MBL International Corporation

QuickSwitch™ Quant Tetramer Kit

- QuickSwitch™ Quant HLA-DRB1*04:01 Tetramer Kit-PE (Code No: TB-7502-K1)
- QuickSwitch™ Quant HLA-DRB1*04:01 Tetramer Kit-APC (Code No: TB-7502-K2)
- QuickSwitch™ Quant HLA-DRB1*04:01 Tetramer Kit-BV421 (Code No: TB-7502-K4)

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INTENDED USE

The QuickSwitch™ Class II Tetramer Peptide Exchange and Quantitation Kit utilizes a patented technique for exchanging up to ten peptides on MHC class II molecules. Components for quantifying the extent of peptide exchange by flow cytometry are included. Peptide exchanged monomers are tetramerized with fluorochrome conjugated streptavidin. New specificity tetramers obtained by peptide exchange can then be used for identification of antigen-specific CD4+ T lymphocytes in staining assays[1].

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. MHC class II α and β chains assemble with a third non-MHC encoded glycoprotein chain, the invariant chain, early during biosynthesis in the endoplasmic reticulum. Later during biosynthesis in the endosomal compartment, the invariant chain is eventually degraded by proteases, leaving a small remnant, named CLIP, within the MHC class II antigen-binding groove. In the same endosomal compartments, peptide fragments of either foreign or self-antigens undergo proteolysis and free peptides become available for MHC class II binding. In acidic endosomes, peptide exchange occurs as CLIP is replaced by antigenic peptides, a reaction that is catalyzed by HLA-DM[2]. The QuickSwitch™ Tetramer Peptide Exchange and Quantitation Kit is based on the capacity of MHC class II molecules to exchange peptides.

PRINCIPLE

The kit contains three modules: (1) MHC class II monomer units loaded with an irrelevant exchangeable peptide, for the generation of tetramers loaded with specific peptides of interest (2) a flow cytometry-based sandwich immunoassay consisting of streptavidin-conjugated magnetic beads to capture MHC class II monomers and a FITC-labeled antibody for detecting the Exiting Peptide and (3) a tetramerization module. This assay measures the percentage of original peptide replaced by a peptide of interest to help determine whether the resulting tetramer will be suitable for antigen-specific CD4+ T cell staining (Note 1).

KIT COMPONENTS

QuickSwitch™ Biotinylated Monomer (1x)

MHC class II biotinylated monomer is at 100 µg/mL, in a buffered solution that contains ≤0.09 % sodium azide (250 µL x 1 amber vial with amber cap). Keep away from direct light. Store at ≤ -20°C.

Emulsifier (1x)

The Emulsifier is a sodium-azide free solution (25 µL x 1 clear vial with pink cap). Store at ≤ -20°C.

Neutralizer (1x)

The Neutralizer is a sodium azide-free solution (100 µL x 1 clear vial with blue cap). Store at 2-8°C.

Streptavidin Magnetic Capture Beads (1x)

Magnetic beads conjugated with streptavidin 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.

Streptavidin-Fluorochrome (1x)

The fluorochrome (either PE, APC or BV421) conjugated to streptavidin is in a buffered solution with added protein stabilizers and ≤0.09 % sodium azide (100 µL x 1 amber vial with green cap). Store at 2-8°C.

Exiting Peptide Antibody (1x)

FITC conjugated antibody reacting against the Exiting Peptide in a buffered solution with added protein stabilizers and ≤0.09 % sodium azide (500 µL x 1 amber vial with yellow cap). Store at 2-8°C. Do not freeze.

Reference Peptide 10 mM

$CLIP_{86\text{-}100}$ peptide (PVSKMRMATPLLMQA) dissolved in DMSO at a 10 mM concentration (13 µL x 1 clear vial with black cap). Store at ≤ -20°C.

Peptide Exchange Assay Buffer #2 (10x)

Buffered solution containing ≤0.09 % sodium azide (1.5 mL x 1 clear vial with clear cap). Store at 2-8°C.

CONJUGATES

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

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

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

STORAGE CONDITIONS

The QuickSwitch™ monomer, the Reference Peptide and the Emulsifier must be frozen at ≤-20°C upon kit arrival. The remaining components of the kit as well as the generated tetramers are stored at 2-8ºC.

WARNINGS AND PRECAUTIONS

- The Reference Peptide and concentrated Assay Buffer must be brought to room temperature (20-25ºC) before use.
- QuickSwitch™ Monomer and Exiting Peptide Antibody are light sensitive and therefore should be protected from light during storage and during all the steps of the assay.
- When Assay Buffer (10x) is stored at 2-8ºC, some reversible precipitation or turbidity may appear. Incubation at 37ºC for a few minutes prior to use is recommended to re-solubilize salts.
- Avoid microbial contamination of all reagents involved in the testing procedure or incorrect results may occur.
- Incubation times or temperatures other than those specified may give erroneous results.
- Care should be taken to avoid splashing and well cross-contaminations.
- Some 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.

- Do not mix components from other kits and lots as this creates potential inaccuracy in results.
- Follow **exactly** the protocol specific for this MHC class II allele as some alleles require different peptide concentrations for exchanges and different peptide exchange reaction times.

SYMBOL DEFINITIONS

= Consult Directions for Use
= Store Away From Direct Light
= Storage Temperature
= Expiration Date
= Number of Tests
= Amount
REF = Code Number
LOT = Lot Number

PROCEDURE

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

MATERIALS REQUIRED FOR PEPTIDE EXCHANGE AND CAPTURE ASSAY 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 3)
- 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
- PBS

TEST PROCEDURE

Carefully read this protocol before performing an assay. This protocol allows generation of 10 peptide exchanged MHC class II tetramers (Note 4).

A. Generation of New Specificity Monomer Using Peptide Exchange

Prior to performing the assay, bring to room temperature the QuickSwitch™ Monomer, the Emulsifier and peptides to be used in the assay.

1. Dissolve each lyophilized peptide to be assayed in DMSO to a 10 mM solution (~16-18 mg/mL for a 15 amino acid peptide) and further dilute a portion of it to a 1 mM solution. Freeze the remaining 10 mM stock solution for later use. (Note 5)
2. Spin QuickSwitch™ Monomer and Reference Peptide vials briefly to bring liquid down.
3. For each peptide exchange, pipet 25 µL of QuickSwitch™ Monomer from amber capped vial into a PCR tube or any microtube that seals tightly to prevent evaporation during incubation. Pipet an additional 25 µL for the no exchange control (Fig 1).

   If the user plans to perform cell stainings it is suggested to include 25 µL of QuickSwitch™ Monomer for exchange with the CLIP reference peptide (Fig 1) as the HLA DRB1*04:01 CLIP tetramer is used as negative tetramer (Note 6, Fig 8).
4. Add 2 µL of Emulsifier from pink capped vial and mix gently with pipetting.
5. Add 3 µL of 1 mM peptide. Mix gently with pipetting. The final peptide concentration is 100 µM for tested peptides (Note 7).The CLIP reference peptide is taken directly from the black cap vial (3 µL). Mix gently with pipetting. The final CLIP peptide concentration is 1 mM.
6. Repeat steps 1-5 for each additional peptide.
7. The no exchange control used in the capture assay will contain the QuickSwitch™ Monomer, emulsifier and 3 µL of H_2O.
8. Incubate overnight (16-24 h) at 37°C protected from light (Note 8).
9. Monomers are now ready for use in quantitation (see section B) and/or staining assays after tetramerization. (Note 9)
10. Return the unused QuickSwitch™ Monomer to ≤ -20°C. Peptide exchanged monomers can also be stored at -20°C or below.

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 (clear capped vial) with 6.75 mL of distilled water. For 6-10 exchanges, double the volumes.
2. Immediately before use, vortex the monomer capture beads for 30 seconds, followed by a 30-second sonication in a water bath sonicator.

FIG. 1

		Step 1	Step 2 (30 min. incubation)	Step 3 (Rinse)	Step 4 (45 min. incubation)	Step 5 (Rinse)	Step 6 (Resuspension in sheath fluid))
Beads only Control	Well A1	+ 20 µL Streptavidin Capture Beads/well	+1 µL QuickSwitch™ Monomer (well #1)	+ 150 µL Assay Buffer/well	+ 25 µL Assay Buffer/well	+ 150 µL Assay Buffer/well	+ 200 µL Assay Buffer/well
0% Exiting Peptide (100% exchange)	Well A2		+1 µL Assay Buffer (well #2)		+ 25 µL Exiting Peptide Antibody/well		
100% Exiting Peptide (0% exchange)	Well A3		+1 µL QuickSwitch™ Monomer (well #3)				
Test Peptides ($CLIP_{86-100}$ optional)	Well A4		+1 µL QuickSwitch™ Monomer/peptide A				
	Well A5		+1 µL QuickSwitch™ Monomer/peptide B				
	Well A6		+1 µL QuickSwitch™ Monomer/peptide C				
	Well A7		+1 µL QuickSwitch™ Monomer/peptide D				
	Well A8		+1 µL QuickSwitch™ Monomer/peptide E				

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

Step 1 (Dispensing Capture Beads).

1. Vortex and sonicate vial of streptavidin conjugated magnetic beads (red cap vial).
2. Into each of three wells of a conical-bottom 96 well microtiter plate, pipet 20 µL Magnetic Capture Beads for essential controls.
3. Pipet 20 µL Magnetic Capture Beads to additional wells for each peptide-exchanged monomer to test.

Step 2 (Monomer Capture).

1. Pipet 1 µL QuickSwitch™ negative control monomer (no exchange) in wells #A1 and #A3 (see step A.7).
2. In well #4, pipet 1 µL taken from the first peptide exchange microtube. Repeat for each additional peptide exchange in adjacent wells.
3. Shake plate for 30 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 plate magnet, protected from light, and let beads sediment for at least 5 min.
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 the plate upright, vortex for 2 seconds to disperse the beads.

Step 4 (Bead incubation with Exiting Peptide Antibody).

1. Give a quick spin to the yellow capped Exiting Peptide Antibody vial to bring all liquid to the bottom.
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.

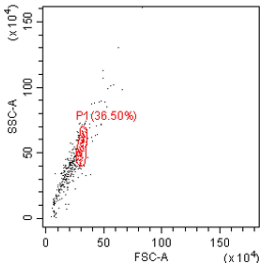
Step 6 (Flow Acquisition).

1. Resuspend beads in Assay Buffer and acquire on a flow cytometer, ideally within 3 hours collecting at least 300 events per sample in order to obtain reliable data.

FLOW CYTOMETRY SET UP

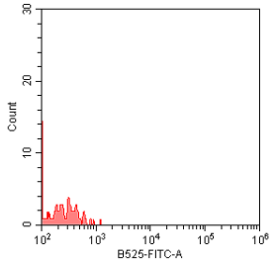
1. Run beads from well A1 on the flow cytometer ("beads only" control #1).
2. Adjust FSC and SSC voltages, gains, and threshold such that bead events are on scale
3. Gate singlet beads based on FSC and SSC parameters, excluding debris, doublets and aggregates (Fig. 2). If acquisition rate is very high, it is likely that debris is collected. In that case the threshold must be increased to exclude low size particles.

FIG. 2



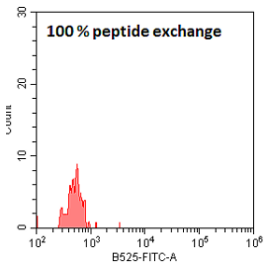
4. Set voltages and gains for FITC such that "beads only" mean fluorescence intensities (MFI) are in the first log decade.

FIG. 3



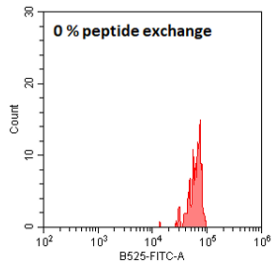
5. Run well B1 beads (or control #2), beads that have not captured any monomer and therefore have no Exiting Peptide. The low MFI_{FITC} corresponds to 0% Exiting Peptide or 100% peptide exchange (Fig. 4).

FIG. 4



6. Run well C1 beads (or control #3), beads that have captured the QuickSwitch™ Monomer, which have an MFI_{FITC} that corresponds to 100% Exiting Peptide or 0% peptide exchange (Fig. 5).

FIG. 5



7. Run samples from well D1 and subsequent peptide exchange samples, noting the MFI_{FITC} of each. Peptide-exchanged monomers will display various Exiting Peptide amounts, which are inversely proportional to the quantity of the newly loaded peptide on the MHC molecules (Fig 6).

FIG. 6

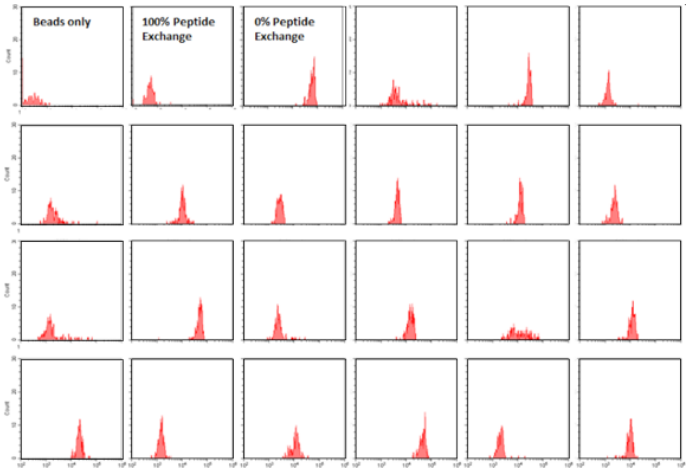


Figure 6 **Flow analysis of various peptide exchanged monomers generated with peptides of different affinities**. The measured MFI_{FITC} associated to the different peaks are intermediary between MFI values obtained with bead controls #2 and #3.

DATA ANALYSIS

1. Collect the different MFI and calculate the different peptide exchanges using the spreadsheet from the MBLI website. The QuickSwitch™ Calculator on the MBLI 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 an HLA-DR QuickSwitch™ Monomer and corresponding peptides (Tables 1-2).

2. 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)	728
Control #3: 100% Exiting Peptide (0% peptide exchange)	85292

3. 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.

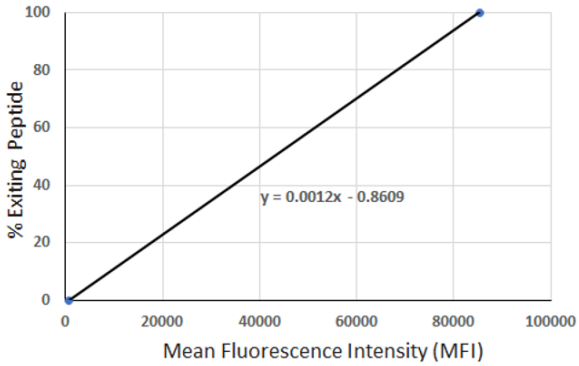
Table 2

Peptide Sample	QuickSwitch™ Monomer MFI_{FITC} after Peptide Exchange	% Peptide Exchange
A	6658.6	92.99
B	16766.9	81.03
C	21835.7	75.04
D	58651.2	31.5
E	86258.6	FALSE
F	3508.2	96.71

4. Alternative calculation of peptide exchanges with excel or other software:

   a. 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 (Fig. 7).

Fig. 7



   b. 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-DR15 QuickSwitch™ Monomer (Table 3).

Table 3

Analyzed sample	MFI_{FITC} (X)	% of Exiting Peptide (Y)	% Peptide Exchange (100-Y)
Control #2: 0% Exiting Peptide (100% peptide exchange)	728	0	100
Control #3: 100% Exiting Peptide (0% peptide exchange)	85292	100	0
Test Peptide	22958	26.69	**73.31**

C. Monomer Tetramerization

Monomers that have undergone peptide exchange are tetramerized according to the procedure below.

1. Spin tubes and vials briefly to bring liquids down: peptide exchanged monomers, Fluorochrome-streptavidin (green cap), Neutralizer (blue cap).
2. To each peptide exchanged monomer (~ 30 µL), pipet 10 µL of Streptavidin-Fluorochrome and mix the solution gently.
3. To the generated tetramer, add 10 µL Neutralizer and mix gently.
4. Use the generated tetramer for cell staining according to the prescribed MBL International staining protocol or store the tetramer protected from light and refrigerated at 2-8°C until use.

D. Cell staining with peptide exchanged MHC class II tetramers

MATERIALS REQUIRED BUT NOT SUPPLIED

- 12x75 mm polypropylene test tubes
- Transfer pipettes
- Pipettors and disposable pipette tips
- Vortex
- Centrifuge capable of 150 x g or 400 x g
- 37°C CO_2 incubator
- Aspirator
- PBS or FACS buffer (e.g. PBS with 0.2–1% BSA and 0.1% Sodium Azide)
- PBS with 0.5% paraformaldehyde or formalin
- RPMI-1640 supplemented with 2 mM L-glutamine/2Mercaptoethanol, 1 mM sodium pyruvate, 1% Non-essential amino acids, 10 mM HEPES, 100 µg/mL penicillin/streptomycin and 10% Human AB Serum
- Lyse Reagent (VersaLyse™ lysing solution, Beckman Coulter, Inc., PN A09777)
- Fixative Reagent (IOTest® 3 10x Fixative Solution, Beckman Coulter, Inc., PN A07800)
- Dasatinib (optional) 1 µM working concentration in media, dilute 1:20 for 50 nM final concentration (Axon Medchem 1392).
- Human anti-CD4 antibody (MBL International clone OKT4 is recommended)
- Viability dyes (Biolegend): 7AA (fixation sensitive) or zombie dye (fixation resistant)

PROCEDURE FOR WHOLE BLOOD

1. Collect blood by venipuncture into a blood collection tube containing an appropriate anti-coagulant.
2. Add 20 µL of peptide exchanged Class II QuickSwitch™ Tetramer. (Notes 1, 2)

3. Add 200 µL of whole blood into each tube. Vortex gently.
4. Incubate for 2 hours 37ºC in 5% CO_2 incubator.
5. Remove from incubator.
6. Add antibodies (e.g. anti-CD4), incubate for 20 minutes at room temperature, protected from light.
7. Lyse red blood cells using 2 mL of VersaLyse™ lysing solution supplemented with 25 µL IOTest® 3 10x Fixative Solution per tube.
8. Vortex for 5 seconds immediately after the addition of the VersaLyse™/ IOTest® 3 10x Fixative Solution.
9. Incubate for a minimum of 10 minutes at room temperature protected from light.
10. Centrifuge tubes at 150 x g for 5 minutes.
11. Aspirate or decant the supernatant.
12. Add 3 mL of PBS or FACS buffer.
13. Centrifuge tubes at 150 x g for 5 minutes.
14. Aspirate or decant the supernatant.
15. Resuspend the pellet in PBS with 0.1% formaldehyde (12.5 µL Fixative Reagent / 1 mL PBS).
16. 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 PERIPHERAL BLOOD MONONUCLEAR CELLS (classical protocol)

1. Prepare mononuclear cell suspension according to established procedures. For staining, cells should be resuspended in RPMI complete media at a final concentration of 5 x 10^6 cells/mL. 200 µL of sample is required for each determination.
2. Add 20 µL of peptide exchanged MHC Tetramer (Notes 1, 2).
3. Add 200 µL (1 x 10^6) PBMC into each tube.
4. Vortex gently.
5. Incubate for 2 hours 37ºC in 5% CO_2 incubator‡.
6. Remove from incubator
7. Add antibodies (e.g. anti-CD4), incubate for 20 minutes at room temperature, protected from light.
8. Add 3 mL PBS or FACS buffer.
9. Centrifuge at 400 x g for 5 minutes.
10. Aspirate the supernatant.
11. Resuspend the pellet in PBS with 0.5% formaldehyde (or 62.5 µL Fixative Reagent /1 mL PBS).
12. Store prepared samples at 4°C protected from light for a minimum of 1 hour (maximum 24 hours) prior to analysis by flow cytometry.

‡Staining conditions may require optimization

PROCEDURE FOR PERIPHERAL BLOOD MONONUCLEAR CELLS (96-well plate format)

1. Prepare peripheral blood mononuclear cells (PBMCs).
   Resuspend cells at 5 x 10^6 cells/mL in complete medium.
   Distribute 50 µL or 250,000 cells per well.
2. To each well add 5 µL of QuickSwitch™ peptide exchanged tetramer (Notes 1, 2).
3. Mix with pipetting or gentle vortexing at low speed.
4. Incubate for 2 hours 37°C in 5% CO_2 incubator and prepare surface antibody cocktail.
5. Remove plate from incubator.
6. Add antibodies (e.g. anti-CD4), mix with pipetting or gentle vortexing at low speed.
7. Incubate for 20 minutes at room temperature, protected from light.
8. Add 150 µL FACS buffer.
9. Centrifuge at 300 x g for 5 minutes.
10. Aspirate the supernatant and repeat wash with FACS buffer.
11. Resuspend the pellet in 200 µL FACS buffer containing 5 µL 7AAD or zombie dye if cells will be fixed.
    For fixing: Resuspend the pellet in PBS with 0.5% formaldehyde (or 62.5 µL Fixative Reagent/1 mL PBS).
12. Store fixed cells at 4°C protected from light for a minimum of 1 hour (maximum 24 hours) prior to analysis by flow cytometry.

‡Staining conditions may require optimization.

CELL EXPANSION

Cell expansion, with or without CFSE for precursor frequency, is performed according to established protocols. Cells should be resuspended after expansion and harvest, at a final concentration of 5 x 10^6 cells/mL. 200 µL of sample is required for each determination [3,4].

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 CD4+ T cell population in donor leukocytes. These tetramers are not intended to be a substitute for classically manufactured tetramers which undergo an additional purification step.
2. The 1x Assay Buffer must be used only on the day it is prepared.
3. Do not mix components from other kits and lots as compositions and concentrations of components are different from kit to kit.
4. For optimal results with whole blood, retain specimens in blood collection tubes at room temperature, while rocking, prior to staining and analyzing. Refrigerated or frozen specimens may give aberrant results.
5. Recommended cell viability for venous blood specimens is > 90%.
6. Prolonged exposure of cells to lytic reagents may cause white blood cell destruction and loss of cells in the population of interest.
7. All red blood cells may not lyse under the following conditions: nucleated red blood cells, abnormal protein concentration or hemoglobinopathies. This may cause falsely decreased results due to unlysed red blood cells being counted as leukocytes.
8. Although QuickSwitch™ reagents are held to strict quality control and purity standards, suitability for the end user's particular experimental system cannot be guaranteed (Note 10)

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NOTES

Note 1. Tetramers bind to T cell receptors via three MHC/peptide monomers to stabilize MHC binding to TcRs. Therefore, the minimal recommended peptide exchange percentage for generating a functional tetramer is 75%. The tetramerized and peptide exchanged MHC class II QuickSwitch™ tetramer has a concentration of 50 µg/mL, measured by MHC monomer content. The prescribed final concentration in staining mixtures is 5 µg/mL. However, depending on samples, this concentration may have to be lowered or increased for optimal staining.

Note 2. Dasatinib can be optionally added at a 50 nM final concentration to the cell/tetramer mixture as well as to solutions used in the subsequent steps of the T cell staining procedure. Dasatinib increases tetramer staining intensity by preventing TCR triggering and internalization. Another optional way to boost staining is to include antibodies against tetramers during the tetramer staining step[5].

Note 3. This assay uses a magnet to pellet the streptavidin conjugated capture beads. If a plate magnet is unavailable, it is possible to pellet magnetic beads by centrifugating conical 96 well microtiter plates at 300 g for 10 minutes. Supernatants can then be discarded by flicking plates. Centrifugating beads is not recommended because this could result in monomer aggregation and/or bead clumping and also bead loss.

Note 4. The assay setup presented here allows to generate 10 peptide exchanged tetramers but their number can be increased or decreased at will so long as ratios of QuickSwitch™ monomer, emulsifier, peptide, neutralizer and streptavidin-fluorochrome are unchanged and control samples are always included.

Note 5. Most peptides are soluble in DMSO. However, some highly basic, acidic or hydrophobic peptides may precipitate in DMSO and would require alternative solvents. Peptide manufacturers generally indicate the solvent most adapted to each peptide.

Note 6. The reference peptide CLIP typically triggers a > 90 % exchange when used at a final 1 mM solution. It is included in the test for confirming that the QuickSwitch™ procedure has been correctly followed. MHC tetramers built with CLIP-exchanged monomers should be used as negative controls in T cell staining assays.

Note 7. Higher or lower peptide concentrations can be tested as well. Final peptide concentrations up to 1 M will increase peptide exchange rates but may increase the risk of monomer and tetramer aggregation and nonspecific staining, particularly when these peptides are highly hydrophobic.

Note 8. As sample evaporation must be avoided, incubation at 95% relative humidity in a tissue culture incubator at 37ºC provides an additional protection.

Note 9. Tetramers obtained by peptide exchange are used directly for cell staining. However, if there is a sign of aggregation, tetramers should be spun for 1 min at maximal speed in a microcentrifuge to pellet aggregates. Tetramers can further be run on desalting columns to further remove excess peptides.

Note 10. Percentages of tetramer positive cells can sometimes be very low leading to questioning whether these cells are truly positive. Indeed, at low positivity levels nonspecific staining can become preponderant. Including the CLIP tetramer control allows to evaluate the nonspecific staining component (see fig. 8). An additional way to solve this issue consists in staining cells with two tetramers exchanged with the same peptide and conjugated to different fluorochromes. Truly tetramer positive T cells will be the ones that are stained with both tetramers.

REPRESENTATIVE DATA

Fig. 8

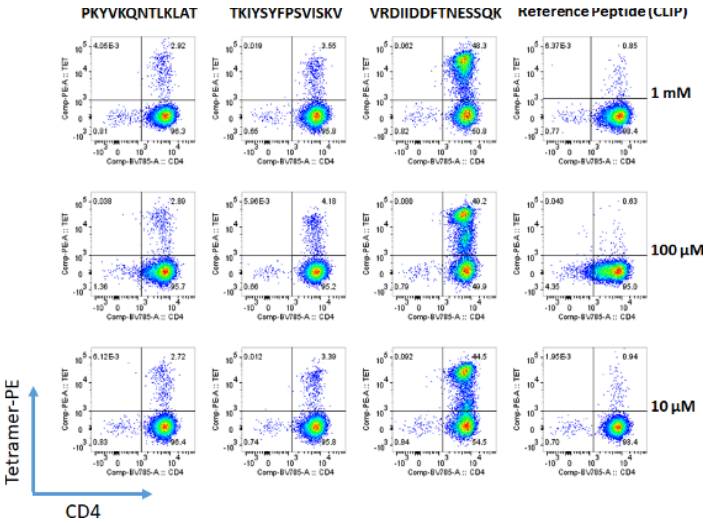

Figure 8. **Staining of CD4 lymphocytes with peptide exchanged HLA-DRB1*04:01 PE conjugated tetramers**. 25 µL of HLA-DRB1*04:01 QuickSwitch™ monomer were mixed with 2 µL of emulsifier and 3 µL peptides at 3 different concentrations. These peptides were respectively PKYVKQNTLAT (Flu Peptide), TKIYSYFPSVISKV, VRDIIDDFTNESSQK (2 Tetanus Toxin Peptides) and PVSKMRMATPLLMQA (CLIP, negative control). Each peptide-exchanged monomer (30 µL) was mixed with 10 µL streptavidin-PE. The tetramers were then mixed with 10 µL Neutralizer. HLA-DRB1*04:01 PBMCs were cultured at 3 x 10^5 cells per well and stimulated with the tested peptides except CLIP. Cells were stained with tetramers for 2 hours at 37ºC followed by a 20 min staining with anti CD3, CD4 & CD8 antibodies (anti CD3 FITC, anti CD8 BV510, anti CD4 BV785). All four peptides which bind to HLA-DRB1*04:01 with high affinity can exchange at final concentrations as low as 10 µM. CD4 T cells stained with CLIP exchanged tetramers display here a faint nonspecific signal (note 10).

REFERENCES

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TRADEMARKS

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US Patent Nos.: 5,635,363; 5,723,584; 5,874,239; 5,932,433 and 6,265,552. French Application No. FR 9911133.

For more information or if damaged product is received, contact MBL International Customer Service at 1–800–200–5459 (U.S. & Canada) or by email at **tetramer@mblintl.com**. Other countries should contact their local distributor found on our website, **mblintl.com**.

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TROUBLESHOOTING PEPTIDE EXCHANGE AND CAPTURE ASSAY

Problems	Potential causes	Ways to solve issue
Very high acquisition rate	Acquisition of debris and very small particles	Increase the flow cytometer acquisition threshold.
Beads not found	Threshold/trigger on cytometer set too high.	Refer to your Flow cytometer manual to adjust threshold/trigger appropriate for microparticles.
	Interfering substances in samples	Make sure solutions are not contaminated.
	Flow cytometer instrument is out of calibration	An uncalibrated machine will give erroneous results. Follow the manufacturer's calibration recommendations.
High Background	Solutions contaminated by exogenous material	Try to use as much as possible single use tubes, vials...
	Spillover from adjacent well(s) if exchange was performed in a plate	Use individual tubes instead of plate for exchange.
	Reagents contaminated	Store in a cool, dry place and do not pipet into vials.
	Improper washing	Use clean tips for washing and make sure not to cross-contaminate wells.
		Make sure washing protocol is followed strictly and that all wells are emptied before moving to the next step.
	Peptides may aggregate	Work with lower peptide concentrations.
Low Bead Count	Improper bead preparation	Make sure to vortex and sonicate beads right before use.
	Incorrect bead density	Make sure that right volumes of beads are correctly dispensed into wells
	Insufficient time for bead sedimentation on the magnet	Be sure to let the beads sediment for at least 5 minutes.
		If using more than 150 µL for washes, the bead sedimentation time must be increased
	Sample lost during washing and flicking	Maintain close contact between the microplate and the magnet.
No Signal or Low Signal	Monomer or Exiting Peptide Antibody are too dilute or absent.	Make sure that the right volumes and the right dilutions of MHC Monomer or Exiting Peptide Antibody are used.
	Incubation times incorrect.	Follow exactly the incubation times indicated in the protocol.
	Degraded reagent(s) are used in the assay.	Make sure that all reagents are stored in the right temperature and refrigerated if required.

TROUBLESHOOTING TETRAMER STAINING

Problems	Potential Causes	Potential Solutions
High Background	Spillover from adjacent well(s) if exchange was performed in a plate	Use individual tubes instead of plate for exchange.
	Reagents contaminated	Store in a cool, dry place and do not pipet into vials.
	Peptide aggregation	Work with lower peptide concentrations.
	Cell aggregation	Perform doublet discrimination (e.g. plot FSC-H x FSC-A and gate on diagonal population representing single cell events).
		Dialyze tetramer using a desalting column.
		Decrease peptide concentration in exchange reaction.
	Incorrect compensation	Check compensation controls and resulting comp matrix.
	Impure T cell population	Perform positive gating (e.g. CD3, CD4) to identify T cells and/or incorporated a dump channel to eliminate non-T cells (e.g. B cell and monocyte markers).
	Contamination with dead cells	Use a viability dye and gate out dead/dying cells.
No Signal or Low Signal	MHC tetramer is too dilute or absent	Perform cross-titration of tetramer and CD4 antibody.
	Incorrect compensation	Check compensation controls and resulting comp matrix.
	Incorrect incubation times	Follow exactly the incubation times indicated in the protocol.
	Degraded reagent(s) are used in the assay	Make sure that all reagents are stored properly.
	No antigen-specific T cells present in sample	Obtain positive control target cells to verify tetramer staining (e.g. generate antigen-specific T cells using mixed lymphocyte peptide cultures method).
	Difficulty to determine whether the low signal is specific or not	Perform comparison stainings with negative control tetramers generated by peptide exchange with irrelevant peptides.