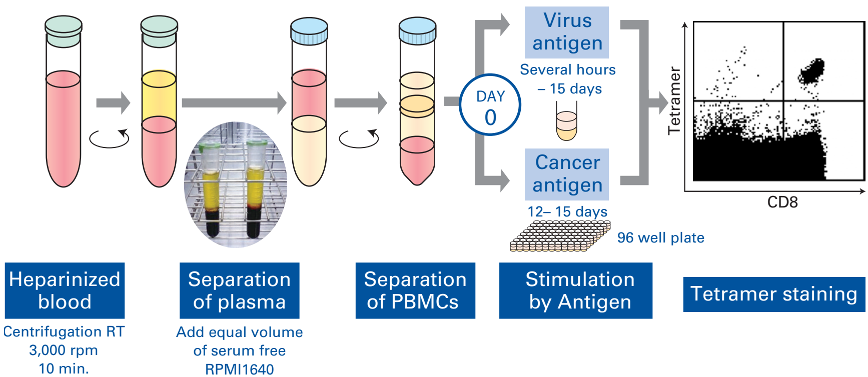
3. Positive control cells can be generated *in vivo*. The literature can be referenced to determine conditions that lead to a tetramer-specific response in a mouse strain and/or vaccination conditions from prior experiments that worked previously.

4. Donors or patients reactive to your tetramer of interest.

5. Some have suggested a tetramer of a different specificity that's positive in a patient population. For example, tetramers to influenza, CMV, or EVB have been recommended as positive controls (in patients known to be reactive) for example, a tetramer study looking at Mart-1-specific T cells. While this is not ideal, as it is not a tetramer positive control, it will serve as an assay positive control to help determine if other parts of the assay worked (wash steps, staining buffers, etc.)

Induction of CTL from peripheral blood using the Mixed Lymphocyte-Peptide Culture method

MHC Tetramers are used as detection reagents for cancer or viral antigen-specific CTL. Because of their relative rarity compared with viral antigen-specific CTL, cancer antigen-specific CTL can be difficult to detect immediately following blood collection. Using the Mixed Lymphocyte-Peptide Cultures (MLPC) method, Karanikas et al. found that increased specific CTL in peripheral blood resulted from vaccine therapy in melanoma patients (J. Immunol, 2003, 171:4898). The MLPC method expands antigen-specific CTL via culture of PBMC with a stimulating peptide, thus allowing for the enumeration of low frequency CTL, which are below detection in freshly isolated blood. Using the following modified MLPC protocol, MBL International has successfully induced cancer antigen-specific CTL from peripheral blood of healthy subjects.



1) Collect blood by venipuncture into a blood collection tube containing heparin sodium. Turn the blood collection tubes over repeatedly to mix. If the blood cannot be treated immediately, allow to stand at room temperature.

2) After low speed centrifugation (-400 xg), aliquot and store the separated plasma at -30°C. Do not repeatedly freeze and thaw.

3) Add back an equal volume of wash medium to blood and isolate PBMC using a Ficoll gradient. Wash cells once.

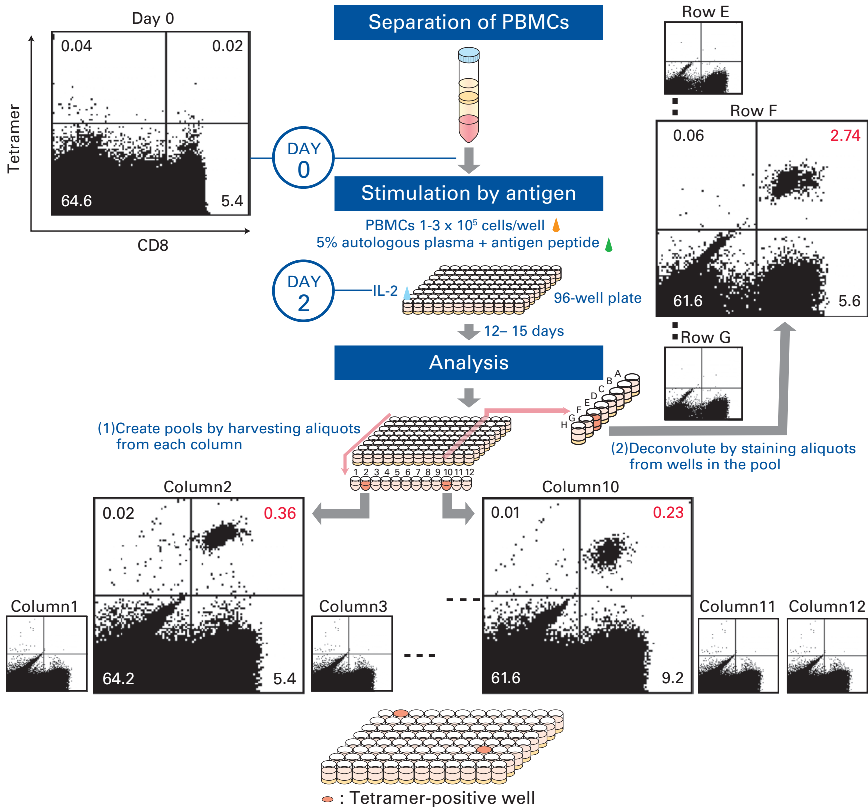
4) Prepare culture medium: 5% autologous plasma/50 U/mL IL-2/50 µM 2-ME/antibiotics/RPMI1640.

5) Resuspend PBMC at 1-3x10^6 cells/ml, add 10 µg/mL of an antigen peptide, and seed 100 µL/well into a 96-well U-bottom plate.

6) After 48 hours, add 100 µL/well IL-2 containing medium. After that, using the color of the medium as a guide, change half of the medium about once in the first week and every 2-3 days in the second week. The optimal concentrations of peptide and IL-2 added may be different for each antigen, depending on solubility of the peptide, epitope prediction score, etc.

Note: Induction of viral antigen-specific CTL can often be achieved by the MLPC method using tubes. In order to induce cancer antigen-specific CTL, the 96-well plate MLPC method is recommended because the frequency of cancer antigen-specific CTL is low.

A) Induction and detection of cancer antigen-specific CTL

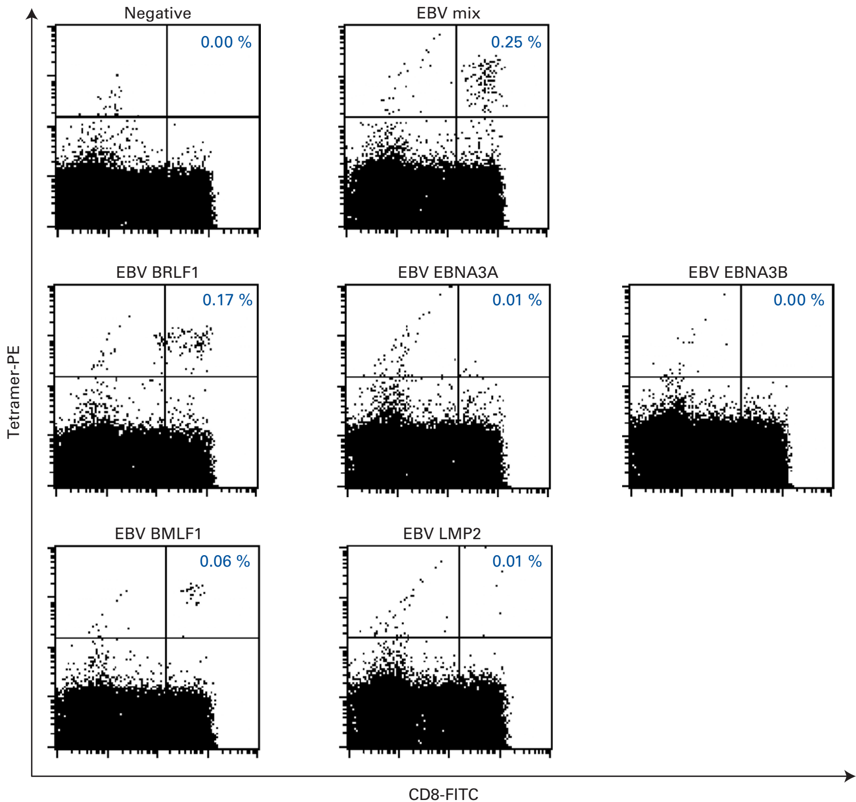


PBMC were separated from peripheral blood of healthy subjects and cancer antigen-specific CTL were induced by the 96-well MLPC method. Fourteen days after peptide stimulation, the cells were stained with Tetramer as follows: First, an aliquot of cells from each of 8 wells in a column of a 96-well plate were collected to make 12 pooled samples. These samples were stained separately with Tetramer. Pools yielding antigen-specific CTL were further deconvoluted by testing for Tetramer binding to cells from individual wells that contributed to the positive pool. Based on the number of positive wells, the frequency of antigen-specific CTL at blood collection was calculated using the following equation.

$$\frac{\text{(Number of Tetramer positive wells)}}{\text{(Cell number/well)} \times \text{(Total well number)} \times \text{(CD8 positive cell rate in day 0)}}$$

EBV

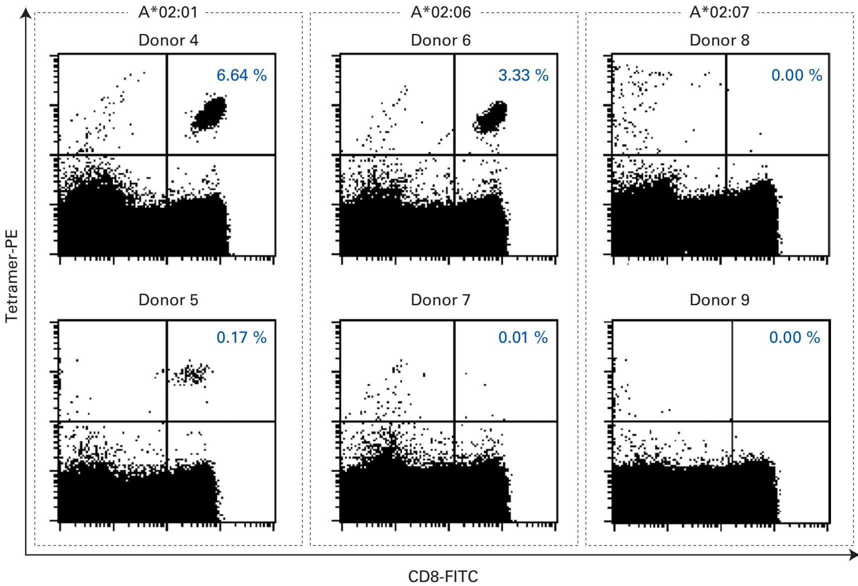
HLA-A*24:02 EBV Tetramer



MBL International offers HLA-A*24:02 Tetramers to five different EBV epitopes. HLA-A*24:02 EBV mix Tetramer (MBLI code no. TS-M009-1, upper middle plot) is a pool of all five Tetramers used to identify multiple types of EBV-specific CTL using a single reagent. Because reactivity of epitopes varies from person to person, the mix Tetramer allows for identification of EBV-specific CTL in HLA-A*24:02 positive individuals. Samples from those testing positive for EBV reactivity can then be selectively stained with the individual Tetramers to determine epitope specificity, if desired. The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.

■ CMV

HLA-A*02:01 CMV pp65 Tetramer



PBMC were separated from peripheral blood of HLA-A2 positive healthy subjects and immediately stained with HLA-A*02:01 CMV pp65 Tetramer (MBLI code no. TB-0010-1). Tetramer positive CTL could be detected in the blood of HLA-A*02:01 and HLA-A*02:06, but not HLA-A*02:07-positive donors. This suggests the CMV peptide is cross-presented by HLA-A*02:01 and HLA-A*02:06 to induce CMV specific CTL and that HLA-A*02:01 CMV pp65 Tetramer can be used to monitor patients of either haplotype. The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.

Figure 1 : HLA-A*24:02 CMV pp65 Tetramer staining immediately after blood collection

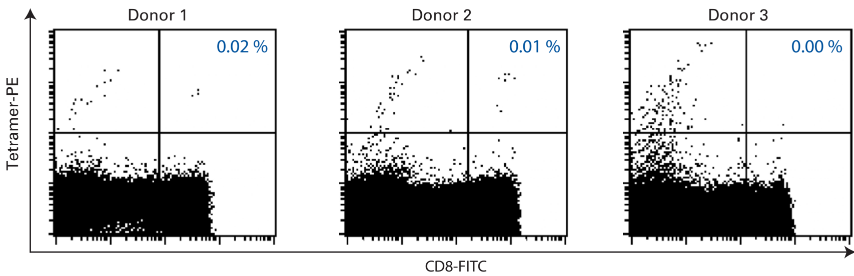
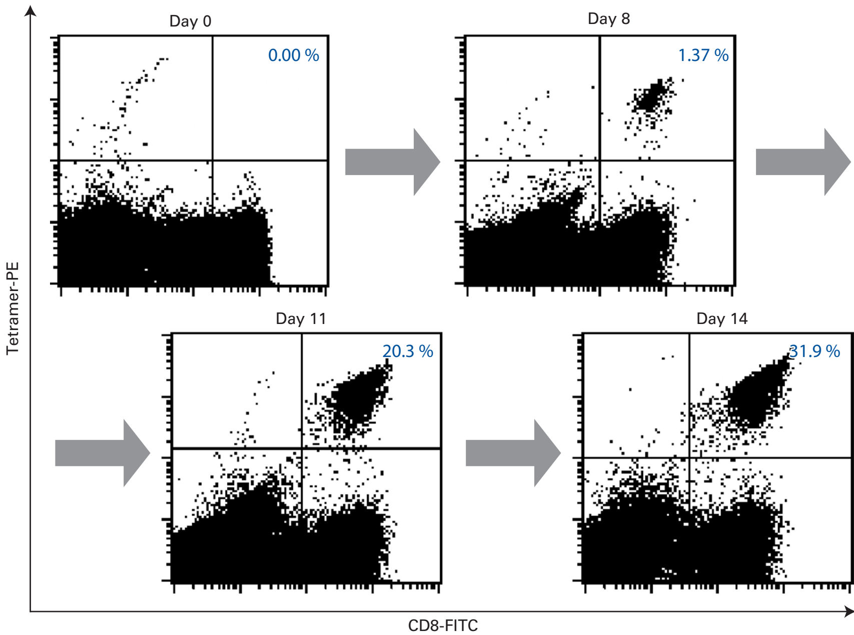


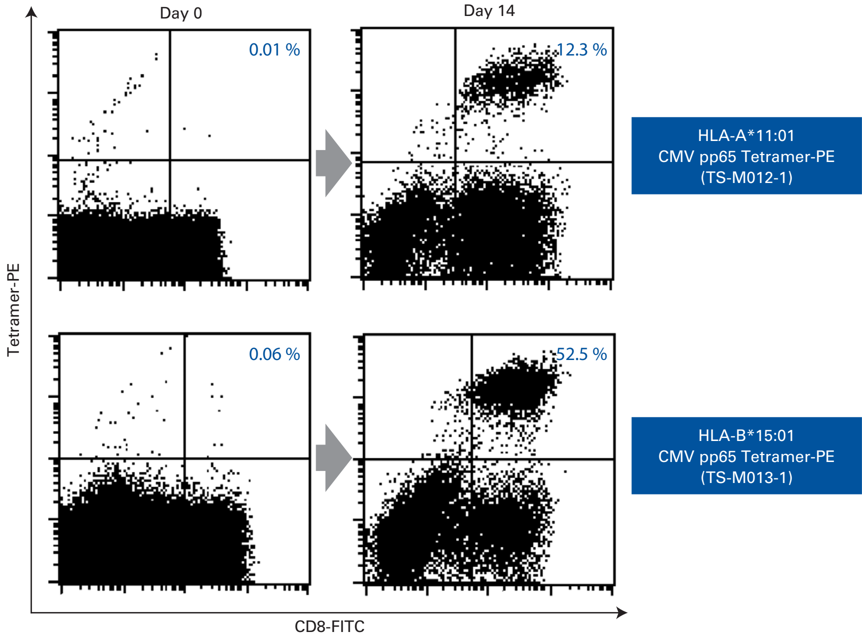
Figure 2 : HLA-A*24:02 CMV pp65 Tetramer staining following MLPC induction



PBMC were separated from peripheral blood of HLA-A*24:02 positive healthy subjects and immediately stained with HLA-A*24:02 CMV pp65 Tetramer (MBLI code no. TB-0020-1) or were cultured to induce antigen-specific T cells using the MLPC method. HLA-A*24:02 CMV pp65 specific CTL responses tend to be more rare and variable than HLA-A*02:01 responses and cannot always be detected immediately after blood collection (Figure 1). However, CMV-specific CTL can be detected by induction using the MLPC method (Figure 2).

CMV-specific T cells could be detected by day 8 of culture from PBMC that had no detectable Tetramer-positive T cells immediately after blood collection. CMV-specific CTL were induced from peripheral blood of ten HLA-A*24:02 positive healthy subjects studied using the MLPC method. The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.

Figure 3: HLA-A*11:01, HLA-B*15:01 CMV pp65 Tetramer

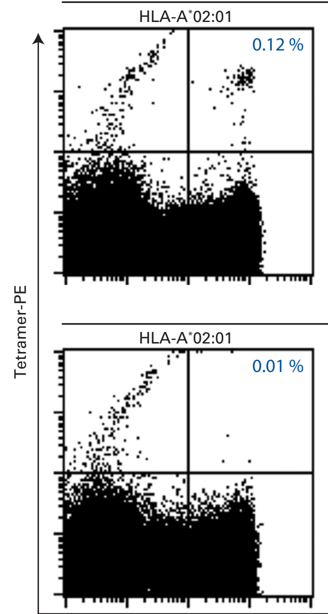


PBMC were separated from peripheral blood of HLA-A*11:01 or HLA-B*15:01 positive healthy subjects and immediately stained with HLA-A*11:01 CMV pp65 Tetramer (MBLI code no. TB-0012-1) and HLA-B*15:01 CMV pp65 Tetramer (MBLI code no. TS-M013-1), respectively (Figure 3). After two weeks of culture with the MLPC method, significant populations of CMV pp65-specific CTL were detected in samples that had only yielded of 0.01 and 0.06% Tetramer-positive T cells immediately after blood collection. The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.

Influenza M1

HLA-A*02:01 Influenza M1 Tetramer

PBMC were separated from peripheral blood of HLA-A2 positive healthy subjects and immediately stained with HLA-A*02:01 Influenza M1 Tetramer (MBLI code no. TB-0012-1). Tetramer positive CTL could be detected in the blood of HLA-A*02:06 and, to a lesser extent, HLA-A*02:07, as well as HLA-A*02:01 donors, suggesting this Tetramer can be used to detect flu-specific responses in subjects of all three haplotypes (Figure 1). The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.



HTLV-1

HTLV-1 Tetramer

HTLV-1 (Human T cell leukemia virus type 1) is known to infect T cells and induce adult T cell leukemia (ATLL) and HTLV-1-associated myelopathy (HAM/TSP). HTLV-1 is one of the viruses receiving extra attention due to the high prevalence of HTLV-1 infected patients and seriousness of symptoms after onset of associated diseases. It is believed that HTLV-1 infection and the corresponding host immune response involving mainly CTL are closely linked to the onset of disease, and HTLV-1-specific CTL epitopes have been described.[1-3] Using sixteen different Tetramer reagents, a group from Kagoshima University showed that the variety and number of HTLV-1-specific CTL was lower in ATLL patients[4-5] and higher in HAM/TSP patients, compared with asymptomatic carries of HTLV-1 (Figure 2 and Table).[6]

Frequency of HTLV-1-specific CTL positivity in asymptomatic HTLV-1 carriers (AC), ATLL, HAM/TSP and carriers with autoimmune diseases (The number of subjects with detectable CTL / Tetramers tested)

allele	Tetramers	AC		ATLL		HAM/TSP		AC with autoimmune diseases	
A*02:01	Tax11-19	73%	(8/11)	33%	(3/9)	90%	(9/10)	55%	(6/11)
A*02:01	Tax123-131	9%	(1/11)	0%	(0/9)	20%	(2/10)	45%	(5/11)
A*02:01	Tax155-163	0%	(0/11)	0%	(0/9)	10%	(1/10)	0%	(0/11)
A*02:01	Tax178-186	9%	(1/11)	0%	(0/9)	20%	(2/10)	18%	(2/11)
A*02:01	Tax307-315	9%	(1/11)	0%	(0/9)	0%	(0/10)	0%	(0/11)
A*02:01	Env175-183	0%	(0/11)	0%	(0/9)	0%	(0/10)	9%	(1/11)
A*02:01	Env239-247	0%	(0/11)	0%	(0/9)	10%	(1/10)	18%	(2/11)
A*02:01	Env442-450	0%	(0/11)	0%	(0/9)	0%	(0/10)	18%	(2/11)
A*24:02	Tax12-20	11%	(2/18)	0%	(0/22)	13%	(2/15)	24%	(4/17)
A*24:02	Tax187-195	11%	(2/18)	0%	(0/22)	7%	(1/15)	18%	(3/17)
A*24:02	Tax289-297	0%	(0/18)	0%	(0/22)	13%	(2/15)	18%	(3/17)
A*24:02	Tax301-309	94%	(17/18)	55%	(12/22)	87%	(13/15)	76%	(13/17)
A*24:02	Tax311-319	0%	(0/18)	0%	(0/22)	7%	(1/15)	6%	(1/17)
A*24:02	Env11-19	6%	(1/18)	0%	(0/22)	40%	(6/15)	47%	(8/17)
A*24:02	Env21-29	0%	(0/18)	0%	(0/22)	13%	(2/15)	35%	(6/17)
A*24:02	Env153-161	0%	(0/18)	0%	(0/22)	13%	(2/15)	24%	(4/17)
Tax CTL positives		22%	(32/145)	10%	(15/155)	26%	(33/125)	26%	(37/140)
Env CTL positives		1%	(1/87)	0%	(0/93)	15%	(11/75)	27%	(23/84)
Total CTL positives		14%	(33/232)	6%	(15/248)	22%	(44/200)	27%	(60/224)

Figure 1

HLA-A*02:01 Influenza M1 Tetramer-PE

HLA-A*02:01 / HLA-A*02:06 0.43 %
HLA-A*02:06 0.19 %
HLA-A*02:07 0.06 %

Negative Tetramer-PE

HLA-A*02:01 / HLA-A*02:06 0.01 %
HLA-A*02:06 0.00 %
HLA-A*02:07 0.01 %

CD8-FITC

Figure 2

HLA-A*24:02 HTLV-1[a]

Tax301-309 (TS-M018-1)
Tax12-20 (TS-M020-1)
Tax187-195 (TS-M021-1)
Env11-19 (TS-M022-1)

HLA-A*02:01 HTLV-1[a]

Tax11-19 (TS-M017-1)
Tax178-186 (TS-M019-1)

HLA-A*11:01 HTLV-1[b]

Tax88-96 (TS-M023-1)
HTLV-1 Tax272-280 (TS-M024-1)

Tetramer-PE

CD8-FITC

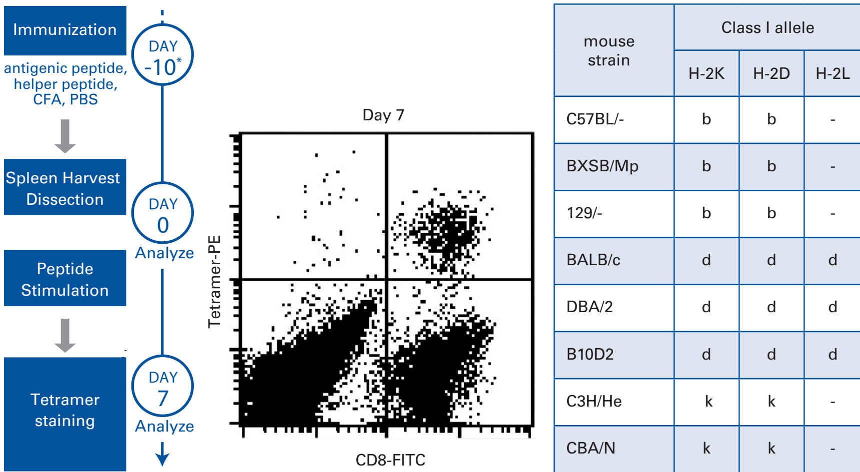
[a]Data provided by Dr. Tomohiro Kozako, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Fukuoka University

[b]Data provided by Dr. Mari Kannagi, Department of Immunotherapeutics, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University.

1) Kannagi M, et al., J. Virol. 66: 2928-2933 (1992)
2) Yashiki S, et al., AIDS Res Hum Retroviruses 17: 1047-1061 (2001)
3) Harashima N, et al., J. Virol. 79: 10088-10092 (2005)
4) Kozako T, et al., J. Immunol. 177: 5718-5726 (2006)
5) Akimoto M, et al., J. Med. Virol. 79: 977-986 (2007)
6) Kozako T, et al., J. Med. Virol. 83: 501-509 (2011)

Induction method for antigen-specific murine CTL

Mouse models are commonly used to study various *in vivo* immune responses, and antigen-specific CTL can be detected using mouse MHC Tetramers. Antigen-specific CTL can be induced rapidly and easily using the following method. First, the antigen peptide of interest is mixed with a second "helper" peptide designed to induce T helper activity. This mixture is emulsified with adjuvant, and injected intraperitoneally (IP). Spleens are harvested 7-11 days after the final immunization, and splenocytes are stimulated by the same peptides *in vitro* for 1 week. The times of immunization depend on the antigen used. In a study conducted by MBL, antigen-specific CTL could be induced in 1-4 rounds of immunization. Immunization of two or more mice per antigen is recommended due to individual differences.



mouse strain	Class I allele		
	H-2K	H-2D	H-2L
C57BL/-	b	b	-
BXSB/Mp	b	b	-
129/-	b	b	-
BALB/c	d	d	d
DBA/2	d	d	d
B10D2	d	d	d
C3H/He	k	k	-
CBA/N	k	k	-

■ Induction of antigen-specific murine CTL by *in vitro* stimulation (influenza virus)

A variety of influenza virus epitopes and variants are reported in mouse models and used for various purposes including structural analysis, MHC binding studies, vaccine development, and infectious disease research. Influenza virus epitopes and variants are among the antigens suitable for CTL induction by peptide immunization.

H-2D^b Influenza NP Tetramer-PE (MBL code no. TS-M505-1)

The antigen peptide derived from H-2D^b-restricted Influenza NP (ASNENMDTM, MBL code no. TS-M502-P) was mixed with I-A^b HBc helper peptide (MBL code no. TS-M701-P), an antigen peptide which induces T cell help. This mixture was emulsified with adjuvant and IP immunization was performed on C57BL/6 mice. The spleen was harvested 11 days later, and spleen cells were stimulated in culture with the antigen peptide derived from Influenza NP (1 μg/mL) for 1 week. Aliquots were stained with the MHC Tetramer reagent on (days 0 and 7 of culture), and live lymphocytes were assessed for Tetramer reactivity. In mice immunized with the antigen peptide derived from influenza NP (ASNENMDTM), expansion of influenza NP-specific CTL by *in vitro* stimulation was observed (Figure 1). Negative staining with a Tetramer to an unrelated epitope (H-2D^b human gp100 Tetramer-KVPRNQDWL-PE, MBL code no. TS-M505-1) demonstrated the specificity of the expanded CTL to NP. The value on them upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.

Figure 2 shows *in vitro* expansion of various Influenza-specific T cells from mice immunized with the corresponding peptide.

*Please refer to the data sheet of each reagent for induction condition for each epitope.

Figure 2

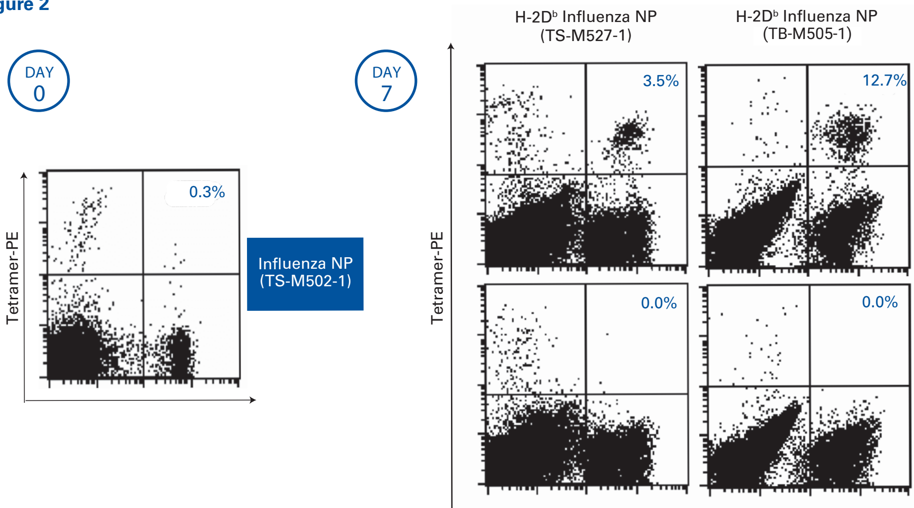
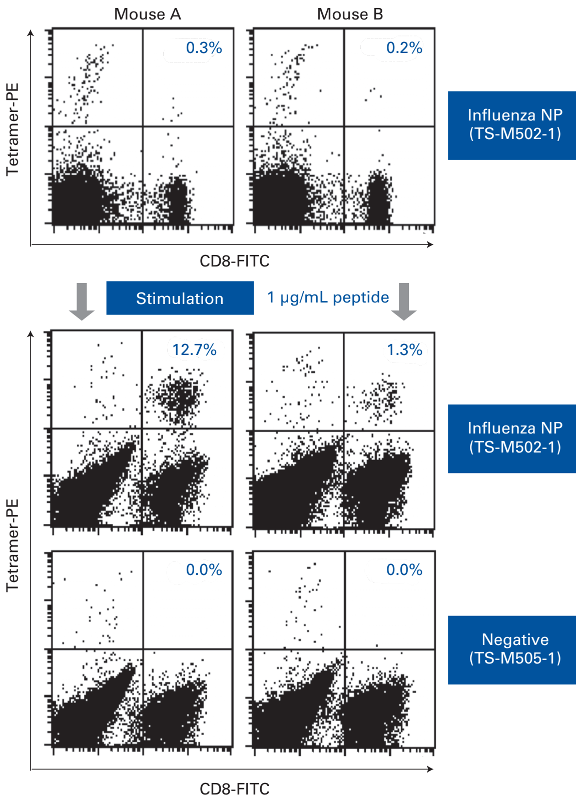
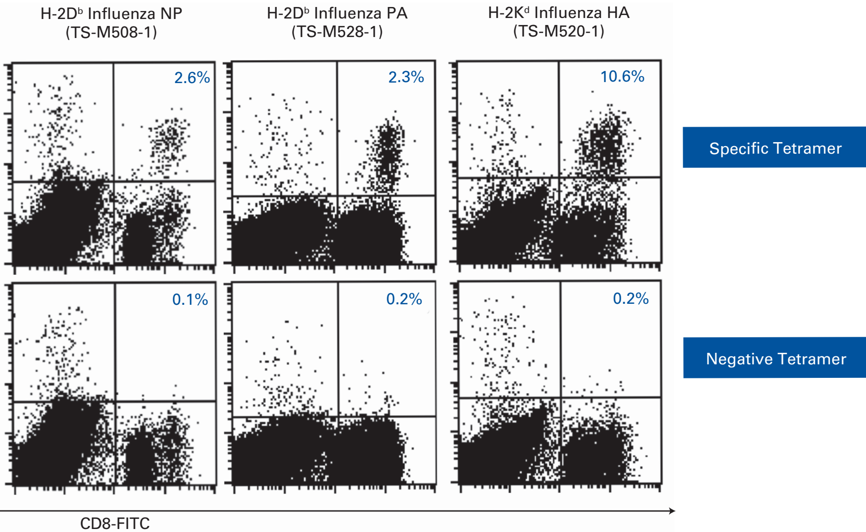


Figure 1





Additional Tips for Tetramer Staining

In cases where an antigen-specific T cell population of interest has a low frequency in a sample or if there are issues obtaining a clear result, the following additional tips discussed in a review in 2015 by Dolton G, Tungatt K, Lloyd A, *et al.*,[1] may help increase the efficacy of tetramer staining:

1. Brighter Fluorochromes
    a. Many fluorochromes exist for use with MHC tetramers and monomers. PE, APC, and BV421 can work very well for many common MHC tetramer and monomer applications.

2. Use of Protein-Kinase Inhibitor
    a. Many T-Cell receptors are internalized after exposure to a cognate antigen. This internalization prevents detection of the T-cell receptor using a MHC tetramer. However, by adding a protein-kinase inhibitor such as dasatinib before MHC tetramer staining, the amount of TCR internalization can be reduced and staining intensity increased.

3. Use of Anti-Coreceptor Antibody
    a. The use of an anti-coreceptor antibody is generally recommended for tetramer staining. However, the clone of the antibody is important to be aware of as certain clones hinder tetramer staining.

4. Signal Boosting with Antibodies
    a. An increased signal has been found in many instances when using an antibody that targets the tetramer allele. For example, certain HLA-A*02:01antibodies have been found to increase the staining of a HLA-A2 tetramer.

[1]Dolton G, Tungatt K, Lloyd A, et al. More tricks with tetramers: a practical guide to staining T cells with peptide-MHC multimers. Immunology. 2015;146(1):11-22.

MBL International offers a MHC tetramer for the violet laser: **Brilliant Violet™ 421**. This laser allows the users to expand detection options to the 405 nm laser and detect antigen-specific T cells of multiple specificities in one tube using PE, APC and BV421 tetramers.

Figure 1: Similar percentages of antigen-specific T cells were detected in PMBC from an EBV+ donor stained with HLA-A*02:01 EBV (GLCTLVAML) tetramer labeled with APC or BV421. Tetramer signal to noise ratios for EBV tetramer was 25.5 for APC and 23.1 for BV421. Fewer background events were observed in the BV421 tetramer sample compared with the APC tetramer sample.

Figure 1

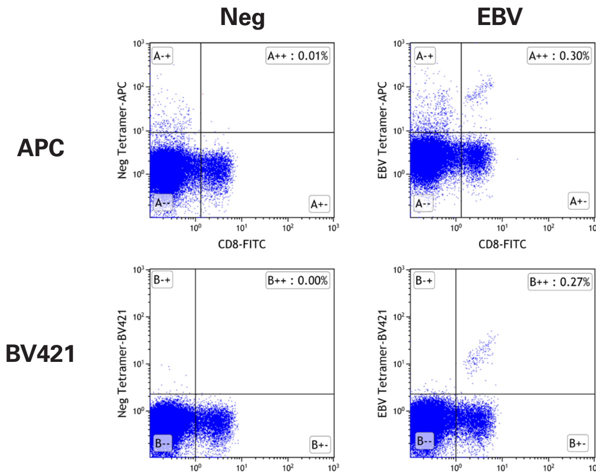
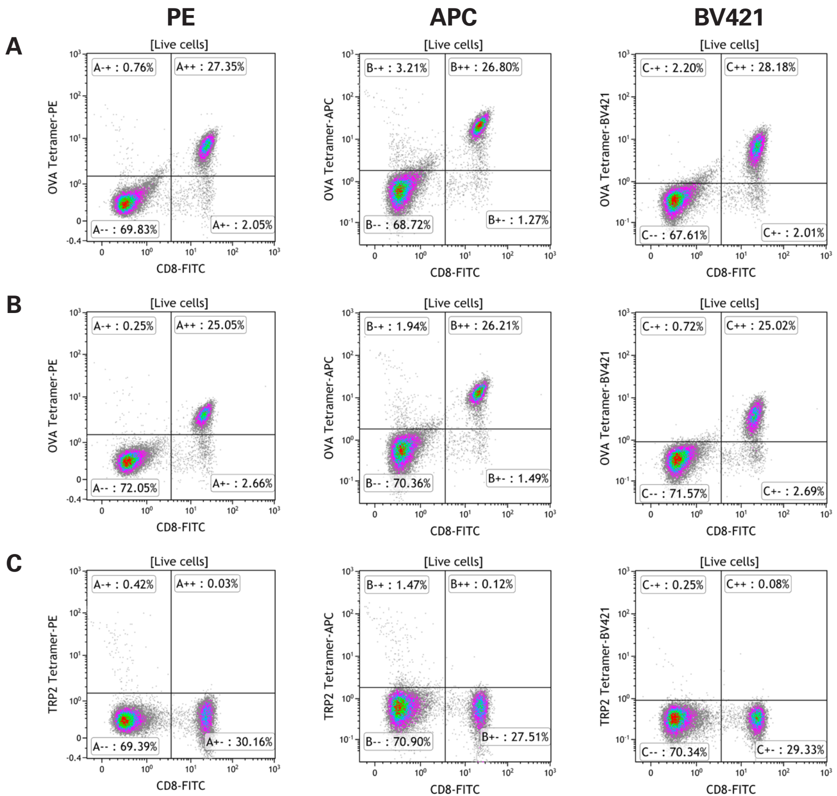


Figure 2: Similar percentages of antigen-specific T cells were detected in OT-I transgenic splenocytes stained individually (A) or simultaneously (B) with H-2 Kb OVA (SIINFEKL) tetramers labeled with PE, APC, or BV421. Tetramer signal to noise ratios for OVA tetramer were 15.6, 31.0, and 15.9 for PE, APC, and BV421, respectively when tetramers were used individually (A), versus 9.5, 21.1, 9.7 when used simultaneously, reflecting the competition of the three tetramers to the same target (B). Regions were set based on TRP2 irrelevant tetramer (C).

Figure 2



Class II Tetramers

CD4+ T cells, which include helper and regulatory T cells, recognize complexes of MHC class II molecules and peptides.

CD4+ T cells play an important role in health and diseases. Analysis of these cells has long been hampered by a lack of suitable assays. Class II tetramers have become an important tool to investigate rare antigen-specific CD4+ T cells such as CD4+CD25+ regulatory T cells. In addition to monitoring disease progression and therapeutic intervention, class II tetramers can be used to study tolerance induction, vaccination efficacy, and autoimmunity.

Class II tetramers bind to a distinct population of CD4+ T cells. MHC class II tetramer staining is more technically challenging than class I tetramer staining.

- Antigen-specific CD4+ T cells are more rare than antigen-specific CD8+ T cells
- Affinity between the T cell receptor and MHC/peptide complex is generally lower
- Acquisition of 100,000-200,000 CD4+ T cells is typically required
- Exclusion gating essential
- T cell expansion and/or pre-enrichment may be required

MHC class II tetramers can be used to monitor antigen specific CD4+ T cell expansion after vaccination (Fig. 1) or after a natural immune response against a pathogen (Fig. 2).

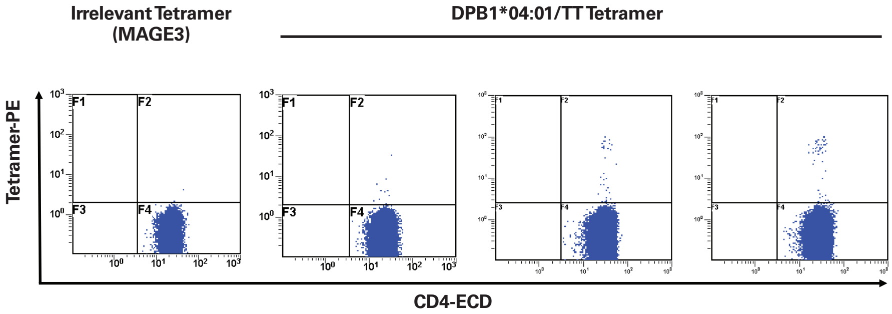


Figure 1 *Ex vivo* measurement of DPB1*04:01/TT947-967-specific CD4+ T cells following vaccination with Tetanus Toxoid.

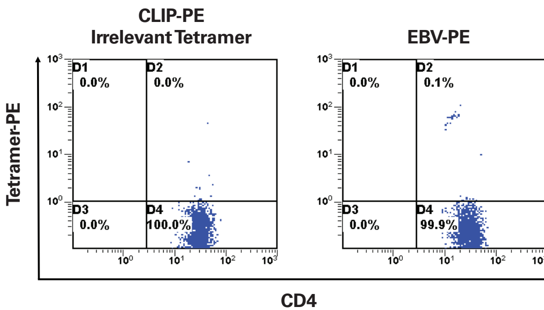


Figure 2 Detection of DRB1*01/EBV-specific CD4+ T cells in whole blood.

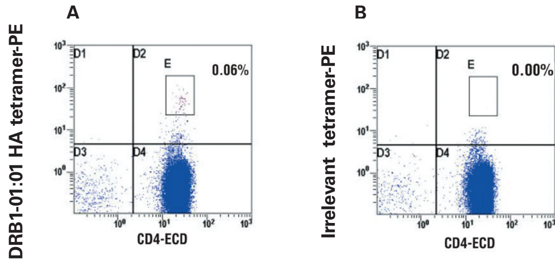


Figure 3 Detection of Influenza HA-specific CD4+ T cells in whole blood
Unstimulated whole blood samples were stained with anti-CD4-ECD and DRB1-01:01 HA tetramer (A) or DBR1-01:01 irrelevant tetramer (B).

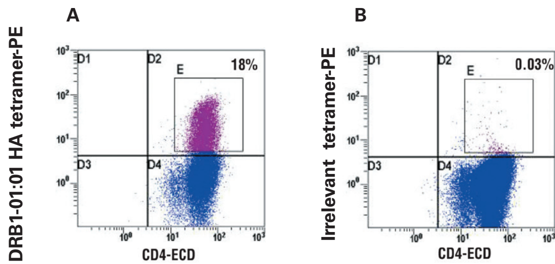
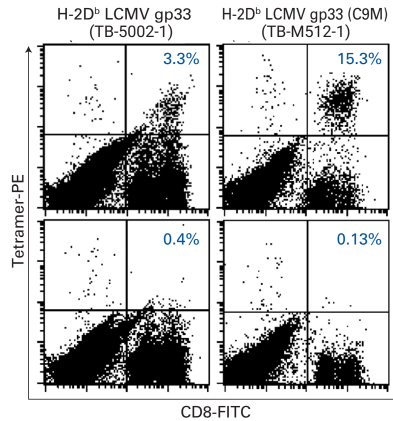


Figure 4 Expansion of Influenza HA-specific CD4+ T cells in peptide-stimulated PBMCs. PBMCs were stimulated for 7 days with HA peptide and then stained with anti-CD4-ECD and DRB1-04:01 HA tetramer (A) or DRB1-04:01 irrelevant (gp39) tetramer (B)

Staining with Mouse MHC class I Tetramer

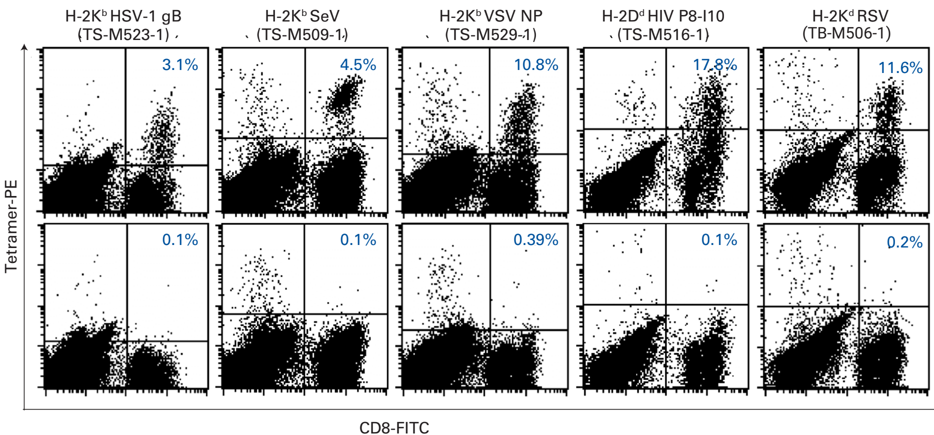
MBL International offers Tetramer reagents for murine model antigens, tumor antigens, viral antigens, etc. All the data shown below are results of staining with a Tetramer reagent (upper column) or a negative Tetramer (lower column) of each antigen peptide-specific CTL expanded by *in vitro* stimulation after peptide immunization. Negative Tetramers were constructed using the matching allele with a different (non-reactive) peptide epitope sequence. Murine spleen cell samples 6 days after peptide stimulation are shown. The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.

♦LCMV

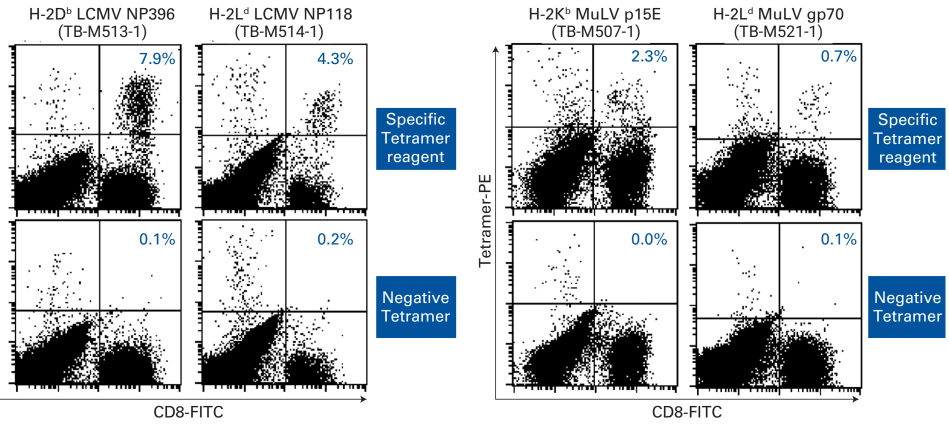


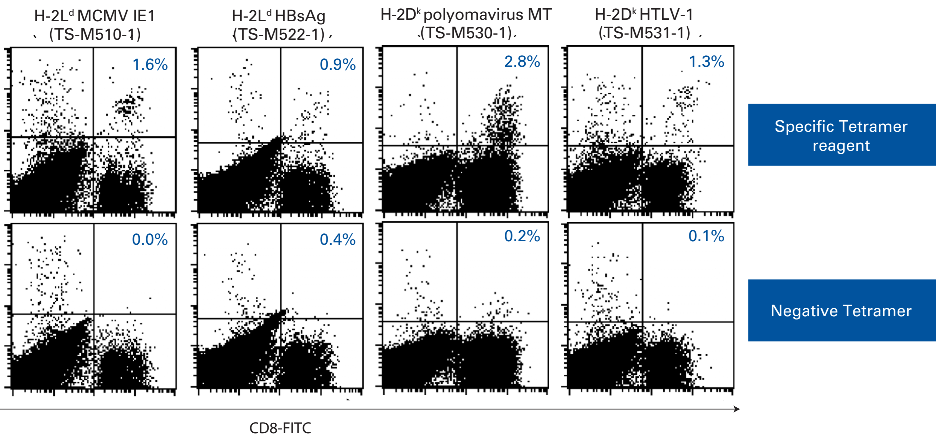
*Please refer to the data sheet of each reagent for induction condition for each epitope.

♦Other Viral antigens

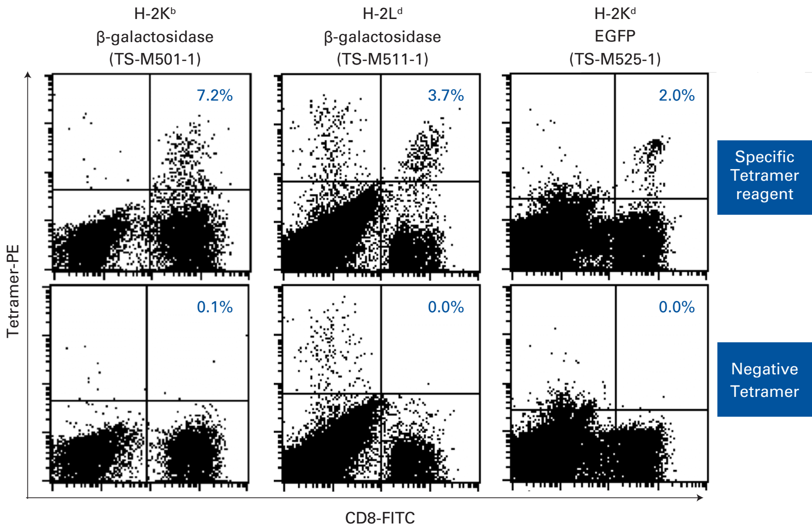


♦MuLV

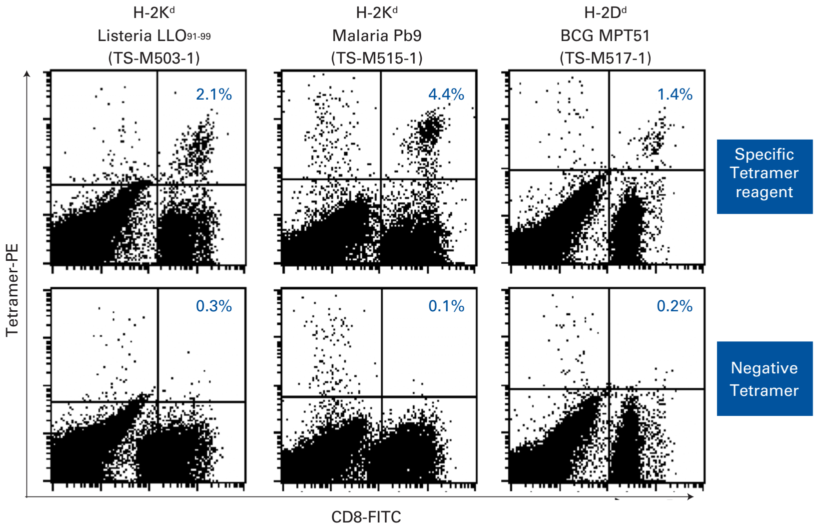




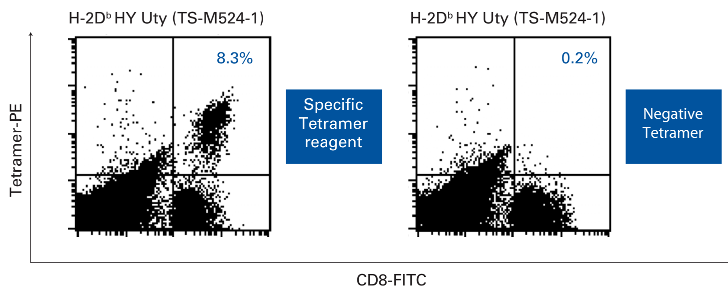
◆Model antigen



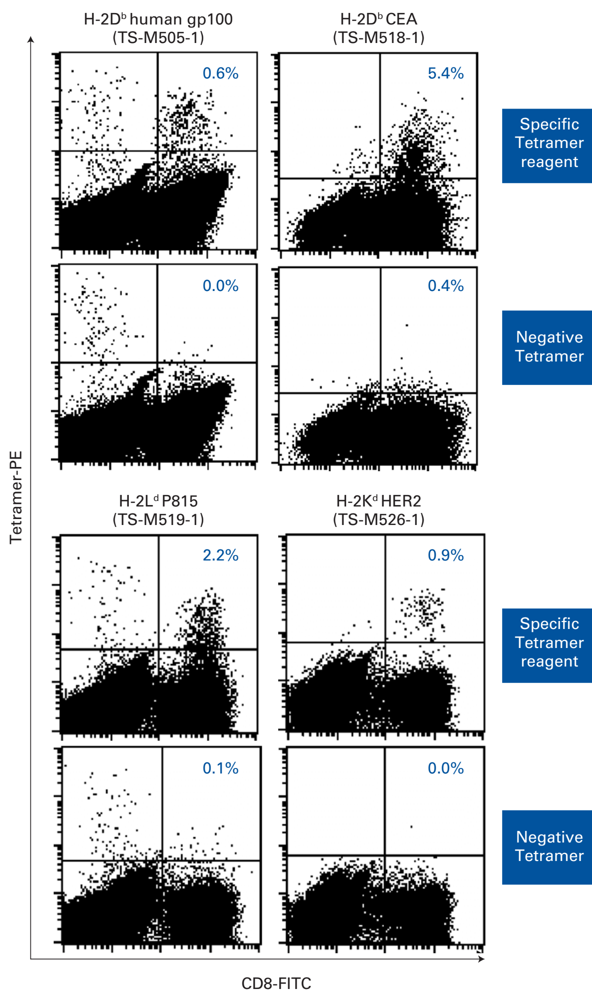
◆Bacterial antigen

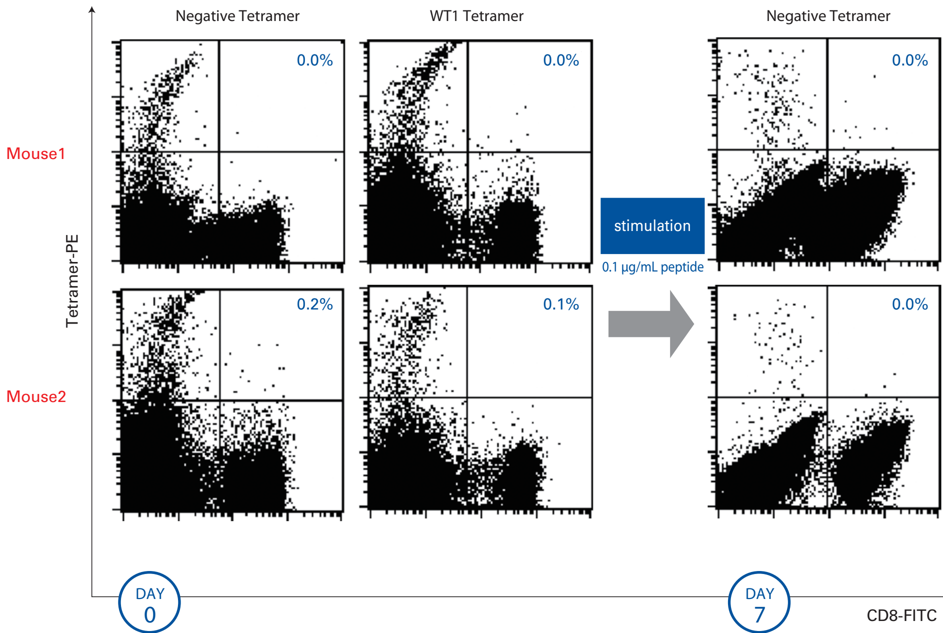


♦Minor antigen



♦Cancer antigen

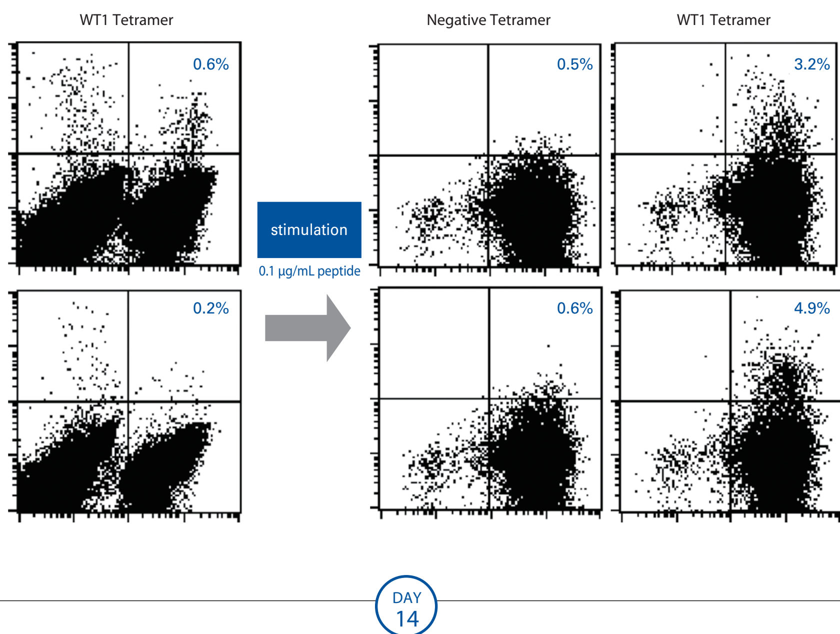




Induction of murine WT1 Tetramer-specific CTL

Wilms tumor factor WT1 is a transcription factor regulating cell growth, differentiation, and apoptosis. The *WT1* gene is highly expressed in leukemia and various solid cancers and is known to contribute to their onset and progression. It has been shown that WT1-specific CTL kill tumor cells with high WT1 expression, but not normal cells. WT1 is receiving widespread attention as a target molecule for cancer immunotherapy and clinical trials are in progress.

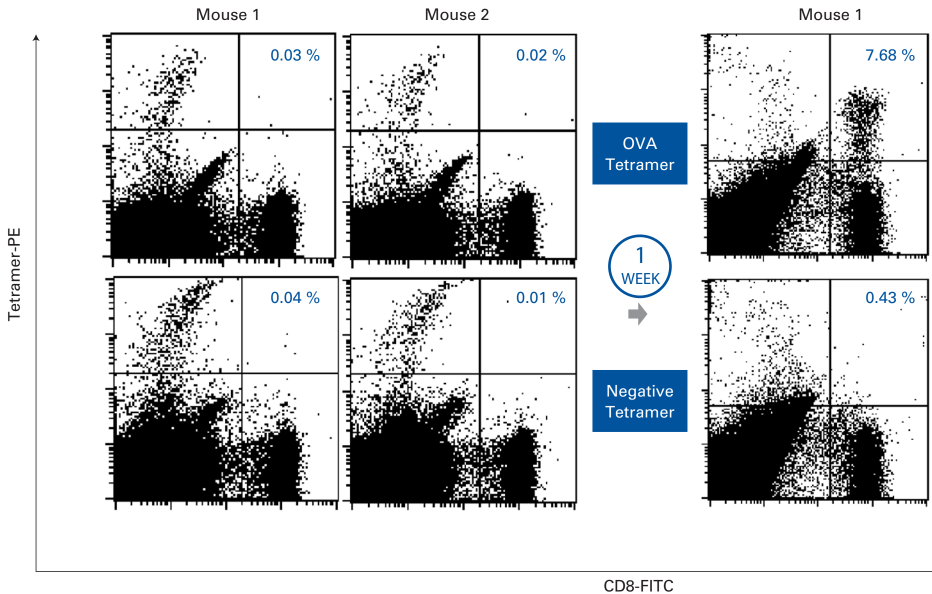
The Figure above shows an increase in H-2D^b WT1 Tetramer positive cells (using MBLI code no. TS-M504-1) in splenocyte cultures from peptide immunized mice stimulated with peptide *in vitro*. The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.



It is sometimes difficult to obtain adequate amounts of antigen-specific CTL with a single round of *in vitro* peptide stimulation. A second peptide stimulation may amplify the specific CTL, but because the peptide is presented on MHC class I molecules of the cells in culture, those cells can become targets of the MHC peptide-specific CTL. This will often cause a significant decrease in CD8 negative cells, as shown in the day 14 plots, or even a decrease in CTL themselves, as they too can become targets of the antigen-specific CTL. To avoid these complications, the addition of peptide-pulsed antigen-presenting cells (such as dendritic cells), rather than soluble peptide, is recommended for the second round of stimulation.

H-2K^b OVA Tetramer

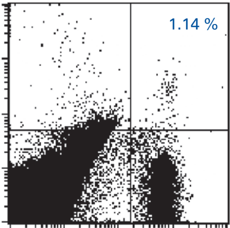
Ovalbumin (OVA), the major protein found in chicken egg whites, is a T cell-dependent antigen commonly used as a model protein for studying antigen-specific immune responses in mice. H-2K^b OVA Tetramer detects OVA-specific T cells in C57BL/6 and other mouse strains expressing the class I allele, H-2K^b.

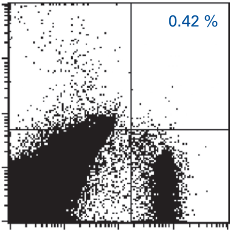


H-2K^b OVA Tetramer staining

An antigenic peptide derived from H-2K^b-restricted OVA was mixed with a T-helper peptide. This mixture was emulsified with adjuvant, and IP immunization was performed on two C57BL/6 mice. Spleens were harvested 10 days later, and spleen cells were prepared. Some cells were sampled and stained with the OVA-specific or negative Tetramers (left figure). Remaining spleen cells were incubated *in vitro* for 1 week with naïve splenocytes pulsed with OVA peptide, and then stained with Tetramers (right figure). OVA-specific T cells were detected one week following *in vitro* stimulation. The value on the upper right of each plot indicates the percentage of Tetramer-positive CD8 T cells.

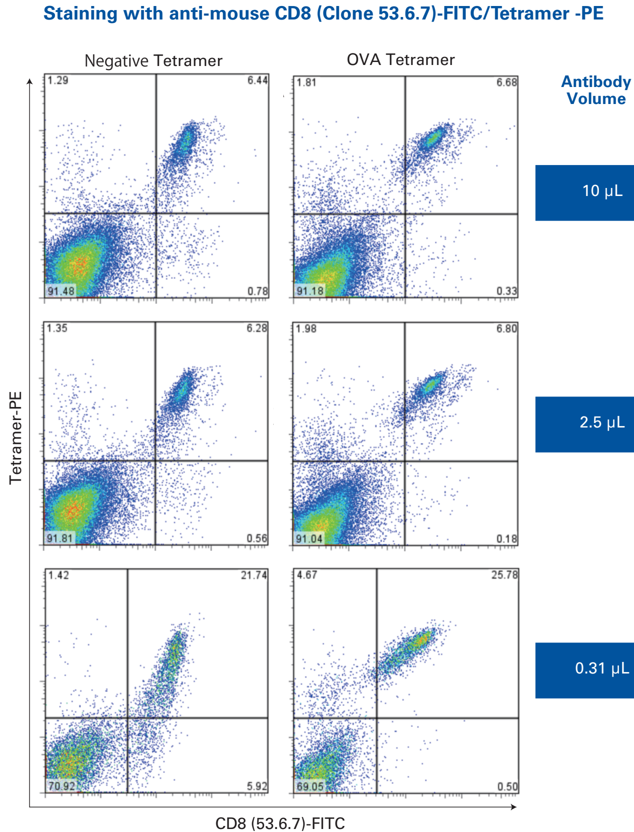
Mouse 2





Effect of CD8 antibody clones on H-2K^b OVA Tetramer staining: Cells from OT-I mice

Figure 1 **Staining with anti-mouse CD8 (Clone 53.6.7)-FITC/Tetramer -PE**



Splenocytes from mice transgenic for OVA-specific T cells (OT-I) were used to explore staining differences among mouse CD8 antibody clones in combination with H-2K^b Tetramers. OT-I mouse spleen cells (1×10^6 cells/sample) were stained with H-2K^b OVA Tetramer-PE (10 µL/sample) and serially diluted anti-mouse CD8 (clone KT15 or clone 53.6.7) antibody in a final assay volume of 100 µL. H-2K^b β-galactosidase (β-gal) Tetramer-PE (10 µL/sample) was used as a negative Tetramer to assess non-specific binding. When anti-CD8 clone 53.6.7 was used, positive staining was observed on CD8 positive cells with both OVA Tetramer and β-gal Tetramer (Figure 1). Even when 53.6.7 was diluted, the cells were stained with both Tetramers similarly, suggesting the Tetramer staining was not specific.

Figure 2 **Staining with anti-mouse CD8 (Clone KT15)-FITC/Tetramer -PE**

Negative Tetramer | OVA Tetramer | Antibody Volume

10 µL

2.5 µL

0.63 µL

Tetramer-PE

CD8 (KT15)-FITC

When anti-CD8 clone KT15 was used, however, CD8 positive cells among OT-I mouse spleen cells were specifically stained only with OVA Tetramer at all antibody concentrations tested (Figure 2). Cells stained with β-gal Tetramer were negative. Titration of KT15 revealed an optimal signal to noise ratio at 0.63 µL/sample for OT-I splenocytes. In summary, when staining cells with H-2K^b Tetramers it is important to use appropriately titrated anti-CD8 clone KT15 and include a negative control Tetramer in the experiment.

The concentration used for this evaluation is only a guide for use. Antibody titrations should be performed to optimize concentration according to the experimental system used.

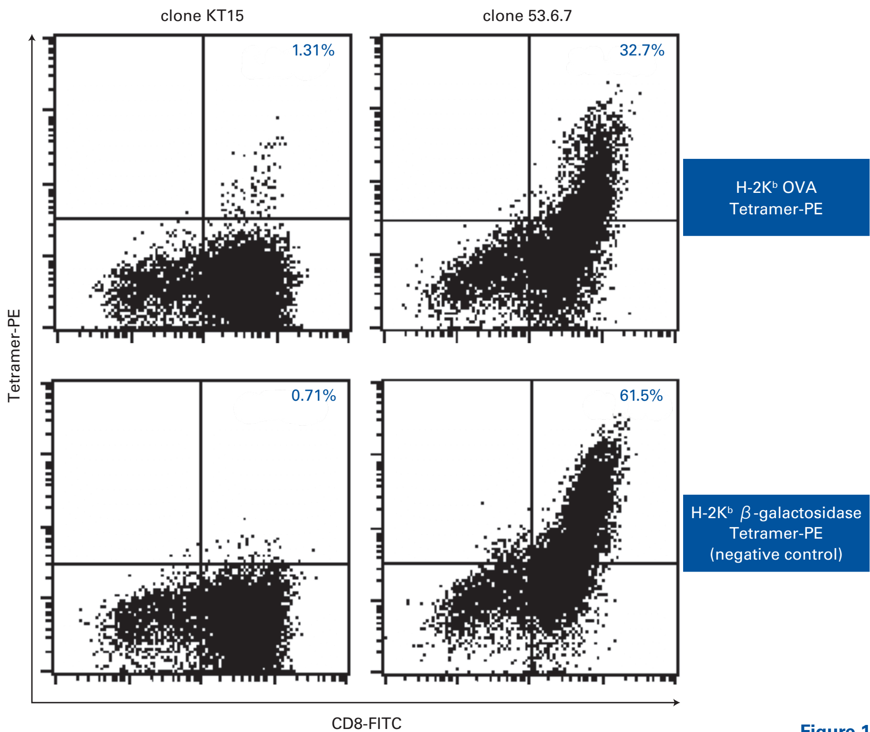
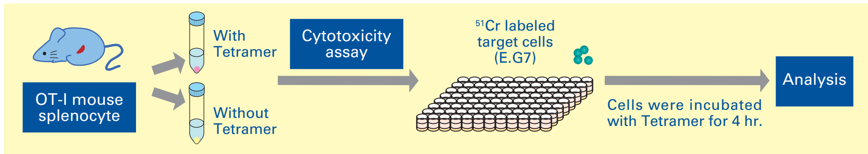


Figure 1

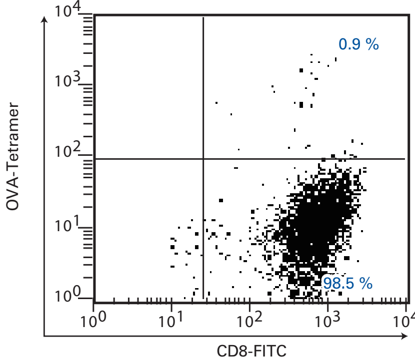
Effect of CD8 antibody clones on H-2Kᵇ OVA Tetramer staining: Cells from peptide-immunized mice

Mouse spleen cells prepared using peptide immunization for OVA-specific CTL induction were stained with H-2Kᵇ OVA Tetramer-PE (10 μL/sample) and anti-mouse CD8 (clone KT15 or clone 53.6.7). H-2Kᵇ β-galactosidase Tetramer-PE (10 μL/sample) was used as a negative Tetramer to assess non-specific staining. When KT15 was used, OVA-specific Tetramer positive cells were observed compared with the negative Tetramer, while when 53.6.7 was used, marked non-specific staining was observed in both H-2Kᵇ β-galactosidase and OVA Tetramer samples. Because clone 53.6.7 is known to have poor compatibility with OVA and other H-2Kᵇ Tetramers, anti-mouse CD8 clone KT15 is recommended.

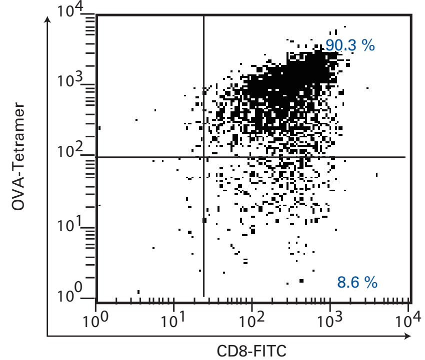
Staining using cancer- related MHC Tetramers



A. Wild type

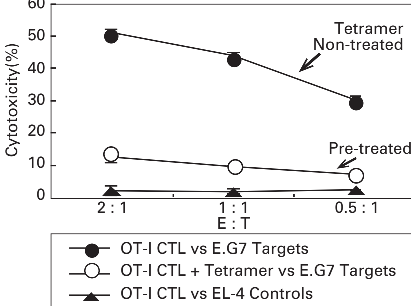


B. OT-I transgenic mice



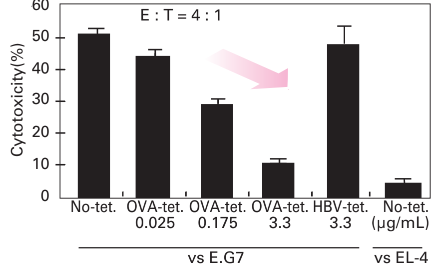
About 90% of CD8+T cells in splenocytes of OT-I mice are OVA Tetramer positive.

C



Tetramer staining inhibits TCR-specific cytotoxic activity

D



Cytotoxic activity is inhibited dependent on concentration of the Tetramer reagent.

Data provided by Dr. Kenji Chamoto and Dr. Takashi Nishimura, Division of Immunoregulation, Institute for Genetic Medicine Hokkaido University.

1) Wakita D, et al., Int. Immunol. 18: 425–434 (2006)
2) Chamoto K, et al., Cancer Res. 66: 1809–1817 (2006)
3) Yokouchi H, et al., Cancer Sci. 97: 148–154 (2006)
4) Yokouchi H, et al., Clin Exp Metastasis 24: 533–540 (2007)

The Tetramer blocking assay is an experimental system showing inhibition of antigen specific cytotoxic activity by blocking TCR using MHC-Tetramer reagents. The assay shows whether or not the cytotoxic activity observed in the ^{51}Cr release assay or the IMMUNOCYTO Cytotoxity Detection Kit is caused by Tetramer positive CTL. Cytotoxic activity of the Tetramer-stained CTL is reported to recover about 24 hours after the blocking assay is completed.

I-A^b OVA$_{323-339}$ Tetramer-PE

OVA is a model antigen commonly used to study immune responses in mice. OT-II transgenic mice express TCR specific for OVA$_{323-339}$ epitope (ISQAVHAAHAEINEAGR) in the context of I-A^b and serve as an important tool to study differentiation and activation of CD4 T cells. I-A^b OVA$_{323-339}$ Tetramer can be used to monitor antigen-specific CD4 T cell responses in OT-II mice and in various experimental systems using OT-II cells for adoptive transfer. Reactivity of I-A^b OVA$_{323-339}$ Tetramer reagent was assessed using freshly isolated spleen cells from OT-II mice. The majority of CD8 T cells were Tetramer positive in mouse 2, but not in mouse 1 (Figure 1). *In vitro* stimulation with specific peptide greatly increased the number of Tetramer positive cells (Figure 2.)

Figure 1: I-A^b OVA$_{323-339}$ Tetramer staining of OT-II splenocytes

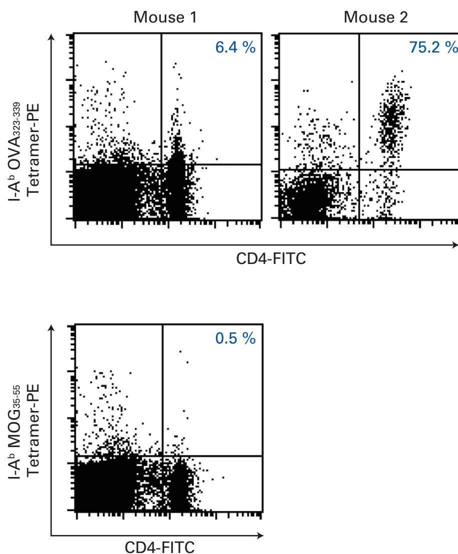
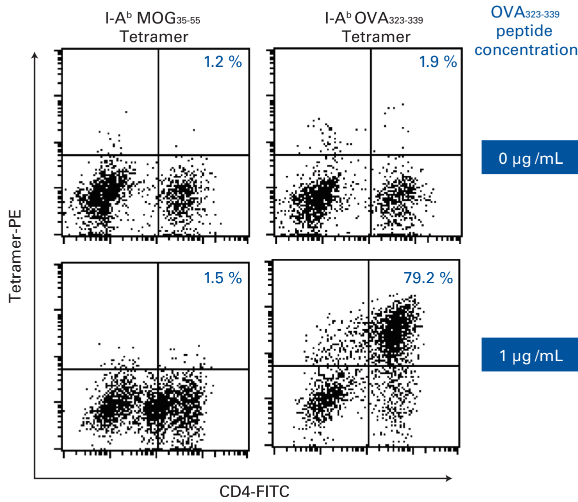
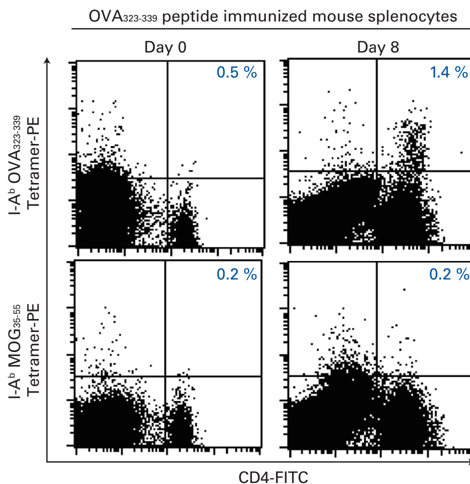


Figure 2: I-A^b OVA$_{323\text{-}339}$ Tetramer staining of peptide stimulated OT-II splenocytes (Day 6).



OVA-specific CD4 T cells can also be induced in wild-type mice. C57BL/6 mice were immunized twice IP with a mixture of 100 nmol of OVA$_{323\text{-}339}$ peptide and 10 µg of cholera toxin emulsified with adjuvant. After 11 days, spleen cells were harvested and cultured with a final concentration of 1 µg/mL OVA$_{323\text{-}339}$ peptide for 8 days. Cells were tested on days 0 and 8 of culture for OVA Tetramer reactivity (Figure 3). In mice immunized with OVA$_{323\text{-}339}$ peptide, induction of specific T cells by *in vitro* peptide stimulation was confirmed using I-A^b OVA$_{323\text{-}339}$ Tetramer. I-A^b MOG$_{35\text{-}55}$ Tetramer, used as a negative control, showed no specific staining.

Figure 3: I-A^b OVA$_{323\text{-}339}$ Tetramer staining of splenocytes from peptide-immunized mice

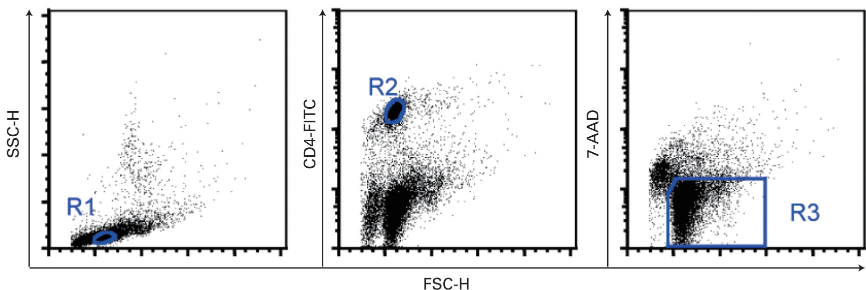


I-A^b ESAT-6$_{1-20}$ Tetramer-PE

Human tubercle bacillus (*Mycobacterium tuberculosis*, Mtb) is well known as a causative agent of pulmonary tuberculosis. ESAT-6 (Early Secreted Antigenic Target 6) is a low molecular weight protein secreted by Mtb and possesses strong antigenicity. *M. bovis* BCG strain used for tuberculosis vaccination lacks the ESAT-6 gene and thus the T cell response specific to the antigen is used for diagnosis of tuberculosis infection in BCG-vaccinated subjects. ESAT-6$_{1-20}$ is an immunodominant I-A^b restricted epitope in the mouse and widely used for monitoring of immune response *in vivo* for research and development of new vaccines. I-A^b ESAT-6$_{1-20}$ Tetramer is anticipated to become an important tool in various experimental systems involving transgenic mice expressing I-A^b ESAT-6$_{1-20}$-specific TCR.

FSC/SSC plot

Live CD4 positive lymphocytes were defined by a FSC/SSC lymphocyte region (R1), CD4 positive cell region (R2), and 7-AAD negative cell region to analyze CD4 positive T cells for Tetramer reactivity.



Human tubercle bacillus H37Rv strain or BCG Pasteur strain was transmitted to mice via the tail vein (5x10^5 CFU/animal). Cells harvested from lung and spleen four weeks later were stained with the MHC Tetramer I-A^b ESAT-6$_{1-20}$. Tetramer positive cells were detected in 7.73% of CD4 positive lymphocytes from lungs of human tubercle bacillus-infected mice (Figure 1). I-A^b ESAT-6$_{1-20}$ Tetramer positive cells were detected in spleen cells of the human tubercle bacillus-infected, but not BCG-infected, mice, (Figure 2).

Figure 1 : Staining of ESAT-6$_{1\text{-}20}$ specific CD4^{+}- T cells harvested from spleen lung

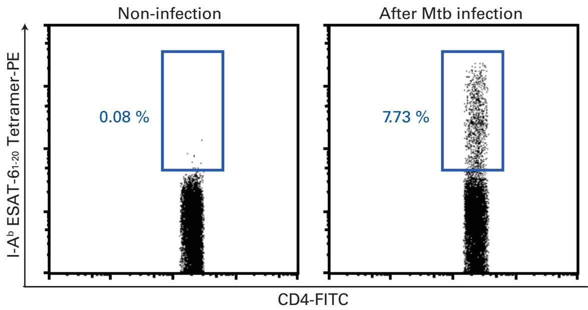


Figure 2 : Staining of ESAT-6$_{1\text{-}20}$ specific CD4^{+} T cells harvested from spleen

I-A^{b} MOG$_{35\text{-}55}$ Tetramer-PE (Negative control)

I-A^{b} ESAT-6$_{1\text{-}20}$ Tetramer-PE

Tetramer-PE

0.02 %

0.03 %

Non-infection

0.01 %

0.02 %

After BCG infection

0.02 %

1.19 %

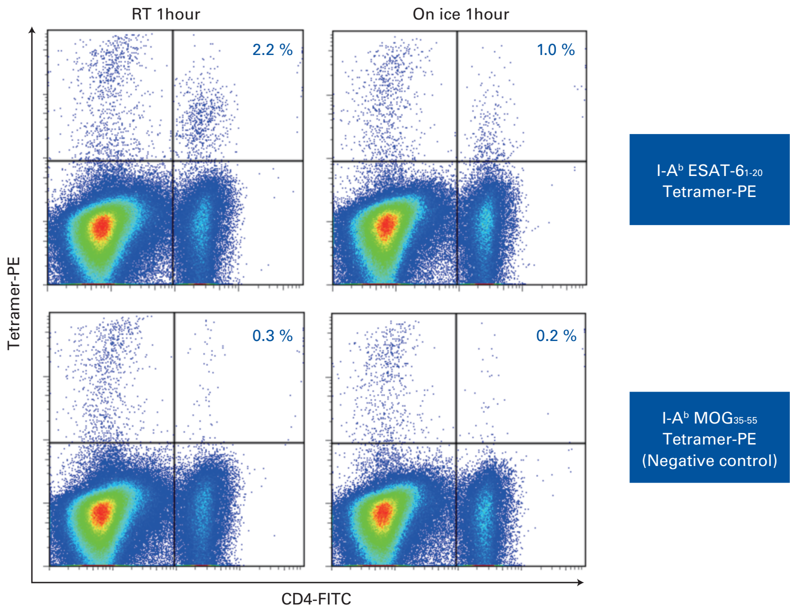
After Mtb infection

CD4-FITC

Data provided by Dr. Shunsuke Sakai and Dr. Masao Mitsuyama, Department of Microbiology, Kyoto University Graduate School of Medicine

■ I-A^{b} EAST-6$_{1\text{-}20}$ Tetramer-PE

Figure 1



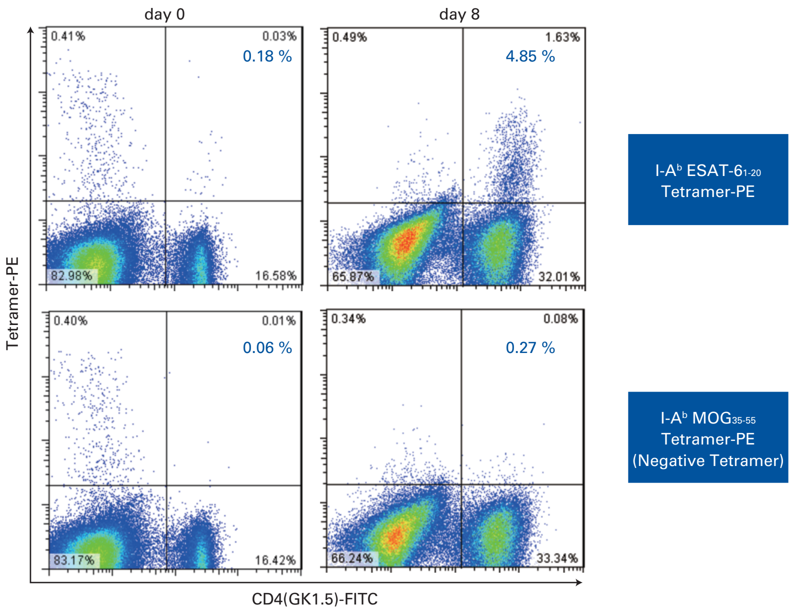
Data provided by Dr. Shunsuke Sakai and Dr. Masao Mitsuyama, Department of Microbiology, Kyoto University Graduate School of Medicine.

Spleen cells from the tubercule bacillus infected mice were stained at room temperature or on ice. The percent positive and fluorescence intensity of I-A^{b} ESAT-6$_{1\text{-}20}$ Tetramer was found to be higher when staining was performed at room temperature for 1 hour, than staining on ice for 1 or 2 hours (Figure 1 and data not shown).

Mice were immunized twice IP with a mixture of 100 nmol of I-A^{b} ESAT-6$_{1\text{-}20}$ peptide (MTEQQNFAGIEAAASAIQG, MBLI code no. TS-M707-P) and 10 µg of cholera toxin emulsified with adjuvant. After 11 days, spleen cells were harvested and cultured with ESAT-6$_{1\text{-}20}$ peptide (0.1 µg/mL) for 8 days. Cells were tested on days 0 and 8 of culture for antigen-specific T cells. Induction of specific CD4+ T cells was confirmed using I-A^{b} ESAT-6$_{1\text{-}20}$ Tetramer. I-A^{b} MOG$_{35\text{-}55}$ Tetramer, (MBLI code no. TS-M704-1), used as a negative Tetramer, showed no specific staining (Figure 2).

*In this data, live lymphocytes were defined by FSC/SSC plot, lymphocyte region and 7-AAD negative cell region to analyze CD4 positive T cells for Tetramer reactivity

Figure 2

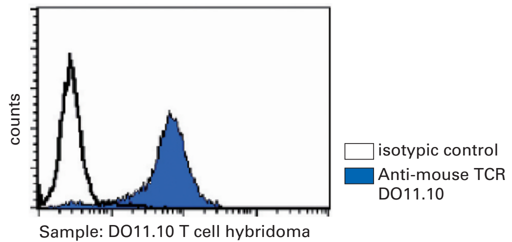


Reagents for studying OVA specificity in the context of I-A^d murine class II molecules

Anti-Mouse TCR DO11.10 (clone KJ1.26, MBLI code no. K0221-5) is an antibody that binds to the TCR specific for OVA$_{323-339}$ presented in the context of I-A^d. This antibody can be used to identify OVA$_{323-339}$-specific CD4 T cells derived from I-A^d mice. OVA$_{323-339}$ peptide (ISQAVHAAHAEINEAGR, MBLI code no. TS-M703-P) is often used as a helper peptide for induction of antigen-specific CTL in I-A^d-expressing mice.

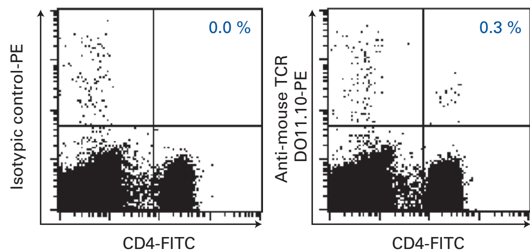
Anti-mouse TCR DO11.10

DO11.10 T cell hybridoma



Sample: DO11.10 T cell hybridoma

OVA$_{323-339}$ peptide BALB/c mouse splenocyte



What is the difference between paraformaldehyde and formaldehyde?

Paraformaldehyde is a form of formaldehyde.

The simple chemical formula for formaldehyde is CH_2O. Paraformaldehyde is polymerized formaldehyde. When paraformaldehyde is dissolved, it becomes formaldehyde. Only the dissolved formaldehyde form is able to fix tissues.

When you are making a paraformaldehyde solution, you should make it in a hood. Mix paraformaldehyde with PBS or TBS at 70°C. Use 5N NaOH to make the solution clear. You can then do a quick spin or use a filter syringe to remove any insoluble impurities. It’s always best to use a fresh solution but aliquots can be stored at -20°C and used over a couple months.

What are some examples of diseases and models that have been studied using MHC tetramers?

- **Infectious Diseases:** HIV, EBV, CMV, HPV, HBV, HCV, Influenza, Measles, Malaria, TB, RSV
- **Cancer:** Breast, Prostate, Melanoma, Colon, Lung, Cervical, Ovarian, Leukemia
- **Autoimmune Diseases:** Diabetes, Multiple sclerosis, Rheumatoid arthritis, Autoimmune vitiligo
- **Transplantation:** EBV and CMV
- **Animal Models:** OVA, E alpha, SIV

What do “Premium”, “Select”, “Custom”, and “T-Select” designation mean?

Premium tetramers are the most commonly ordered PE-labeled tetramers and can be shipped immediately. Select tetramers are PE-, APC- or BV421-labeled tetramers that may need to be assembled from existing monomer stocks. Custom tetramers are manufactured with peptide and allele combinations selected by the customer. While all three classifications of tetramers are all manufactured using the same methods, only Premium tetramers come with a stability claim, which is a minimum of 12 months from the date of manufacture. T-Select tetramers

are manufactured by MBL in Japan and include additional allele choices. T-Select tetramers have a stability claim of a minimum of 90 days from the date of purchase.

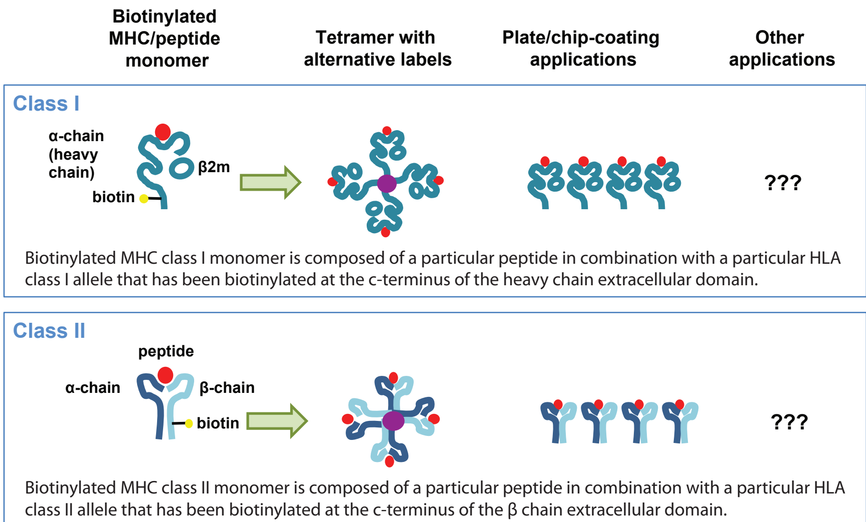
What allele/peptide comprises the Negative Tetramer?

The "Negative Tetramer" is prepared with the HLA-A*02:01 allele and a proprietary peptide whose sequence does not occur in nature. We offer the "Negative Tetramer" conjugated to either PE (PN T01044), APC (PN T01054), and BV421 (PN T01044B).

MHC Monomers

An MHC monomer is a sub-component of an MHC tetramer, made up of the MHC complex (α-chain plus β-2 microglobulin for class I or α-chain plus β-chain for class II) folded together with a specific peptide. Biotinylated monomers are available for most associated tetramer products. Biotinylated MHC monomers, the building blocks of MHC tetramers, are available for tetramer construction flexibility and novel applications.

Investigators often select MHC monomers to create tetramers in their own labs with alternate labels or to generate smaller tetramer batches. Others use them for screening or immunization strategies. The production of metal-labeled tetramers for use with CyTOF instruments using MBL International monomers is becoming increasingly popular.



Biotinylated MHC class I monomer is composed of a particular peptide in combination with a particular HLA class I allele that has been biotinylated at the c-terminus of the heavy chain extracellular domain.

Biotinylated MHC class II monomer is composed of a particular peptide in combination with a particular HLA class II allele that has been biotinylated at the c-terminus of the β chain extracellular domain.

Procedure for Creating MHC Tetramers from MHC Monomers

When an MHC monomer is purchased, the monomer often has to be folded and tetramerized before it can be used for most tetramer applications such as identification of antigen-specific T cells. One procedure is shown below for class I tetramers, developed by the NIH Tetramer Core Facility at Emory University[1].

Reagents & Solutions

Reagents for 500ml reaction:

- 0.76825g reduced glutathione
- 0.15315g oxidized glutathione
- 0.5ml 200mM PMSF
- 15mg peptide
- 500ml DMSO
- 1.5mmol inclusion bodies (heavy chain)
- 1mmol human b2m inclusion bodies (light chain)

Folding buffer (pH 8.3):

- 42.14g L-Arginine (final concentration: 400mM)
- 50ml 1M Tris (final concentration: 100mM)
- 2ml 1.5M EDTA (final concentration: 2mM)
- ddH2O to 500ml
- adjust pH to 8.3

Injection Buffer (pH 4.2):

- 3M Guanidine HCl
- 10mM NaC2H3O2 (Sodium Acetate)
- 10mM EDTA

Day 1

1. Chill folding buffer. In a 1L glass Erlenmeyer flask equipped with a large stir-bar, chill 500ml of folding buffer to 10°C.

[1]http://tetramer.yerkes.emory.edu/support/protocols#4.

2. Add first three reagents to the folding buffer. Add the reduced glutathione, oxidized glutathione and PMSF to the cold folding buffer.
3. Dissolve the peptide in DMSO. Weigh 15mg of peptide and add it to 500ml DMSO in an Eppendorf tube. Note: If the peptide is insoluble in DMSO, add successive small amounts of TCA (100% solution) (~.05ml) until it dissolves. Alternatively, it is possible to use TFA.
4. Add the peptide to the stirring reaction.
5. Load the inclusion bodies into two syringes. Load 500 nmol heavy chain and 1000 nmol hub2m into two separate disposable 3cc syringe using a 20 gauge needle.
6. Place the folding reactions on a stir plate and inject the inclusion bodies. Place the folding reaction on a stir plate set at high speed. Replace the 20g needle with a 26g needle. Forcefully inject the heavy chain and the light chain into the reaction as close to the stirring bar as possible.
7. Incubate the folding reaction overnight at 10°C. Shake the folding reaction in a refrigerated incubator/shaker at 70rpm and 10°C and incubate for 1-3 days. If you do not have access to equipment that can maintain the temperature at 10 °C, you can incubate the folding reactions at 4 °C, such as on a magnetic stir plate in a cold room.

Day 2

1. In the morning, load and inject heavy chain into the folding reaction. Load 500nmol heavy chain into a 3cc syringe using a 20gauge needle. Inject the heavy chain forcefully into the reaction as close to the stirring bar as possible.
2. Store the folding reaction at 70rpm and 10°C all day.
3. In the evening, load and inject heavy chain into the folding reaction. In the evening, load 500nmol heavy chain into a 3cc syringe using a 20g needle. Inject the heavy chain into the reaction as described above.
4. Incubate the folding reaction overnight at 10°C. Shake the folding reaction at 70rpm and 10°C. Store overnight. It is possible to incubate the folding reactions for a few additional days; deleterious side reactions—such as proteolysis of the BSP tag—appear to be very slow in folding buffer.

Example Monomer Application: CyTOF

Metal-Conjugated NeutrAvidin for MHC Multimer Assays Using Mass Cytometry.

Evan Newell et al.[2] adapted MHC-peptide tetramer technology to mass cytometry[3] for the purpose of screening of up to 109 different peptide–MHC tetramers in a single human blood sample, as well as analyzing another 23 markers of T cell phenotype and function using a recombinant form of streptavidin conjugated to metal tags (Fluidigm Maxpar® kits). The workflow is described below for enumeration and identification of CMV-specific CD8+ T cells with a) NeutrAvidin TM metal reagent complexed with HLA-A*0201 CMV pp65 biotinylated monomer (MBL International), b) metal-labeled surface markers, c) cisplatin dead cell identifier, and d) metal barcoding (4) of several samples. There are 198 different biotinylated monomers, commercially available from MBL International, which can be combined with up to 35 isotope-tagged NeutrAvidinTM reagents to design a highly multiparametric assay.

Within this workflow (see following), 10 markers were used for cell phenotyping and discrimination of dead cells, in combination with metal-tagged tetramer staining, enabled identification of CMV-specific cytotoxic T lymphocytes in donor PBMCs samples. Using the dual-barcoding system enabled a decrease in background staining of metal-tagged tetramers (e.g., B-cells).

[2]Newell, E.W., Sigal, N., Nair, N., et al. "Combinatorial tetramer staining and mass cytometry analysis facilitate T cell epitope mapping and characterization." Nat. Biotech. 31 (2013): 623–629.
[3]Bandura, D.R., Baranov ,V.I., Ornatsky, O.I. et al. "Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry." Anal. Chem. 81 (2009): 6813–22.

Table 1. Mass Cytometry panel

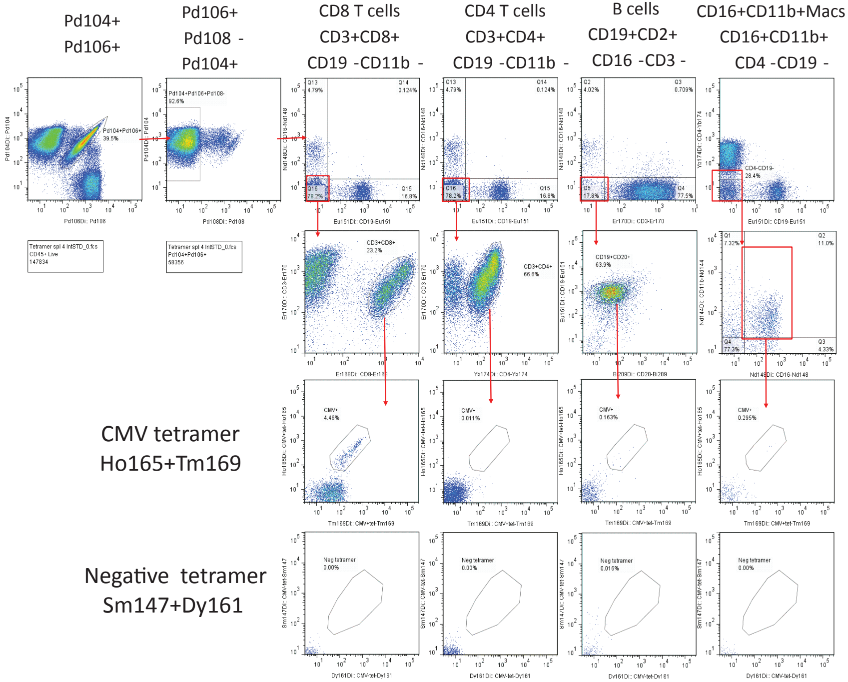
Channel	Isotope	Marker	Marker used for identification
144	Nd	CD11b	Cell population
148	Nd	CD16	Cell population
151	Eu	CD19	Cell population
168	Er	CD8	Cell population
170	Er	CD3	Cell population
174	Yb	CD4	Cell population
209	Bi	CD20	Cell population
147	Sm	CMV-neg.tetramer	Antigen-specific T cell
161	Dy	CMV-neg.tetramer	Antigen-specific T cell
165	Ho	CMV-pos.tetramer	Antigen-specific T cell
169	Tm	CMV-pos.tetramer	Antigen-specific T cell
171	Yb	CMV tetramers	Antigen-specific T cell
104	Pd	Barcode	Sample ID
106	Pd	Barcode	Sample ID
108	Pd	Barcode	Sample ID
198	Pt	Cell-ID stain	Dead cells

Table 2. Barcoding and tetramer staining of PBMCs samples

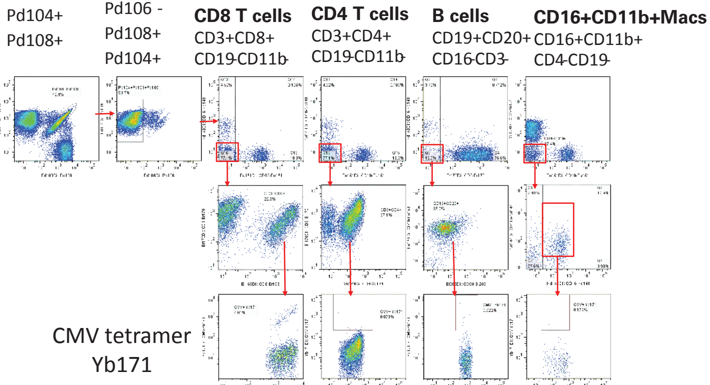
Sample	Barcodes	Tetramer identity	Tetramer tags
SPL1	Pd104, Pd106	CMV	Ho165 + Tm169
		Negative	Sm147 + Dy161
SPL2	Pd104, Pd108	CMV	Yb171
SPL3	Pd106, Pd108	Negative	Yb171

All samples were stained with the same surface markers. Plots are presented with data normalized to EQ beads and gated on CD45-positive, Cisplatin-negative cells

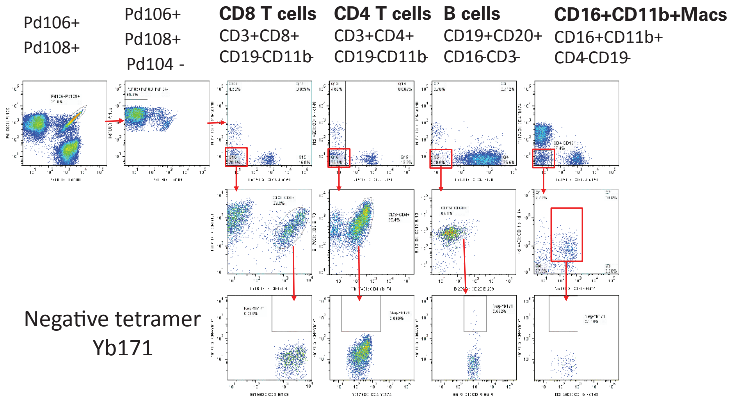
SPL1



SPL2



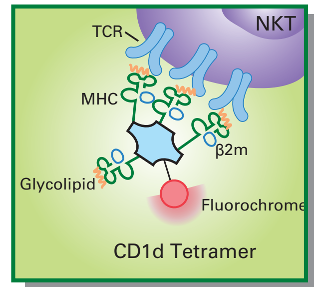
SPL3

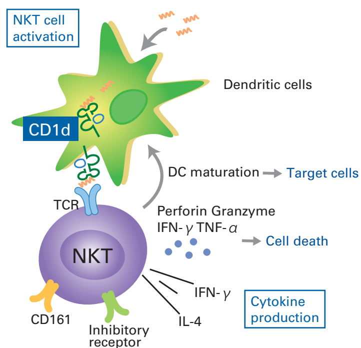


CD1d Tetramer

CD1d is a membrane protein non-covalently bonded to β2-microglobulin (β2m) and shows high homology between human and mouse. CD1d can present α-galactosylceramide (α-GalCer), a glycolipid extracted and isolated from the marine sponge, and this complex can activate human and murine CD1d-restricted NKT cells. CD1d Tetramer is a reagent prepared by tetramerization of complexes of CD1d and β2m by PE- or APC- labeled streptavidin. Binding this reagent to α-GalCer enables highly sensitive detection of CD1d-restricted NKT cells and can be combined with antibodies to study NKT cell function by flow cytometry.

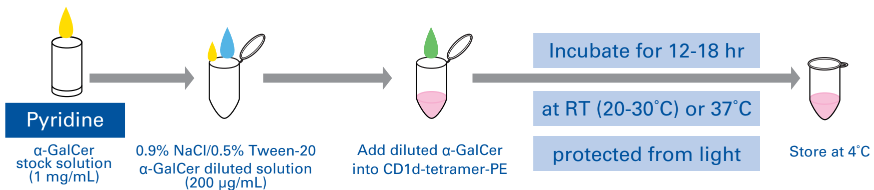
1) Stephane S, et al., J. Immunol. Methods 268: 107–121 (2002)
2) Matsuda JL, et al., J. Exp. Med. 192: 741–754 (2000)
3) Benlagha K, et al., J. Exp. Med. 191: 1895–1903 (2000)





Dissolving and loading α-GalCer on CD1d Tetramer

*This product does NOT include α-GalCer

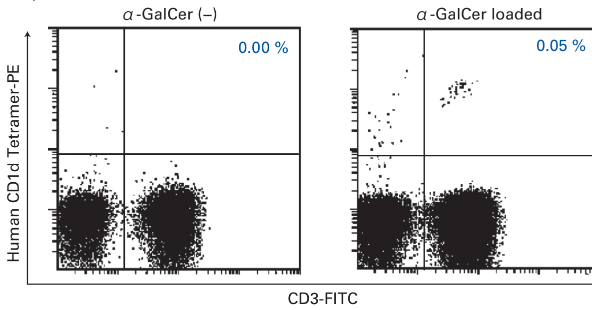


Detection of NKT cells with CD1d Tetramer

Product	α-GalCer unloaded	α-GalCer loaded
	MBL code no.	MBL code no.
Human CD1d Tetramer-PE	TS-HCD-1	TS-HCG-1
Human CD1d Tetramer-APC	TS-HCD-2	TS-HCG-2
Mouse CD1d Tetramer-PE	TS-MCD-1	TS-MCG-1
Mouse CD1d Tetramer-APC	TS-MCD-2	TS-MCG-2

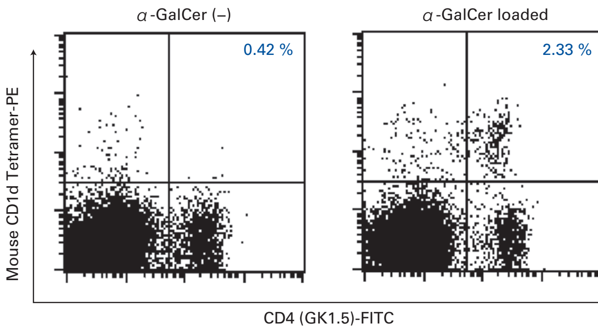
Detection of human NKT cells with CD1d Tetramer

PBMC were separated from peripheral blood of healthy subjects and incubated at room temperature for 5 minutes with 40 μL of Clear Back (Human Fc receptor blocking reagent, MBLI code no. MTG-001). CD3-FITC and human CD1d Tetramer-PE (with or without binding of α-GalCer) were added and incubated for 30 minutes at 4°C protected from light, and cells were analyzed by flow cytometry. Results showed total cells contained 0.05% NKT cells, as defined by CD1d/CD3 dual positivity.



Detection of murine NKT cells with CD1d Tetramer

C57BL/6 mouse splenocytes were stained with CD4-FITC and mouse CD1d Tetramer-PE (with or without binding of α-GalCer) for 30 minutes at 4°C protected from light, and analyzed by flow cytometry. Results showed splenocytes contained 2.33% NKT cells, as defined by CD1d/CD4 dual positivity.



Can I dissolve α-GalCer in DMSO instead of Pyridine?

Pyridine is recommended, as it has been shown that tetramer staining can be lost when the tetramer is loaded with α-GalCer dissolved in DMSO.

Can α-GalCer be stored when diluted to 200 ug/ml?

Storing α-GalCer diluted to 200 μg/mL is not recommended, as reactivity has been shown to decrease.

What is the shelf life of the CD1D tetramer?

CD1d tetramers are stable for at least 12 months when properly stored, and should be discarded when increased background or other signs of deterioration are noted.

What percent positive is expected for CD1d?

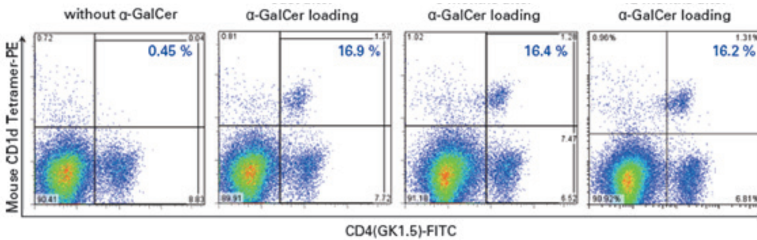
In a study of 20 healthy donors, Gumperz et al. reported a CD1d-positive range of undetectable (<0.01%) to 2.34% of CD3+ cells, with a median of 0.034% and a mean of 0.194%, when the tetramer was loaded with α-GalCer (J. Exp. Med. 2002; 195: 625). In a comparison of various mouse strains, Hammond et al. showed CD1d tetramer-positive cells varied depending on the tissue source, ranging from 0.5-1.4% in spleen/lymph node samples and higher (≥20%) in liver (J. Immunol.2001; 167:1164-1173).

What blocking reagents are recommended for use with cd1d tetramers?

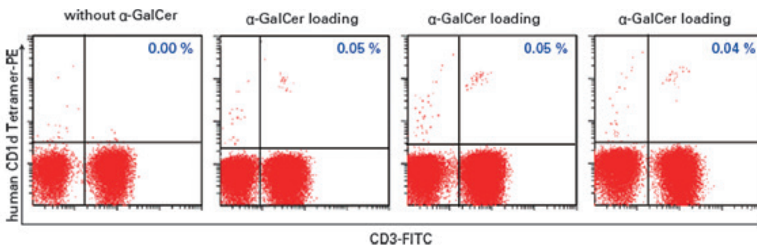
We recommend using a blocking reagent such as Clear Back (MBLI code no. MTG-001) to block non-specific binding of monocyte-derived cells in both human and mouse specimens.

Can α-GalCer loaded CD1d tetramer be stored at -4°C?

Yes. The reactivity of α-GalCer is stable for at least 12 months for both the human and mouse reagents, as shown in the figure below.



The value at the top right of the figure indicates the percentage of Mouse CD1d Tetramer positive cells (%) of CD4 positive cells



The value at the top right of the figure indicates the percentage of Mouse CD1d Tetramer positive cells (%) of CD4 positive cells.

In both cases, reactivity was similar to that of the CD1d Tetramer stored without loading of α-Galcer at 4°C and loaded with α-Galcer on the day before the reactivity estimation.

High Throughput Immunogenic Peptide Discovery and Validation in 90 min

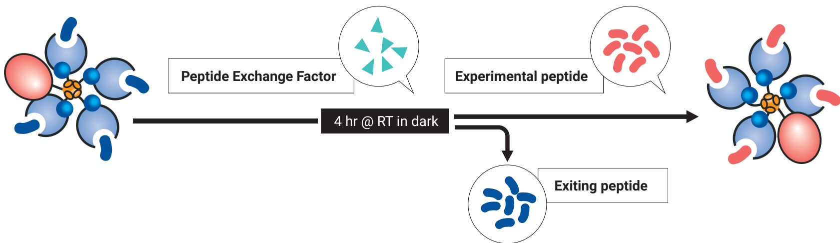
Functional screening of peptides for MHC class I binding is essential for vaccine design and immune monitoring. A rapid, high throughput and user friendly assay system that has the potential for clinical immune monitoring is needed now more than ever.

QuickSwitch™ is a 90-min-assay system that allows discrimination of MHC binding from non-binding peptides. This is particularly essential for the screening of immunogenic peptides from infectious agents or cancer neoantigens. Tetramers resulting from peptide exchange with selected peptides can then be used for immune monitoring.

QuickSwitch™ Platform Capabilities:

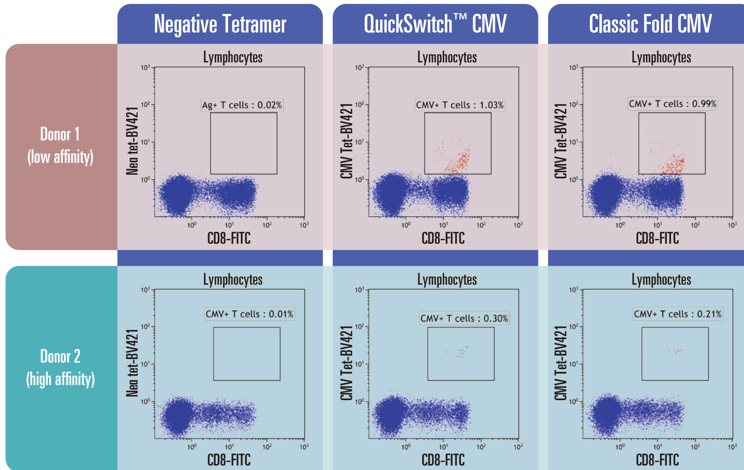
- Validate MHC binding peptides from in silico selected list of candidate peptides
- Generate new specificity tetramers for immune monitoring
- Perform functional stability studies for MHC binding peptides
- Compare epitopes to rank better binders and perform epitope mapping

Peptide exchange, quantification, cell staining, and flow cytometry analysis can all be performed in one day!



Peptide exchange to generate new specificity tetramers	Peptide exchange quantitation assay	Flow analysis of peptide exchange	Cell staining with new specificity tetramers	Flow analysis
4 hours	1.5 hours	45 min	45 min	1.5 hours
8 hours				
Hands on time				
15 min	20 min	20 min	15 min	30 min

QuickSwitch™ tetramers detect similar percentages of low and high affinity CMV responses in PBMCs as classically folded tetramers

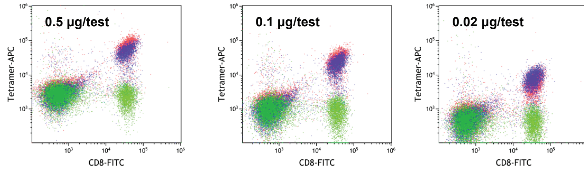


QuickSwitch™ Quant Kit can be used to assess peptide exchange so that you can select peptides with appropriate affinities to make functional tetramers prior to cell staining. In a study where HLA-A*02:01 QuickSwitchTM tetramer was incubated for 4 hours with two Mart-1 related peptides at a final concentration of 20 µM in presence of peptide exchange factor #1, peptide exchange correlated with the theoretical peptide affinity of each peptide towards HLA-A*02:01.

QuickSwitch™ Quant kit components:

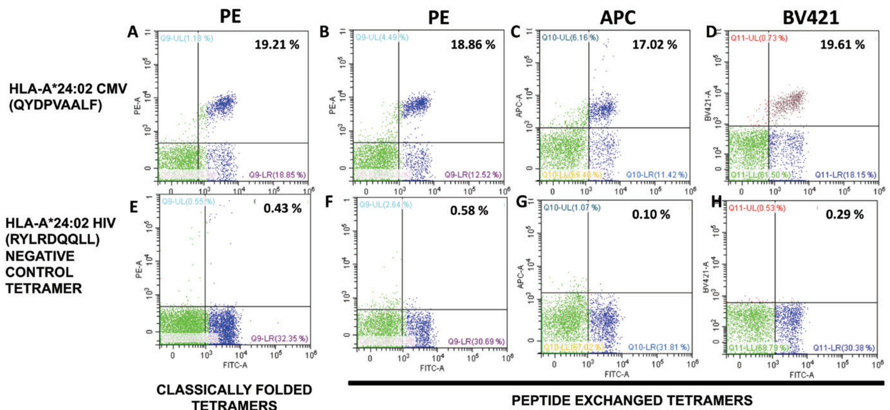
- Tetramer with an irrelevant exchangeable peptide in the MHC groove
- A peptide exchange factor for catalyzing the peptide exchange reaction
- A high affinity MHC-binding reference peptide used as peptide exchange positive control
- FITC conjugated antibody specific to the exiting peptide
- Magnetic beads conjugated with anti-MHC antibody for tetramer capture
- An Assay Buffer for diluting reagents and washing steps
- Aluminum foil
- Distilled or purified water
- DMSO
- Peptides for new specificity tetramers

H2-Kb Peptide-exchanged tetramers perform similarly to classically folded tetramers



H-2 Kb TRP2 used a negative control (#TB-5004-2; green), classically folded H-2 Kb OVA (#TB-5001-2; blue), and H-2 Kb QuickSwitch™ OVA (red) tetramer staining.

QuickSwitch™ HLA-A*24:02 tetramers perform similarly to classically folded tetramers



Data show CD3+ PBMCs stained with classically folded tetramers (A,E) or with QuickSwitch™ tetramers obtained by peptide exchange with the HLA-A*24:02 CMV peptide (B,C,D) or the HIV negative control peptide (F,G,H). 2 x 105 cells in 50 µL PBS-BSA-NaN3 buffer were stained for 30 min at RT with 1 µL of anti CD3-PC5.5 mAb (clone OKT3), 1 µL anti CD8-FITC (clone RPA-T8) (MBL) and 0.25 µg tetramer. Cells were fixed with a 0.5% formaldehyde PBS solution. Cells were analyzed on a Cytoflex S flow cytometer (Beckman Coulter). Cell doublets were discriminated using SSC-W/SSC-A gating.

MATERIALS REQUIRED BUT NOT SUPPLIED

- Flow cytometer
- Plate shaker (Labline model 4625 or equivalent)
- Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
- Magnetic tray for microplate (Note 2)
- Vortex
- Calibrated adjustable precision single channel micropipettes (for volumes between 1 μL and 1000 μL) with disposable tips
- Round or conical bottom microplates
- Microtubes
- Aluminum foil
- Distilled or purified water
- DMSO
- Peptides for new specificity tetramers

TEST PROCEDURE

Carefully read this protocol before performing an assay. Bring all the reagents to room temperature prior to start and centrifuge briefly to pull liquid to the bottom of the tubes.

A. Generation of New Specificity Tetramer Using Peptide Exchange

Prior to performing the assay, bring to room temperature Peptide Exchange Factor and peptides to be used in the assay.

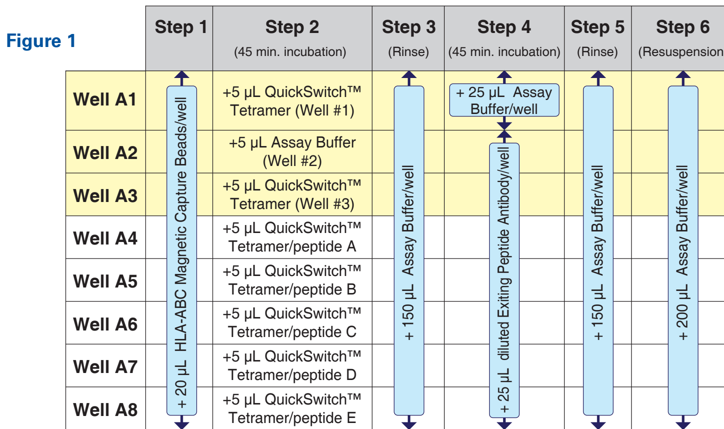
1. Dissolve each lyophilized peptide to be assayed in DMSO to a 10 mM solution (~10 mg/mL for a 9 amino acid peptide). (Note 3) Aliquots of this peptide solution can be further diluted in water to the desired concentration. For high affinity peptides, a 1 mM stock solution is a reasonable starting concentration for the assay. For lower affinity peptides, a higher concentration may be necessary, but may cause tetramer aggregation.
2. Pipet 50 μL of QuickSwitch™ Tetramer into a microtube or well of round- or conical-bottom 96 well microtiter plate.
3. Add 1 μL of peptide and mix gently with pipetting. (Note 4)
4. Add 1 μL of Peptide Exchange Factor from green capped vial and mix gently with pipetting.
5. Repeat steps 1-4 for each additional peptide, including the Reference Peptide, if desired. (Note 5)
6. Incubate at least for 4 hours at room temperature protected from light.
7. Tetramers are now ready for use in quantitation (see section B) and/or staining assays. (Note 6) Tetramers generated with the Reference Peptide are used as a positive control for exchange quantitation (see Section B).
8. Refrigerate tetramers at 2-8°C protected from light when not used.

Note that peptide exchange reaction volumes can be scaled up or down, so long as reagent proportions are maintained.

B. Quantification of Peptide Exchange Using Flow Cytometric Sandwich Immunoassay

1. Prepare 1x Assay Buffer as follows: for 1-5 peptide exchanges, prepare 7.5 mL by mixing 750 µL of 10x concentrated Assay Buffer with 6.75 mL of distilled water. For 6-10 exchanges, double the volumes.
2. Immediately before use, vortex the tetramer capture beads for 30 seconds, followed by a 30-second sonication in a water bath sonicator. If no sonicator is available, vortex an additional 30 seconds.

Figure 1 describes a capture assay in which five peptide-exchanged tetramers are tested. The yellow-filled wells are dedicated to controls which must be included in every assay.



Step 1 (Dispensing capture beads).

1. Into each of three wells of a round or conical-bottom 96 well microtiter plate, pipet 20 µL Magnetic Capture Beads for essential controls.
2. Pipet 20 µL Magnetic Capture Beads to additional wells for each peptide-exchanged tetramer to test.

Step 2 (Tetramer capture).

1. Pipet 5 µL 1x Assay Buffer in well #2.
2. Pipet 5 µL QuickSwitch™ Tetramer in wells #1 and #3.
3. In well #4, pipet 5 µL taken from the first peptide exchange microtube. Repeat for each additional peptide exchange in adjacent wells.
4. Shake plate for 45 min. at 550 rpm, protected from light with an opaque cover such as a piece of aluminum foil.

Step 3 (Rinse).

1. Dispense 150 µL of 1x Assay Buffer in each well.
2. While holding microplate tightly to the magnet, flick the plate and blot on a paper towel to minimize cross-contamination of wells. After returning plate upright, vortex for 2 seconds to disperse the beads.

3. While holding microplate tightly to the magnet, flick the plate. After returning plate upright, vortex for 2 seconds to disperse the beads.

Step 4 (Bead incubation with Exiting Peptide Antibody).

1. Dilute 25x Exiting Peptide Antibody to 1x as follows: Determine the number (n) of samples to stain with the antibody, including controls #2 and #3. Add one (+1), to account for pipetting errors. In a microtube, pipet (n+1) x 24 µL of Assay Buffer and then add (n+1) x 1 µL of Exiting Peptide Antibody. Mix by pipetting.

2. Pipet 25 µL of 1x Exiting Peptide Antibody in all wells, except well #1.

3. Pipet 25 µL of 1x Assay Buffer in well #1.

4. Shake plate for 45 min. at 550 rpm, protected from light.

Step 5 (Rinse).

1. Wash with 150 µL/well of 1x Assay Buffer as in Step 3.

Step 6 (Flow Acquisition).

1. Resuspend beads in 200 µL 1x Assay Buffer buffer and acquire on a low cytometer, ideally within 3 hours, collecting at least 300-500 events per sample in order to obtain reliable data.

FLOW CYTOMETRY SET UP AND DATA ANALYSES

1. Pipet 5 µL of Magnetic Capture Beads from the red cap vial to a llow cytometer tube containing 200 µL 1x Assay Buffer and run as a "beads only" control.

2. Adjust FSC and SSC voltages, gains, and threshold such that bead events are on scale.

3. Gate singlet beads based on FSC and SSC parameters, excluding doublets and aggregates (Fig. 2).

4. Set voltages and gains for FITC and second fluorochrome (PE, APC or BV421) such that "beads only" mean fluorescence intensities (MFI) are in the first log decade (Fig. 3). Note the MFI of the FITC channel (MFI^{FITC}).

5. Run control #1 (bead-captured QuickSwitch™ Tetramer), adjusting compensation such that the MFI^{FITC} of bead control #1 equals the MFI^{FITC} of the "Beads Only" control (see Fig. 4A, uncompensated, and Fig. 4B, compensated). Values shown are for demonstration purposes only and will vary based on experiment and low cytometer.

FIG. 2

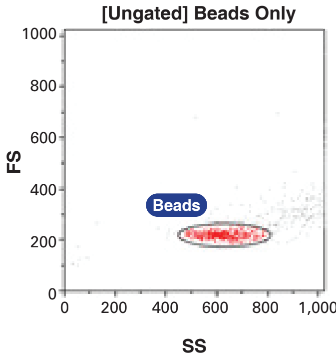
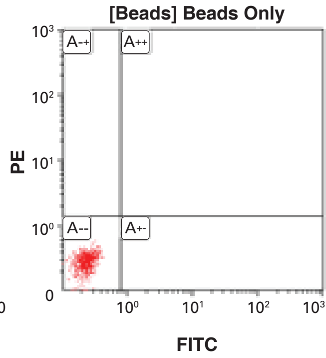
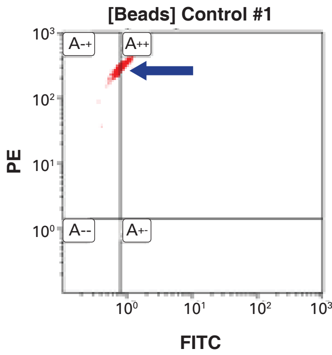


FIG. 3



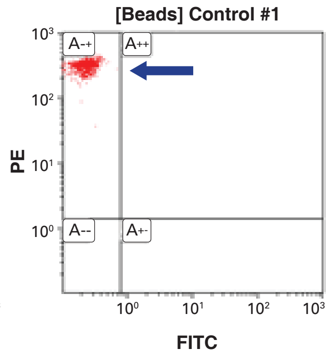
Gate	X-G Mean	Y-G Mean
A--	0.23	0.27
A-+	N/A	N/A
A+-	N/A	N/A
A++	N/A	N/A

FIG. 4A



Gate	X-G Mean	Y-G Mean
A--	N/A	N/A
A-+	0.70	251.43
A+-	N/A	N/A
A++	0.91	328.63

FIG. 4B



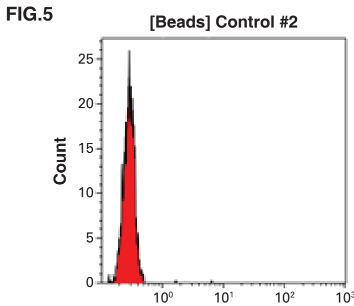
Gate	X-G Mean	Y-G Mean
A--	N/A	N/A
A-+	0.23	305.33
A+-	N/A	N/A
A++	N/A	N/A

6. Run control #2, beads that have not captured any tetramer and therefore have no Exiting Peptide. The low MFI^{FITC} corresponds to 0% Exiting Peptide or 100% peptide exchange (Fig. 5). Note the MFI^{FITC}.

7. Run control #3, beads that have captured the QuickSwitch™ Tetramer, which have an MFI^{FITC} that corresponds to 100% Exiting Peptide or 0% peptide exchange (Fig. 6). Note the MFI^{FITC}.

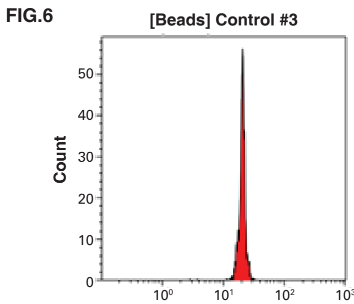
8. Run samples from well #4 and subsequent peptide exchange samples, noting the MFI^{FITC} of each. Peptide-exchanged tetramers will display various Exiting Peptide amounts, which are inversely proportional to the newly loaded peptide on the MHC molecules. Consequently the measured MFI^{FITC} will be intermediary between MFI values obtained with bead controls #2 and #3 (Fig. 7).

FIG.5



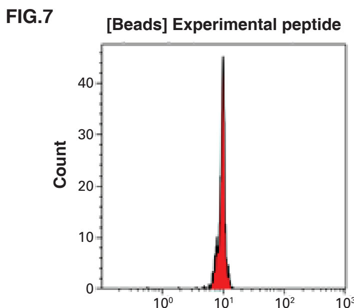
Gate X-GMean
All 0.28

FIG.6



Gate X-GMean
All 20.38

FIG.7



Gate X-GMean
All 9.37

CALCULATION OF RESULTS USING QUICKSWITCH™ DOWNLOADABLE CALCULATOR

The QuickSwitch™ Calculator on the MBL International website (https://www.mblintl.com/quickswitch-peptide-exchange-calculator/) can be downloaded for determining percentages of peptide exchange, as shown in the example below using HLA-A*02:01 QuickSwitch™ Tetramer and corresponding peptides (Tables 1-2).

1. Enter the MFI^{FITC} associated with bead controls #2 and #3.
   Table 1

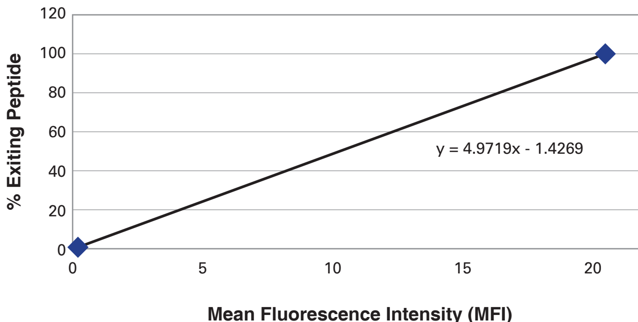
Analyzed sample	MFI_{FITC}
Control #2: 0% Exiting Peptide (100% peptide exchange)	0.28
Control #3: 100% Exiting Peptide (0% peptide exchange)	20.38

2. Enter the MFI obtained with the different tests (2nd column) to obtain the percentages of peptide exchange. Note that the calculator provides results only for MFI values below control #3. Higher values will return a "FALSE" response, as indicated in row E.
   Table 2

Peptide Sample	QuickSwitch Tetramer MFI_{FITC} after Peptide Exchange	% Peptide Exchange
A	9.37	54.78
B	5.29	75.07
C	2.12	90.85
D	1.29	94.98
E	22	FALSE
F	0.11	100.45

CALCULATION OF RESULTS USING EXCEL OR OTHER SOFTWARE

1. Generate a linear curve by plotting the MFI^{FITC} obtained with controls #2 and #3 against percent Exiting Peptide detected, 0% and 100%, respectively, as shown in the example below using the HLA-A*02:01 QuickSwitch™ Tetramer (Fig. 8).
   Fig. 8



2. Use the linear curve equation for calculating the percentages of peptide exchange by entering the MFI^{FITC} of each peptide-exchanged sample as the variable (X), as shown in the example below using the HLA-A*02:01 QuickSwitch™ Tetramer (Table 3).
   Table 3

Analyzed sample	MFI (X)	% of Exiting Peptide (Y)	% peptide exchange (100-Y)
Control #2	0.28	0	100
Control #3	20.38	100	0
Test peptide	9.37	**45.22**	**54.78**

USE OF THE REFERENCE PEPTIDE

The Reference Peptide included in the kit serves as a positive control for peptide exchange of the QuickSwitch™ Tetramer.

Percentage of peptide exchange obtained with the Reference Peptide for HLA-A*02:01 is shown in Table 4.

	Reference Peptide
Stock Concentration	1 mM
Final Concentration	20 µM
Peptide Exchange (N=3)	91.9 ±1.8 %

LIMITATIONS

1. The QuickSwitch™ Quant Tetramer Kit has been devised mainly for exploratory research such as testing whether a peptide binds to MHC or for quickly determining presence/absence of an MHC/peptide specific CD8+ T cell population in donor leukocytes. These tetramers are not intended to be a substitute for tetramers classically manufactured by folding of peptide with MHC and tetramerization with fluorochrome-conjugated streptavidin (Note 7).

2. Once diluted, the Exiting Peptide Antibody is stable at room temperature for up to 6 hours (protect from light).

3. Do not mix components from other kits and lots.

NOTES

Note 1. Tetramers bind to T cell receptors via three MHC/peptide monomers. Therefore the minimal recommended peptide exchange percentage should be 75%. The QuickSwitch™ Tetramer concentration is 50 µg/mL, measured by MHC monomer content. Depending on the T cell receptor affinity towards the MHC/peptide complex, cell stainings require 0.5 ng to 2 µg tetramer per reaction.

Note 2. This current protocol uses a magnet to pellet the beads. It is possible to pellet by centrifugation using a plate holder or by suction using filter plates. The user will then have to modify the protocol accordingly.

Note 3. Most of peptides are soluble in DMSO. However some highly basic or acidic peptides may precipitate in DMSO and would require alternative buffers.

Note 4. The final peptide concentration is 20 µM in this assay. The user may want to test higher or lower peptide concentrations as well. Higher concentrations may increase the percentage of peptide exchange but have the risk to trigger tetramer aggregation. In some cases, working with concentrations lower than 20 µM may be beneficial.

Note 5. The Reference Peptide can be included as a positive control for peptide exchange. As an example, the high affinity binding HLA-A*02:01 Reference Peptide typically undergoes a > 90% exchange when used a final 20 µM solution (see Table 4).

Note 6. Tetramers obtained by peptide exchange are used directly for cell staining. However, the user may want to dialyze the tetramers to remove excess peptide, which may interfere with staining or cause tetramer aggregation. MBL International recommends simultaneous staining of class I tetramer with anti-CD8 and other antibodies for 30 minutes at room temperature.

Determining Biological Activity of a Vaccine

As discussed in a scientific poster presented at the 2016 American Association of Immunologists conference, the QuickSwitch™ Kit was used to determine the biological activity of a vaccine, DPX-Survivac, an ovarian cancer vaccine candidate. The QuickSwitch™ Quant HLA-A*02:01-PE Kit was used to assess the biological activity of SurA2.M, a HLA-A2 restricted peptide, in DPX-Survivac. The detection of the peptide was tested individually in a buffered solution or in the DPX-Survivac vaccine prepared in an aqueous formulation. Results indicate that the peptide exchange of SurA2.M is similar whether it is dissolved individually in a buffered solution or mixed with other components of the vaccine. Results may be dependent on the affinity of the peptide for HLA-A2. By optimizing a concentration curve using individual peptides, the QuickSwitch™ Quant HLA-A*02:01-PE Kit can be used to quantify the concentration of HLA-A2 restricted peptides in simple solutions or more complex formulations.

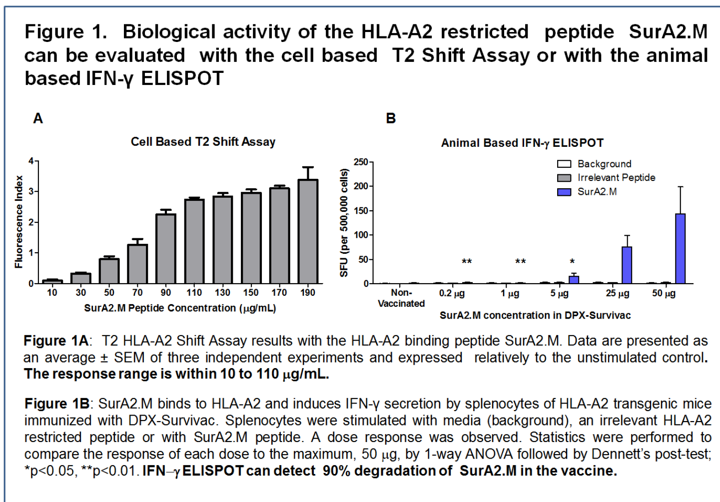


Figure 1. Biological activity of the HLA-A2 restricted peptide SurA2.M can be evaluated with the cell based T2 Shift Assay or with the animal based IFN-γ ELISPOT

Figure 1A: T2 HLA-A2 Shift Assay results with the HLA-A2 binding peptide SurA2.M. Data are presented as an average ± SEM of three independent experiments and expressed relatively to the unstimulated control. **The response range is within 10 to 110 µg/mL.**

Figure 1B: SurA2.M binds to HLA-A2 and induces IFN-γ secretion by splenocytes of HLA-A2 transgenic mice immunized with DPX-Survivac. Splenocytes were stimulated with media (background), an irrelevant HLA-A2 restricted peptide or with SurA2.M peptide. A dose response was observed. Statistics were performed to compare the response of each dose to the maximum, 50 µg, by 1-way ANOVA followed by Dennett's post-test; *$p<0.05$, **$p<0.01$. **IFN–γ ELISPOT can detect 90% degradation of SurA2.M in the vaccine.**

Figure 3. QuickSwitch™ assessment of HLA-A2 binding peptides as individual reagents or as constituents of Survivac vaccine.

A

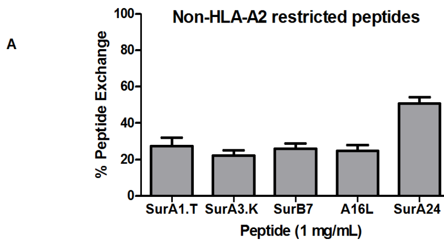


Figure 3A: QuickSwitch™ Quant Tetramer assay was performed using 1 mg/mL of non-HLA-A2 restricted peptides SurA1.T, SurA3.K, SurB7, A16L and SurA24. Note: the SurA24 peptide is an HLA-A24 high affinity binder and displays an intermediate binding affinity towards HLA-A2. Data shown as an average ± SEM of three independent repeats.

B

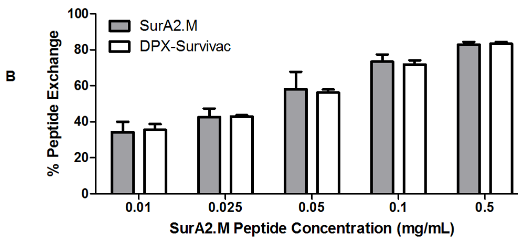


Figure 3B: Specificity and Selectivity of QuickSwitch™ Quant Tetramer assay was evaluated using DPX-Survivac vaccine. DPX-Survivac contains HLA–A2 restricted (SurA2.M) and non-HLA-A2 restricted peptides (SurA1.T, SurA3.K, SurA24, SurB7, A16L) in the lipid environment. Results demonstrate that QuickSwitch™ Quant Tetramer assay can be used to evaluate HLA-A2 restricted peptide SurA2.M in the presence of a vaccine matrix. Data shown as an average ± SEM of three independent repeats. Student's t-test was performed to compare the levels of peptide exchange for DPX-Survivac to that for SurA2.M peptide at each concentration level; no statistical differences detected $p<0.05$.

Developing CD8+T-Cell Based Immunotherapies

There is currently a great interest in discovering novel peptide sequences from pathogens or tumors (neo-epitopes) that can be used for developing various CD8+T cell-based immunotherapies. The combination of whole-exome and transcriptome sequencing analysis with mass spectrometry now permits rapid identification of a myriad of peptide candidates. As a first screening step, potential binders to MHC molecules can be determined using MHC class I prediction algorithms. However, these algorithms typically generate a large proportion of false binders, therefore requiring experimental validation. To make this task easier, the QuickSwitch™ Quant kit can both help determine binding of peptides to MHC class I molecules and generate MHC class I tetramers with these peptides for detection of peptide specific T cells. If the peptide is able to bind to the MHC molecule, then the neo-epitope could potentially be used in a CD8+ T cell based immunotherapy.

Figure 4. Peptides of various HLA-A2 affinities assessed with the QuickSwitch™ Quant assay.

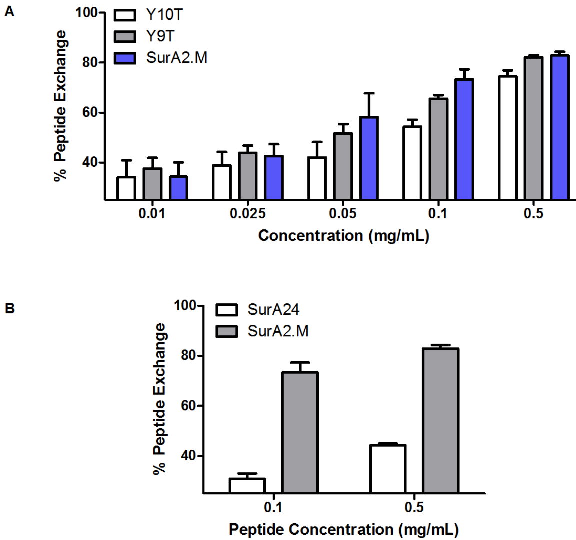


Figure 4A, B: The HLA-A2. QuickSwitch™ Quant Tetramer assay was performed using indicated peptide amounts. Data shown as an average ± SEM of three independent repeats. Predicted binding affinity was calculated using the IEDB artificial neural network method and presented in Table1. **Peptide Y9T binds to HLA-A2 with a higher affinity than Y10T which is reflected on peptide exchange yields across multiple concentrations. Peptide SurA2.M which behaves like peptide Y9T also displays a very high affinity towards HLA-A2. Peptide SurA24, an HLA-A24 high affinity binder, displays an intermediate binding affinity towards HLA-A2, in agreement with the QuickSwitch™ Quant assay data.**

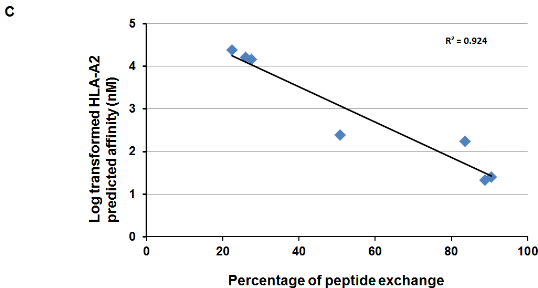


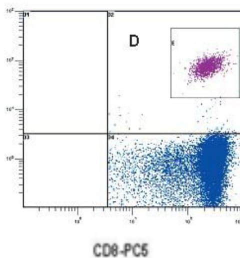
Figure 4C: Log transformed peptide HLA-A2 binding affinities from table 1 were plotted against the measured peptide exchanges at the concentration of 1 mg/mL. **Predicted HLA-A2 binding affinities correlate with values obtained with the QuickSwitch™ Quant assay.**

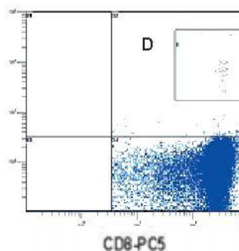
MBL International has extensive expertise in tetramer and monomer development and strives to meet the unique and evolving needs of our customers. MBL International offers custom allele development, custom specificities and other services to support our customers. Please do not hesitate to reach out to us to inquire about our capabilities and how we can support your research.

- Custom Tetramers for Novel Applications
- Custom Specificity Development
- Custom Allele Development
- Novel Immune Monitoring and Screening Solutions

Detection of gag-specific CD8+ T cells in whole blood

Whole blood samples were stained with anti-CD8-PC5 (clone B9.11) and PE-labeled Mamu-A*01 SIV gag (CTPYDINQM) or Irrelevant tetramer. Gating strategy included a singlet gate based on FSC-H and FSC-A, followed by a CD3+ gate and FSC x SSC gate. Region D represents the antigen-specific CD8+ T cells detected by the tetramer.





Other Applications of MHC Tetramers

Eliminating Antigen-Specific T Cells Using Tetramers

Ellen M. Leitman et al. 5 describes the use of tetramers coupled to a toxin (saporin) to selectively kill antigen-specific T cells. These tetramers have the potential to be used in immunotherapy and vaccine development.

MHC Microarrays for Antigen-Specific T Cell Detection

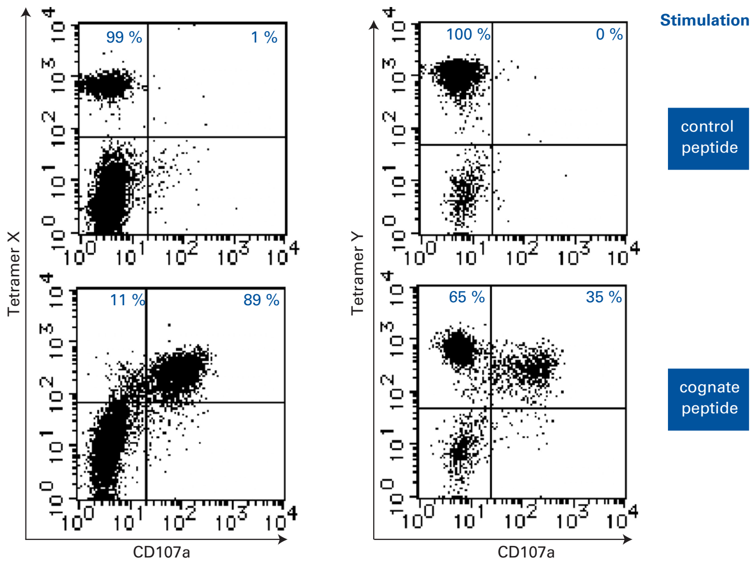
A scientific review authored by Amalie Kai Bentzen and Sine Reker Hadrup of the Technical University of Denmark[6] analyzes the use of pMHC microarrays to screen in parallel multiple antigen-specific T cell specificities. One researcher documented the ability to screen 30-40 different antigen-specific T cell specificities in parallel.

DNA Barcoding for Antigen-Specific T Cell Detection

Amalie Kai Bentzen and Sine Reker Hadrup of the Technical University of Denmark[6] describe the use of DNA tags instead of fluorescent or metal tags. DNA barcodes can give up to 1010 unique tags. This method has been used to screen 1000 different specificities within a single sample. However, a limitation of this methodology is that it can only provide an estimate of the number of antigen-specific T cells rather than a precise number.

[5]Leitman EM, Palmer CD, Buus S, et al. Saporin-conjugated tetramers identify efficacious anti-HIV CD8+ T-cell specificities. PLoS ONE. 2017;12(10):e0184496.
[6]Bentzen AK, Hadrup SR. Evolution of MHC-based technologies used for detection of antigen-responsive T cells. Cancer Immunol Immunother. 2017;66(5):657-666.

Measurement methods for antigen-specific CTL using MBL International Kits



*This kit does not include CFSE

IMMUNOCYTO CD107a Detection Kit

When activated by antigen stimulation, CTL release cytotoxic factors including perforin and granzyme from intracellular granules. During this process, CD107a (LAMP-1) in the inner membrane of intracellular granules is exposed on the cell membrane. IMMUNOCYTO CD107a Detection Kit (MBLI code no. 4844) enables indirect measurement of CTL cytotoxicity by detecting CD107a mobilization. While intracellular flow staining for IFN-γ has been found to be more sensitive than CD107a mobilization, a recent study conducted by MBL International identifying new CT epitopes showed the latter better correlated with MHC Tetramer staining. In addition to being more specific, the CD107a mobilization assay is more rapid than cytokine flow cytometry.

IMMUNOCYTO Cytotoxity Detection Kit*

T cells and NK cells (effector cells) directly recognize virus-infected and tumor cells (target cells) and can respond with cytotoxic activity to injure or kill target cells. While the ^{51}Cr release assay has been a reliable method to measure cytotoxic activity, special training and facilities for radioisotopes are required. Therefore, methods to measure cytotoxic activity without using radioisotopes have been developed. The principle to detect cytotoxic activity of effector cells using IMMUNOCYTO Cytotoxity Detection Kit (MBLI code no. AM-1005) is to double stain the target cells with both CFSE and Kusabira-Orange labeled Annexin V, and measure cytotoxic activity by flow cytometry. Target cell death (as a consequence of effector cell cytotoxicity) is measured based on the ratio of cells stained with Annexin V to cells stained with CFSE, as described below. Because the effector cells are not stained with CFSE, they are easily distinguished by flow cytometry for analysis. This non-radioactive method is reported to have high correlation with the ^{51}Cr release assay making it a safer alternative.

Principle of apoptosis detection by Annexin V

Phosphatidylserine (PS) located on the inner side of the lipid bilayer is exposed on the cell surface in apoptotic cells due to change in membrane structure. Annexin V binds with high affinity to PS in a Ca^{2+}-dependent manner, effectively labeling apoptotic cells (Figure 1). By using fluorescently-labeled Annexin V, the target cells damaged by effector cells can be detected by flow cytometry. IMMUNOCYTO Cytotoxity Detection Kit contains fluorescent protein Kusabira-Orange (KO; Excitation Max 548 nm/Emission Max 559 nm)-labeled Annexin V.

Data analysis by flow cytometry

Cytotoxic activity measured by IMMUNOCYTO Cytotoxity Detection Kit is shown for specific and negative control targets. Effector cell (E) to target cell (T) ratios are indicated in Figure 2. When killing takes place, CFSE-single positive target cells become dual positive for CFSE and Annexin V. Cytotoxic activity can be calculated from percentages of single and dual positive cells using the following equation:

$$\text{Cytotoxicity (\%)} = [(ET-T_0)/(100-T_0)]\times 100$$

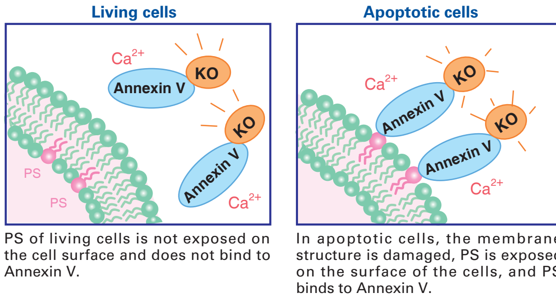
ET: % CFSE+ Annexin V+ cells when effector cells and target cells are co-cultured.
T_0: % CFSE+ Annexin V+ cells when only target cells are cultured.

*This kit does not include CFSE

Graphs showing cytotoxic activity

Graphs showing cytotoxic activity by HLA-A*24:02 CMV pp65 CTL line against various targets illustrate the specificity of CTL killing, as well as the effect of different E:T ratios (Figure 3).

Figure 1



PS of living cells is not exposed on the cell surface and does not bind to Annexin V.

In apoptotic cells, the membrane structure is damaged, PS is exposed on the surface of the cells, and PS binds to Annexin V.

Figure 2

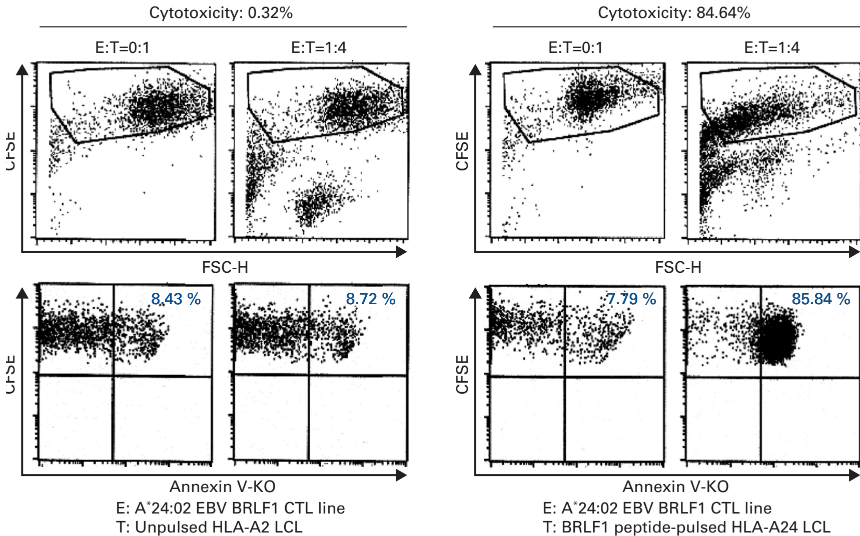
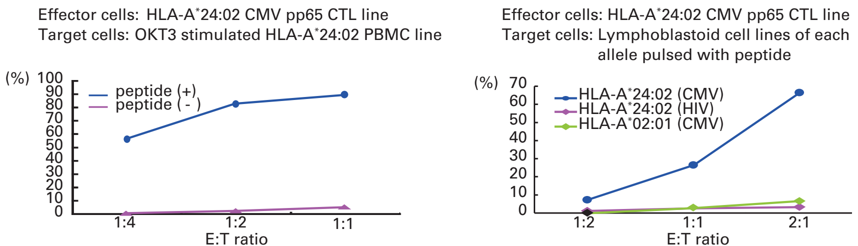


Figure 3



MBL International offers stock and custom peptides that can be used for stimulation assays along with custom tetramer generation using the QuickSwitch™ custom tetramer kits. All peptides undergo a rigorous quality control process to ensure they are high grade including MS, LC-MS and HPLC analysis as well as COA documentation to ensure at least 95% purity.

A variety of sizes are available for custom peptides, from 1mg quantities up to 500mg and above. Peptide lengths can range from 2 amino acids to 100 amino acids depending on the peptide of interest.

MBL International offers over 150 stock peptides that are specific to the MHC tetramer product line and contain peptides for popular disease targets such as CMV, MuLV, melanoma, prostate cancer and other virology and cancer targets.

Flow Cytometry Reagents

MBL International offers over 2000 flow cytometry antibodies, isotype controls, RBC lysis buffers, permeabilization buffers and viability dyes. Popular targets such as CD4 and CD8 are available. MBL International antibodies have a high signal to noise ratio with bright fluorochromes.

Tetramer References

Human

Reagent	Reference
BMLF1	Ezinne CC, et al., PLoS One 9: e87631 (2014)
	Kühtreiber WM, et al., J Diabetes Metab 4: 9 (2013)
	Shultz LD, et al., PNAS 107: 13022–13027 (2010)
	Tomiyama M, et al., Anticancer Res. 24: 3327–3334 (2004)
	Sato K, et al., A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. Blood 117: 5663-5673 (2011)
	Watanabe K, et al., CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy. Int J Hematol. 88: 311-320 (2008)
BRLF1	Ezinne CC, et al., PLoS One 9: e87631 (2014)
	Sato K, et al., Blood 117: 5663–5673 (2011)
	Watanabe K, et al., Int J Hematol. 88: 311–320 (2008)
	Shultz LD, et al., PNAS 107: 13022–13027 (2010)
	Sato K, et al., A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. Blood 117: 5663-5673 (2011)
	Watanabe K, et al., CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy. Int J Hematol. 88: 311-320 (2008)
EBNA3A	Watanabe K, et al., CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy. Int J Hematol. 88: 311-320 (2008)
	Sato K, et al., A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. Blood 117: 5663-5673 (2011)
EBNA3B	Watanabe K, et al., CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy. Int J Hematol. 88: 311-320 (2008)
	Sato K, et al., A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. Blood 117: 5663-5673 (2011)
env	Tezuka K, et al., 123: 346-55 (2014)
	White Y, et al., Leuk Lymphoma 54: 2243-50 (2013)
	Tanaka T, et al., Clin Dev Immunol. 2013: 262967 (2013)
	Akiyama Y, et al., Anticancer Res. 29: 647–655 (2009)
	Tsukahara T, et al., Cancer Sci. 99: 368–375 (2008)
	Akimoto M, et al., J. Med Virol. 79: 977–86 (2007)
	Kozako T, et al., Hum Immunol. 72: 1001–1006 (2011)
	Kozako T, et al., J. Immunol. 177: 5718–5726 (2006)
	Kozako T, et al., Reduced frequency, diversity, and function of human T cell leukemia virus type 1-specific CD8+ T cell in adult T cell leukemia patients. J. Immunol. 177: 5718-5726 (2006)
	Akimoto M, et al., Anti-HTLV-1 tax antibody and tax-specific cytotoxic T lymphocyte are associated with a reduction in HTLV-1 proviral load in asymptomatic carriers. J. Med Virol. 79: 977-86 (2007)
	Akiyama Y, et al., Characterization of a MAGE-1-derived HLA-A24 epitope-specific CTL line from a Japanese metastatic melanoma patient. Anticancer Res. 29: 647-655 (2009)
	Akiyama Y, et al., Clinical response in Japanese metastatic melanoma patients treated with peptide cocktail-pulsed dendritic cells. J. Transl. Med. 3: 4-13 (2005)
	Akimoto M, et al., Anti-HTLV-1 tax antibody and tax-specific cytotoxic T lymphocyte are associated with a reduction in HTLV-1 proviral load in asymptomatic carriers. J. Med Virol. 79: 977-86 (2007)
	Tsukahara T, et al., Prognostic impact and immunogenicity of a novel osteosarcoma antigen, papillomavirus binding factor, in patients with osteosarcoma. Cancer Sci. 99: 368-375 (2008)

Human

Reagent	Reference
gag	Pahwa R, et al., J Infect Dis 193: 879-887 (2006)
gp100	Frleta D, et al., J Immunol 182: 2766-2776 (2009)
	Shi H, et al., J Immunol 176: 2134-2141 (2006)
	Morgan RA, et al., J Immunol 15: 3287-3295 (2003)
	Walker EB, et al., Clin Cancer Res 15: 2541-2551 (2009)
	Walker EB, et al., Clin Cancer Res 14: 5270-5283 (2008)
IE1	Gratama JW, et al., Blood 116: 1655-1662 (2010)
LMP1	Shultz LD, et al., Generation of functional human T-cell subsets with HLA-restricted immune responses in HLA class I expressing NOD/SCID/IL2r gamma(null) humanized mice. PNAS 107: 13022-13027 (2010)
LMP2	Sato K, et al., A novel animal model of Epstein-Barr virus-associated hemo-phagocytic lymphohistiocytosis in humanized mice. Blood 117, 5663-5673 (2011)
	Watanabe K, et al., CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy. Int J Hematol. 88, 311-320 (2008)
M1	Frleta D, et al., J Immunol 182: 2766-2776 (2009)
	Walker EB, et al., Clin Cancer Res 14: 5270-5283 (2008)
	He XS, et al., J Infect Dis 197: 803-811 (2008)
	Akiyama Y, et al., J. Transl. Med. 3: 4–13 (2005)
Mart-1	Uzana R, et al., J Immunol 188: 632-640 (2012)
	Favre D, et al., Blood 117: 2189-2199 (2011)
	Frleta D, et al., J Immunol 182: 2766-2776 (2009)
	Takahara M, et al., J. Leukoc. Biol. 83: 742–754 (2008)
	Weishaupt C, et al., Clin Cancer Res 13: 2549-2556 (2007)
	Shi H, et al., J Immunol 176: 2134-2141 (2006)
	Akiyama Y, et al., J. Transl. Med. 3: 4–13 (2005)
	Tomiyama M, et al., Anticancer Res. 24: 3327–3334 (2004)
pp50	Gratama JW, et al., Blood 116: 1655-1662 (2010)
	Brooimans RA, et al., Cytometry A 73: 992-1000 (2008)
pp65	Ezinne CC, et al., PLoS One 9: e87631 (2014)
	White Y, et al., Leuk Lymphoma 54: 2243-50 (2013)
	Chan WK, et al., J Immunol 191: 1625-1636 (2013)
	Favre D, et al., Blood 117: 2189-2199 (2011)
	Wang X, et al., Blood 117: 1888-1898 (2011)
	Brooimans RA, et al., Cytometry A 73: 992-1000 (2008)
	Walker EB, et al., Clin Cancer Res 14: 5270-5283 (2008)
	Watanabe K, et al., Int J Hematol. 88: 311–320 (2008)
	Sabouri AH, et al., Blood 112: 2411–2420 (2008)
	Heijnen IA, et al., Cytometry B Clin Cytom 62: 1-13 (2004)
	Mohty M, et al., J Immunol 171: 3385-3393 (2003)
	Gratama JW, et al., Blood, 98: 1358-1364 (2001)
	Watanabe K, et al., Int J Hematol. 88: 311–320 (2008)
	Ezinne CC, et al., PLoS One 9: e87631 (2014)
	White Y, et al., Leuk Lymphoma 54: 2243-50 (2013)
	Haruta M, et al., Hum Immunol 74: 1400-1408 (2013)
	Gratama JW, et al., Blood 116: 1655-1662 (2010)
	Brooimans RA, et al., Cytometry A 73: 992-1000 (2008)
	Watanabe K, et al., CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy. Int J Hematol. 88: 311-320 (2008)
	Sabouri AH, et al., Impaired function of human T-lymphotropic virus type 1 (HTLV-1)-specific CD8+ T cells in HTLV-1-associated neurologic disease. Blood 112: 2411-2420 (2008)

Human

Reagent	Reference
PR-1	Morita Y, et al., Int. J. Cancer 119: 1360–1367 (2006)
	Burchert A, et al., Blood 101: 259-264 (2003)
	Morita Y, et al., Monitoring of WT1-specific cytotoxic T lymphocytes after allogeneic hematopoietic stem cell transplantation. Int. J. Cancer 119: 1360-1367 (2006)
Survivin-2B	Kameshima H, et al., Immunogenic enhancement and clinical effect by type-I interferon of anti-apoptotic protein, survivin-derived peptide vaccine, in advanced colorectal cancer patients. Cancer Sci. 102: 1181-1187 (2011)
	Miyazaki A, et al., Phase I clinical trial of survivin-derived peptide vaccine therapy for patients with advanced or recurrent oral cancer. Cancer Sci. 102: 324-329 (2011)
	Kameshima H, et al., Immunogenic enhancement and clinical effect by type-I interferon of anti-apoptotic protein, survivin-derived peptide vaccine, in advanced colorectal cancer patients. Cancer Sci. 102: 1181-1187 (2011)
Tax	Ezinne CC, et al., PLoS One 9: e87631 (2014)
	White Y, et al., Leuk Lymphoma 54: 2243-50 (2013)
	Masaki A, et al., J Immunol 191: 135-144 (2013)
	Kozako T, et al., J. Med. Virol. 83: 501–509 (2011)
	White Y, et al., Leuk Lymphoma 54: 2243-50 (2013)
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	Ezinne CC, et al., PLoS One 9: e87631 (2014)
	Tanaka Y, et al., Cancer Res. 70: 6181–6192 (2010)
	Sabouri AH, et al., Blood 112: 2411–2420 (2008)
	Akimoto M, et al., J. Med Virol. 79: 977–986 (2007)
	Kozako T, et al., Programmed death-1 (PD-1)/PD-1 ligand pathway-mediated immune responses against human T-lymphotropic virus type 1 (HTLV-1) in HTLV-1-associated myelopathy/tropical spastic paraparesis and carriers with autoimmune disorders. Hum Immunol.
	Kozako T, et al., Target epitopes of HTLV-1 recognized by class I MHC-restricted cytotoxic T lymphocytes in patients with myelopathy and spastic paraparesis and infected patients with autoimmune disorders. J. Med. Virol. 83: 501-509 (2011)
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	Sabouri AH, et al., Impaired function of human T-lymphotropic virus type 1 (HTLV-1)-specific CD8+T cells in HTLV-1-associated neurologic disease. Blood 112: 2411-2420 (2008)
	Akimoto M, et al., Anti-HTLV-1 tax antibody and tax-specific cytotoxic T lymphocyte are associated with a reduction in HTLV-1 proviral load in asymptomatic carriers. J. Med Virol. 79: 977-86 (2007)
TRP2 (mouse)	Sato K, et al., Blood 117: 5663–5673 (2011)
	Watanabe K, et al., Int J Hematol. 88: 311–320 (2008)
Tyrosinase	Shi H, et al., J Immunol 176: 2134-2141 (2006)
	Akiyama Y, et al., J. Transl. Med. 3: 4–13 (2005)
WT-1 (mutant)	Saitoh A, et al., WT1 peptide vaccination in a CML patient: induction of effective cytotoxic T lymphocytes and significance of peptide administration interval. Med. Oncol. 28: 219-230 (2011)
	Chiba Y, et al., Effects of concomitant temozolomide and radiation therapies on WT1-specific T-cells in malignant glioma. Jpn. J. Clin. Oncol. 40: 395-403 (2010)
	Narita M, et al., WT1 peptide vaccination in combination with imatinib therapy for a patient with CML in the chronic phase. Int J Med Sci. 7: 72-81 (2010)
	Tsuboi A, et al., Wilms tumor gene WT1 peptide-based immunotherapy induced a minimal response in a patient with advanced therapy-resistant multiple myeloma. Int. J. Hematol. 86: 414-417 (2007)

Reagent	Reference
HLA-A*24:02 Negative Tetramer	Akiyama Y, et al., Anticancer Res. 29: 647–655 (2009)
	Tsukahara T, et al., Cancer Sci. 99: 368–375 (2008)
	Akimoto M, et al., J. Med Virol. 79: 977–86 (2007)
	Kozako T, et al., J. Immunol. 177: 5718–5726 (2006)
	Akiyama Y, et al., J. Transl. Med. 3: 4–13 (2005)

Mouse

Reagent	Reference
OVA Tetramer	Kurachi S, et al., J. Exp. Med. 208: 1605–1620 (2011)
	Nierkens S, et al., Cancer Res 71: 6428-6437 (2011)
	Yanai H, et al., PNAS 108: 11542–11547 (2011)
	Crozat K, et al., J Exp Med 207: 1283-1292 (2010)
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	Asano J, et al., J. Immunol. 184: 736–745 (2010)
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	Lou Y, et al., Clin Cancer Res 14: 1494-1501 (2008)
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	Yajima T, et al., J. Immunol. 174: 3590–3597 (2005)
	Li W, et al., Infect. Immun. 72: 7005–7011 (2004)
	Teramoto K, et al., Cancer Res. 63: 7920–7925 (2003)
	Kelly J, et al., 170: 210-217 (2003)
TRP2 Tetramer	Kuwada E, et al., Anticancer Res. 31: 881–891 (2011)
	Takeshima T, et al., Cancer Res. 70: 2697–2706 (2010)
	Okano F, et al., J. Immunol. 174: 2645–2652 (2005)

Reagent	Reference
Influenza Tetramer	Nakamura R, et al., J. Virol. 84: 5574–5582 (2010)
	Koyama S, et al., J. Immunol. 179: 4711–4720 (2007)
	Seo S–U, et al., J. Virol. 84: 12713–12722 (2010)
	Kayamuro H, et al., J. Virol. 84: 12703–12712 (2010)
LLO Tetramer	Ozawa Y, et al., Am. J. Respir. Cell Mol. Biol. 41: 440–448 (2009)
	Hayashi T, et al., J. Immunol. 182: 6360–6368 (2009)
LCMV Tetramer	Takagi A, et al., Clin. Vaccine Immunol. 16: 1383–1392 (2009)
	Jung A, et al., J. Virol. 82: 196–206 (2008)
	Kawagoe T, et al., J. Exp. Med. 204: 1013–1024 (2007)
	Gil MP, et al., Blood 107: 987–993 (2006)
H-2K^b Negative Tetramer	Asano J, et al., J. Immunol. 184: 736–745 (2010)
	Chamoto K, et al., Cancer Res. 66: 1809–1817 (2006)
	Wakita D, et al., Int. Immunol. 18: 425–434 (2006)
Mouse CD1d Tetramer	Yoshiga Y, et al., Clin Exp Immunol. 164: 236–247 (2011)

Non-Human Primate

Reagent	Reference
Mamu-A*01 SIV Tetramer	Cecchinato V, et al., J. Immunol. 180: 5439–5447 (2008)
	Mao H, et al., J Virol 79: 14887-14898 (2005)

Custom

Reagent	Reference
Class I Custom Tetramer	Hofer MJ, et al., J Virol 86: 6932-6946 (2012)
	Luo M, et al., J Virol 86: 1166-1180 (2012)
	Takatsuka N, et al., Int. Immunol. 21: 1089–1100 (2009)
	Tsukamoto T, et al., J. Virol. 83: 9339–9346 (2009)
	Yoshizaki M, et al., Blood 114: 1518–1527 (2009)
	Suemori K, et al., J. Gen. Virol. 90: 1806–1811 (2009)
	Akiyama Y, et al., Anticancer Res. 29: 647–655 (2009)
	Loisel-Meyer S, et al., Mol Cancer Ther 8: 692-702 (2009)
	Jeng RR, et al., Blood 113: 1574-1580 (2009)
	Harao M, et al., Int J Cancer 123: 2616–2625 (2008)
	Vasir B, et al., J Immunol 181: 808-821 (2008)
	Wakabayashi A, et al., J. Immunol. 180: 4000–4010 (2008)
	Tsukahara T, et al., Cancer Sci. 99: 368–375 (2008)
	Akimoto M, et al., J. Med Virol. 79: 977–86 (2007)
	Kozako T, et al., J. Immunol. 177: 5718–5726 (2006)
	Shi H, et al., J Immunol 176: 2134-2141 (2006)
	Jalili A, et al., Blood 106: 3538–3545 (2005)
	Akiyama Y, et al., J. Transl. Med. 3: 4–13 (2005)
Class II Custom Tetramer	Hofer MJ, et al., J Virol 86: 6932-6946 (2012)
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Inventors: Lang F, *et al.*, (INSERM)
"Means for detecting and for the purifying CD8+ T-lymphocyte populations specific for peptides presented in the HLA context."

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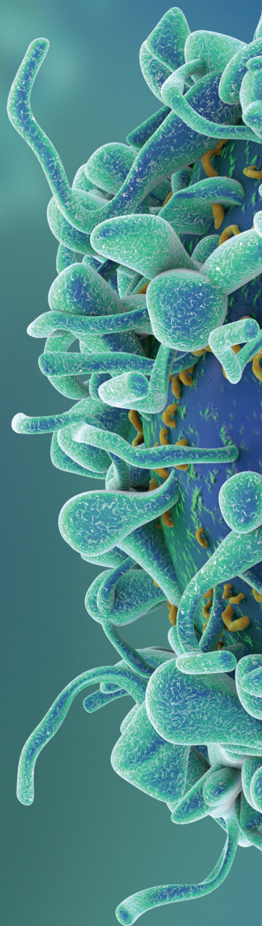

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