For Research Use Only.
Not for use in diagnostic procedures.

QuickSwitch™ Quant Tetramer Kit

QuickSwitch™ Quant H-2 Kb Tetramer Kit

APPLICATION

The QuickSwitch™ Quant Tetramer Kit utilizes a patented technique for exchanging up to ten peptides on an MHC class I tetramer. Components for quantifying the extent of peptide exchange by flow cytometry are included. New specificity tetramers obtained by peptide exchange can then be used for identification of antigen-specific $CD8^+$ T lymphocytes in staining assays.

SUMMARY AND EXPLANATION

Major histocompatibility complex (MHC)-encoded glycoproteins bind peptide antigens through non-covalent interactions to generate complexes that are displayed on the surface of antigen-presenting cells for recognition by T cells. Peptide-binding site occupancy is necessary for stable assembly of newly synthesized MHC proteins and export from the endoplasmic reticulum. During this stage peptides produced in the cytosol compete for binding to MHC molecules, resulting in extensive peptide exchanges that are regulated by accessory molecules, such as tapasin.[1,2] The QuickSwitch™ Quant Tetramer Kit is based on the capacity of MHC class I molecules to exchange peptides.

PRINCIPLE

The kit contains two modules: 1) MHC class I tetramer made from monomer units folded with an irrelevant exchangeable peptide, along with a proprietary Peptide Exchange Factor, for the generation of tetramers loaded with specific peptides of interest and 2) a flow cytometry-based sandwich immunoassay containing antibody-conjugated magnetic beads to capture MHC class I tetramers and a FITC-labeled antibody recognizing the Exiting Peptide. This assay measures the percentage of original peptide replaced by a competing peptide to help determine whether the resulting tetramer is suitable for antigen-specific $CD8^+$ T cell staining (note 1).

KIT COMPONENTS

QuickSwitch™ Tetramer
MHC class I tetramer, whose monomer content is 50 µg/mL, in a buffered solution with added protein stabilizers and ≤0.09 % sodium azide (500 µL x 1 amber vial with amber cap). Keep away from direct light. Store at 2-8°C.

Peptide Exchange Factor
The proprietary Peptide Exchange Factor contains ≤0.09 % sodium azide (13 µL x 1 clear vial with green cap). Store at ≤ -20°C.

Magnetic Capture Beads
Magnetic beads conjugated with a capture antibody specific for tetramers in a buffered solution with added protein stabilizers and ≤0.09 % sodium azide (500 µL x 1 clear vial with red cap). Store at 2-8°C.

Exiting Peptide Antibody-FITC (25x)
FITC conjugated antibody reacting against the Exiting Peptide in a buffered solution with added protein stabilizers and ≤0.09 % sodium azide (25 µL x 1 amber vial with yellow cap). Store at 2-8°C protected from light. Do not freeze.

Reference Peptide 1 mM
Peptide dissolved in DMSO at a 1 mM concentration (13 µL x 1 vial with black cap). Store at ≤ -20°C.

Assay Buffer (10x)
Buffered solution with added protein stabilizers and ≤0.09 % sodium azide (1,700 µL x 1 vial with natural cap). Store at 2-8°C.

CONJUGATES

PE tetramers are labeled with Streptavidin-Phycoerythrin (SA-PE), excitation 486–580 nm/emission 586–590 nm.

APC tetramers are labeled with Streptavidin-Allophycocyanin (SA-APC), excitation 633–635 nm/emission 660–680 nm.

BV421 tetramers are labeled with Streptavidin-Brilliant Violet™ 421 (SA-BV421), excitation maximum 405 nm/emission maximum 421 nm.

STORAGE CONDITIONS

Kit components in the bag (Code: TS-7400-1F, -2F, or -4F) store frozen at ≤ -20°C upon kit arrival: Reference Peptide and Peptide Exchange Factor.
Kit components in the box (Code: TS-7400-1R, -2R, or -4R) are stored at 2-8°C: Tetramer, Magnetic Capture Beads, Exiting Peptide Antibody-FITC, and Assay Buffer.

WARNINGS AND PRECAUTIONS

1. The Reference Peptide and concentrated Assay Buffer must be brought to room temperature (20-25°C) before use.
2. QuickSwitch™ Tetramer and Exiting Peptide Antibody are light sensitive and therefore should be protected from light during storage and during all the steps of the assay.
3. When Assay Buffer (10x) is stored at 2-8°C, some reversible precipitation or turbidity may appear. Incubation

at 37°C for a few minutes prior to use is recommended to re-solubilize salts.
4. Avoid microbial contamination of all reagents involved in the testing procedure or incorrect results may occur.
5. Incubation times or temperatures other than those specified may give erroneous results.
6. Care should be taken to avoid splashing and well cross-contaminations.
7. All solutions contain sodium azide (≤0.09 %) as preservative. Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be flushed with running water while being discarded. These precautions are recommended to avoid deposits in metal piping in which explosive conditions can develop. If skin or eye contact occurs, wash excessively with water.
8. Diluted solutions (antibody and assay buffer) have to be used on the same day as they are prepared. Therefore it is advised to prepare the exact required volumes just before using them.

PROCEDURE

This assay has been optimized for medium affinity and high affinity peptides.

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. issolve 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.

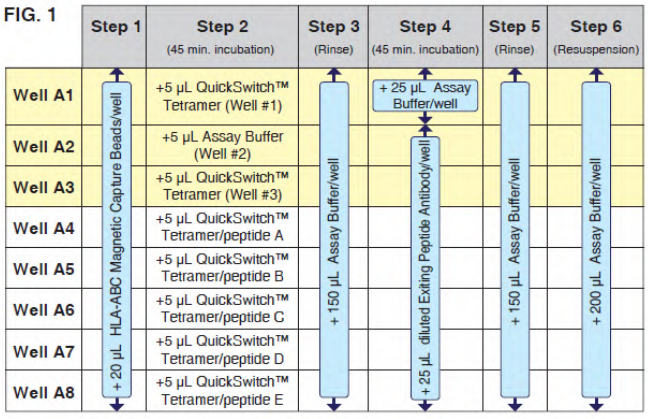
Note that peptide exchange reaction volumes can be scaled up or down, so long as reagent proportions are maintained.

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.

Fig. 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.

FIG. 1



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 or well of 96 well microtiter plate. 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. Place the plate on a magnetic tray, protected from light, and let beads sediment for at least 5 min. Meanwhile, prepare a dilution of the Exiting Peptide Antibody.
3. 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.

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 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 flow 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 flow cytometer.*
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.2

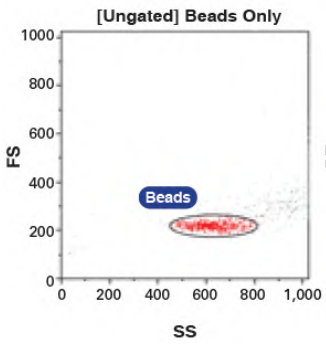
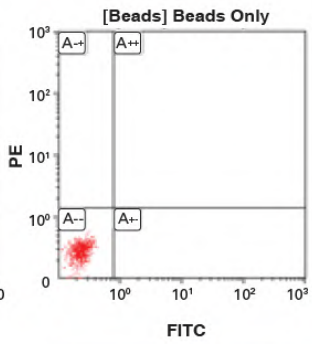
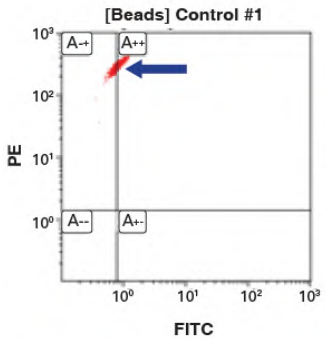


FIG. 3



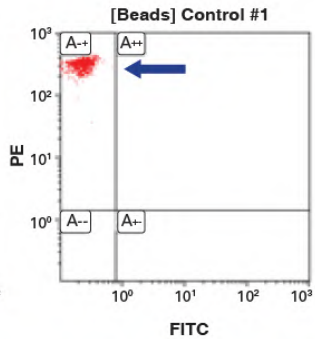
Gate	X-G Mean	Y-G Mean
A--	0.23	0.27
A-+	N/A	N/A
A+-	N/A	N/A
A++	N/A	N/A

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

FIG.5

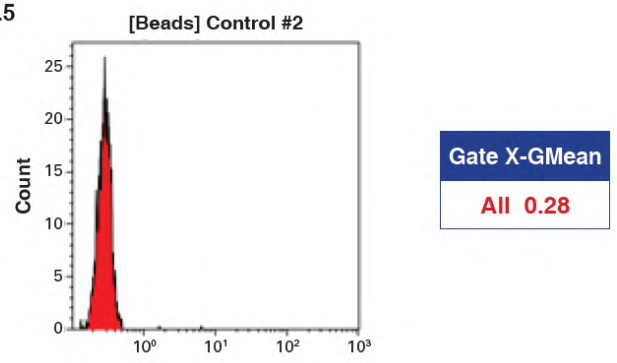


FIG.6

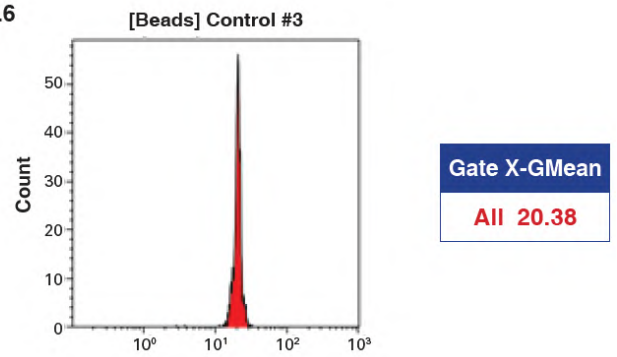
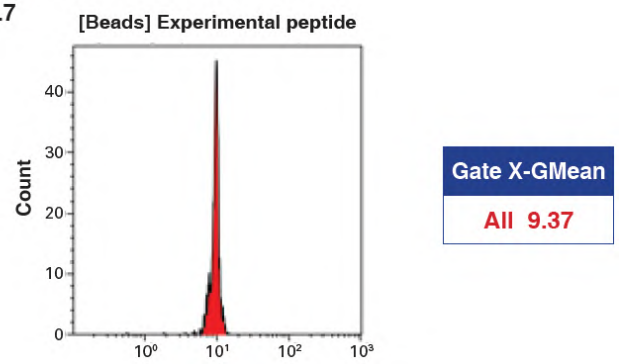


FIG.7



CALCULATION OF RESULTS USING QUICKSWITCH™ DOWNLOADABLE CALCULATOR

The QuickSwitch™ Calculator on the MBL website (https://ruo.mbl.co.jp/bio/product/allergy-Immunology/images/QuickSwitchTM-Quant-Peptide-Exchange-Calculator.xlsx) can be downloaded for determining percentages of peptide exchange, as shown in the example below using a 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.

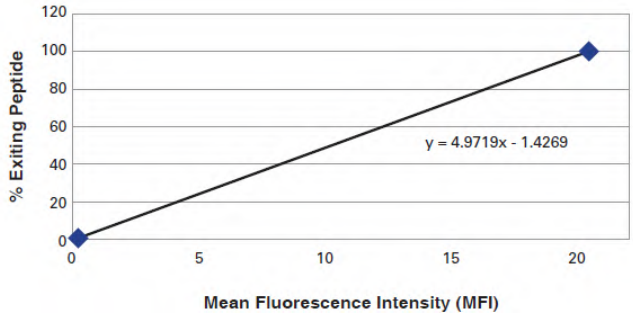
Peptide Sample	QuickSwitch Tetramer MFI_{FITC} after Peptide Exchange	% Peptide Exchange
A	9.37	54.78
B	5.29	75.07
C	2.12	90.85
D	1.29	94.98
E	22	FALSE
F	0.11	100.85

Table 2

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 a 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 a 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 H-2 Kb is shown in Table 4.

Table 4

	Reference Peptide
Stock Concentration	1 mM
Final Concentration	20 µM
Peptide Exchange (N=3)	94.8 ± 1.3 %

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

1. Tetramers bind to T cell receptors via three MHC/peptide monomers.[3,4] 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.[5,6]
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.
3. Most of peptides are soluble in DMSO. However some highly basic or acidic peptides may precipitate in DMSO and would require alternative buffers.
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.
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).
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 recommends simultaneous staining of class I tetramer with anti-CD8 and other antibodies for 30 minutes at room temperature.
7. Avidity of peptide-exchanged tetramers will depend on the percentage of peptide exchange. Classical tetramers made with monomers generated by folding always present 100% specific peptide and therefore display maximal avidity.

REFERENCES

1. Reaper DR, Cresswell P. 2008. Regulation of MHC class I assembly and peptide binding. Annu Rev Cell Dev Biol. 24:343-368.
2. Mayerhofer PU, Tampé R. 2015. Antigen translocation machineries in adaptive immunity and viral immune evasion. J Mol Biol. 427(5):1102-1118.
3. Wooldridge L, van den Berg HA, Glick M, Gostick E, Laugel B, Hutchinson SL, Milicic A, Brenchley JM, Douek DC, Price DA, Sewell AK. 2005. Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor–antigen complexes at the cell surface. J Biol Chem. 280:27491–501.
4. Wooldridge L, Lissina A, Cole DK, van den Berg HA, Price DA, Sewell AK. 2009. Tricks with tetramers: how to get the most from multimeric peptide-MHC. Immunology. 126(2):147-164.
5. Price DA , Brenchley JM, Ruff LE, Betts MR, Hill BJ, Roederer M, Koup RA, Migueles SA, Gostick E, Wooldridge L, Sewell AK, Connors M, Douek DC . 2005. Avidity for antigen shapes clonal dominance in CD8 T cell populations specific for persistent DNA viruses. J. Exp. Med. 202:1349-1361.
6. Mielke S, Nunes R, Rezvani K, et al. 2008. A clinical-scale selective allodepletion approach for the treatment of HLA-mismatched and matched donor-recipient pairs using expanded T lymphocytes as antigen-presenting cells and a TH9402-based photodepletion technique. Blood. 111:4392-4402.
7. Altman, JD, Davis MM. 2003. MHC-peptide tetramers to visualize antigen-specific T cells. Curr Protoc Immunol. 17(17.3 Suppl 53): 23
8. Holman, PO, Walsh ER and Jameson SC. 2005. Characterizing the impact of CD8 antibodies on Class I MHC multimer binding. J Immunol. 174:3986-3991.

RELATED PRODUCTS:

Please visit our website at https://ruo.mbl.co.jp/

REPRESENTATIVE DATA

Peptide Exchange quantitation curve is linear.

Tetramers conftaining various percentages of Exiting Peptide were analyzed using the flow cytometric sandwich immunoassay to quantify peptide exchange. The generated curve was linear, as shown in Fig. 9, indicating that two controls (0% and 100% peptide exchange controls) are sufficient for generating the peptide exchange quantitation curve.

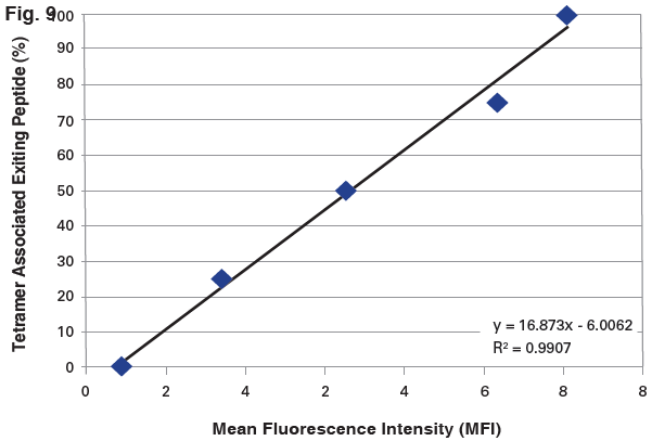
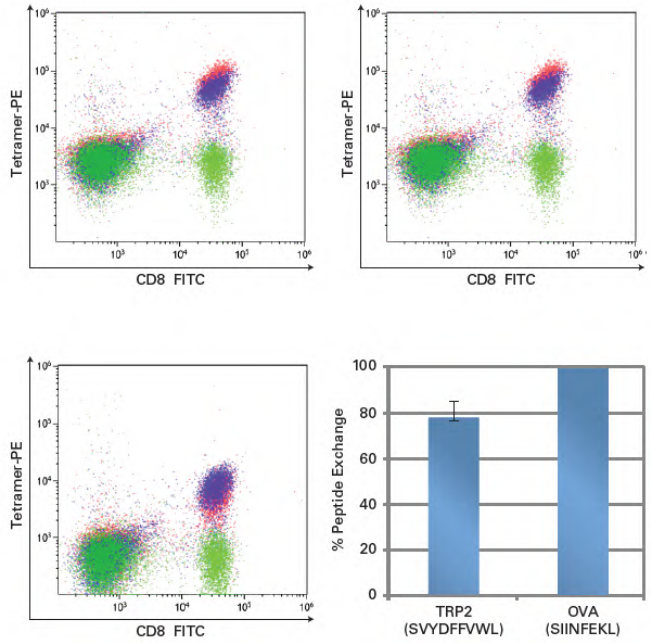


Fig. 10. Peptide-exchanged tetramers perform similarly to classically folded tetramers.

Splenocytes isolated from OT-I TCR- transgenic mice (C57BL/6-Tg(TcraTcrb)1100Mjb/Crl) ($1.2x10^5$/test) were incubated with 0.5 µg, 0.1 µg, or 0.02 µg APC-labeled tetramer along with CD8-FITC (clone KT15; 0.4 µL/test) in 100 µl final assay volume for 30 minutes at room temperature, protected from light. Cells were washed in
1.5 mL/tube cell with cell staining buffer and resuspended in 0.1% PFA. Approximately 10,000 cell events were acquired on a 3 laser/10 color Gallios flow cytometer and analyzed using Kaluza 1.5a. H-2 Kb TRP2 used a negative control (green), classically folded H-2 Kb OVA (blue), and H-2 Kb QuickSwitch™ OVA (red) tetramer staining, as well as peptide exchange quantification, are shown.

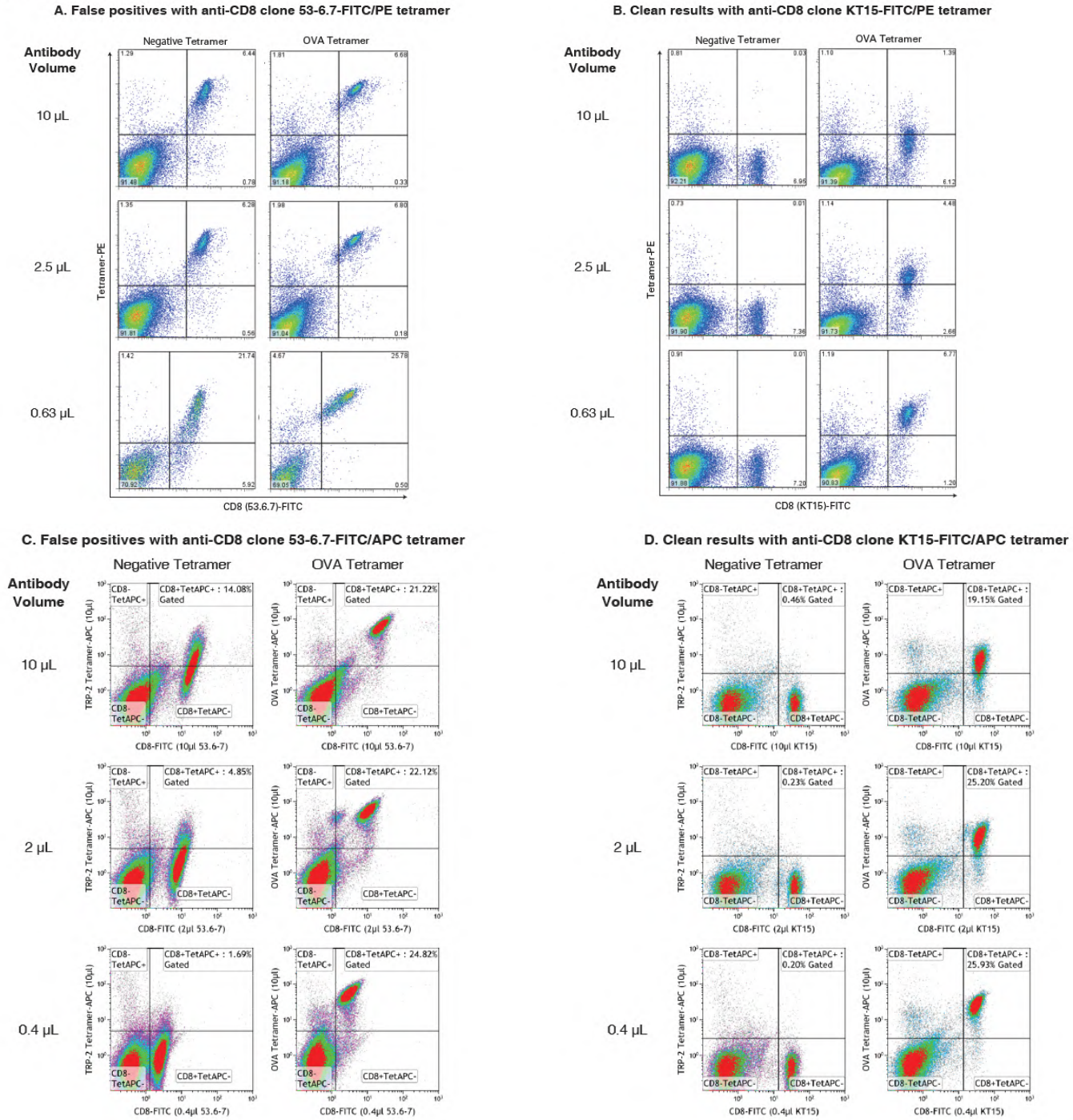


TROUBLESHOOTING H-2 Kb BACKGROUND STAINING

Certain anti-murine CD8 clones have been shown to affect tetramer staining[7-8]. In Current Protocols in Immunology, Altman and Davis caution, "Some MHC tetramers (e.g., H-2 Kb) have been observed to stain all $CD8^+$ T cells, presumably via an interaction between CD8 and the α3 domain of the MHC molecule. In these cases, it is useful to perform a cross-titration series with the MHC tetramer and several distinct anti-CD8 antibodies, looking for CD8 antibodies which block with CD8-mediated interaction, but not the antigen-specific interaction. For example, Kb tetramers bind to all $CD8^+$ T cells in the presence of the anti-CD8 antibody 53-6.74."

Fig. 11 compares two different anti-murine CD8 FITC clones used to stain OT-I transgenic splenocytes with Negative (ß-gal, A-B; TRP2, C-D) or OVAspecific (A-D) H-2 Kb tetramer. The use of anti-murine CD8 clone KT15 (B, D) eliminates confusing diagonals resulting from non-specific binding seen with clone 53-6.7 (A, C) in tetramer assays. PE-labeled tetramers (A, B) and APC-labeled tetramers (C, D) are shown. Titration of anti-murine CD8 antibody is recommended for optimized staining.

Figure 11



MBL manufactures and distributes these products under license from Beckman Coulter Inc.
Brilliant Violet™ 421 is a trademark of Sirigen, and Sirigen is an entity of Becton, Dickinson and Company.