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QuickSwitch™ Quant Tetramer Kit

QuickSwitch™ Quant HLA-A*24:02 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.

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-7302-1F or -4F) store frozen at ≤ -20°C upon kit arrival: Reference Peptide and Peptide Exchange Factor.
Kit components in the box (Code: TS-7302-1R 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. Most 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. 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.

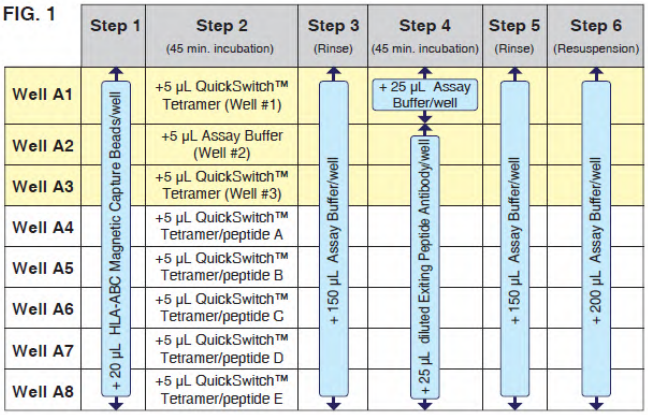
Note that peptide exchange reaction volumes can be scaled up or down, so long as reagent proportions are maintained.

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.

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 flow 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 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

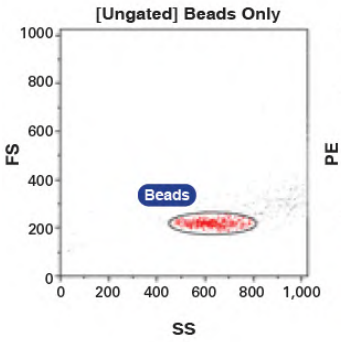
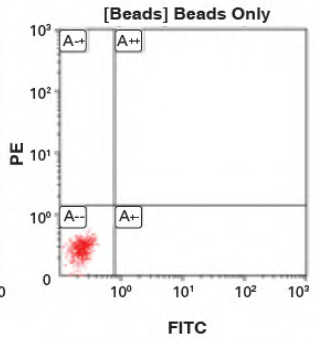
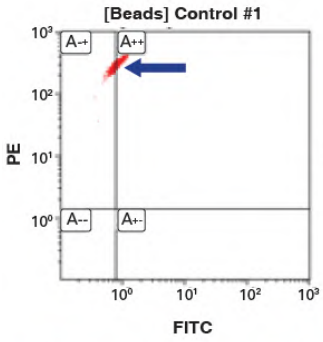


FIG. 3



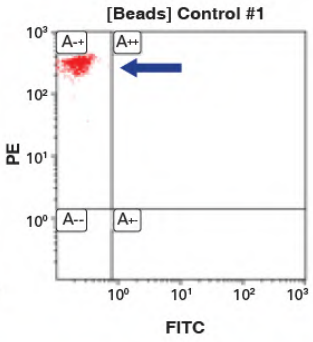
Gate	X-G Mean	Y-G Mean
A--	0.23	0.27
A-+	N/A	N/A
A+-	N/A	N/A
A++	N/A	N/A

FIG.4A



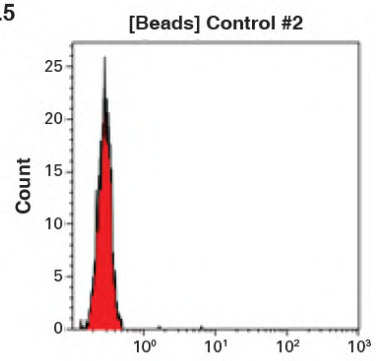
Gate	X-G Mean	Y-G Mean
A--	N/A	N/A
A-+	0.70	251.43
A+-	N/A	N/A
A++	0.91	328.63

FIG.4B



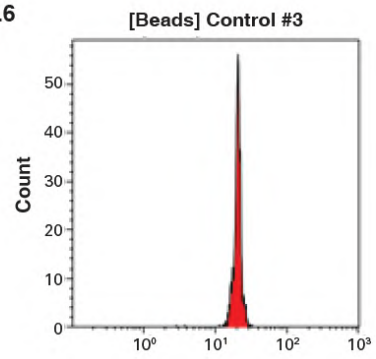
Gate	X-G Mean	Y-G Mean
A--	N/A	N/A
A-+	0.23	305.33
A+-	N/A	N/A
A++	N/A	N/A

FIG.5



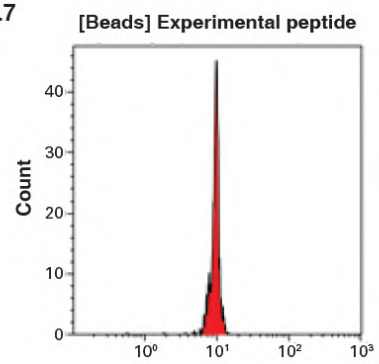
Gate X-GMean
All 0.28

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 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 HLA-A*24:02 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.

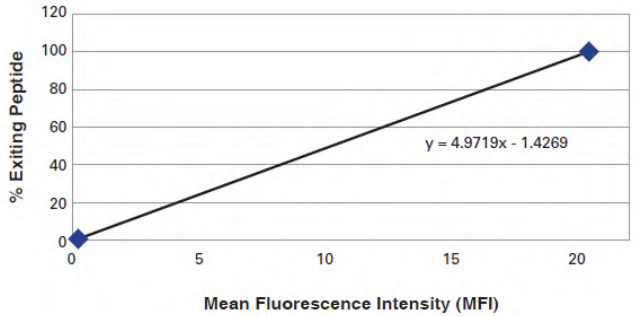
Table 2

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	100.85

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*24:02 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 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*24:02 is shown in Table 4.

Table 4

	Reference Peptide
Stock Concentration	1 mM
Final Concentration	20 µM
Peptide Exchange (N=3)	99.4 ±4.4 %

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 $CD8^+$ 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 binds to HLA-A*24:02 with high affinity and typically undergoes a > 95% exchange when used at a final 20 µM solution (see Table 4). It can be included in the test for confirming that the QuickSwitch™ procedure has been correctly followed.
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.
8. The sequence of the HLA-A*24:02 reference peptide is irrelevant. Exchanged tetramers generated with this peptide can therefore be used as negative controls in tetramer T cell stainings.

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REPRESENTATIVE DATA

Peptide Exchange quantitation curve is linear.

Tetramers containing 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. 9

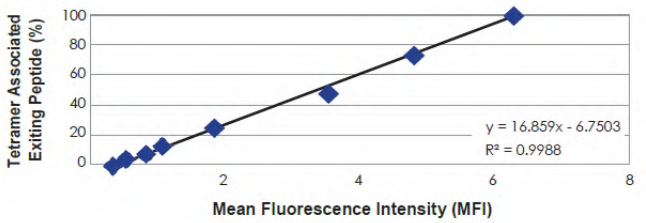


Fig. 10. Peptide-exchanged tetramers perform similarly to classically folded tetramers.

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 10^5 cells in 50 µL PBS-BSA-NaN_3 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) 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.

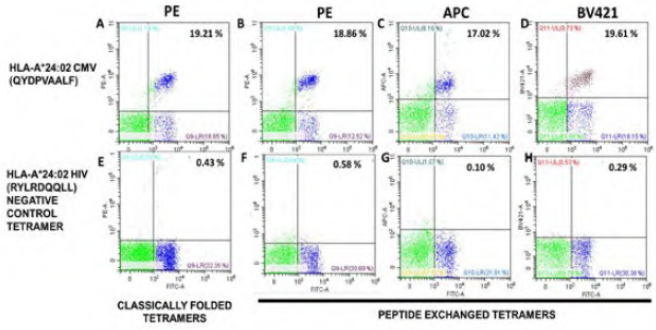
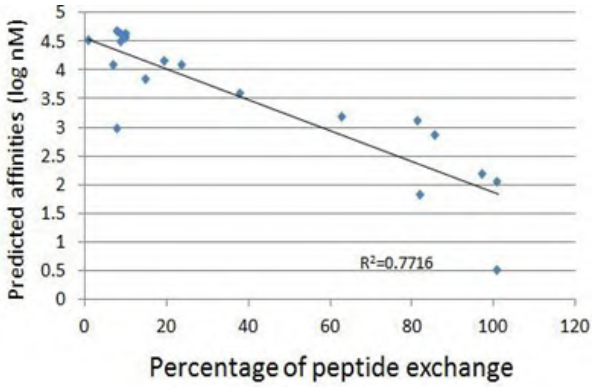


Fig. 11. The HLA-A*24:02 QuickSwitch™ Quant Tetramer assay can be used to validate HLA-A*24:02 binding candidate peptides.

Random peptides of different predicted HLA-A*24:02 binding affinities were tested with the QuickSwitch™ PE HLA-A*24:02 tetramer at a 20 µM final concentration. Peptides with predicted affinities varying from 1 nM to 1000 nM were swapped at a high rate (in the 80-100% range) in proportion to their affinity. These results indicate that the peptide exchange quantification assay can help screen good HLA-A*24:02 binders.



TROUBLESHOOTING BEAD QUANTITATION

Problems	Potential Causes	Potential Solutions
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.
Low Bead Count	Improper bead preparation	Make sure to vortex and sonicate beads immediately before use.
	Incorrect bead density	Make sure correct volumes of beads are 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.
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.
	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.
	Aggregation	Work with lower peptide concentrations.
No Signal or Low Signal	MHC tetramer or Exiting Peptide Antibody are too dilute or absent	Make sure that the correct volumes and dilutions of MHC tetramer and Exiting Peptide Antibody are used.
	Incorrect compensation and/or voltages set too low	Set voltages so that negative control is on scale, in the first decade. 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 correctly.

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.
	Aggregation	Perform doublet discrimination (e.g. plot FSC-H x FSC-A and gate on diagonal population representing single cell events).
		Dialyze tetramer.
		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, CD8) 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 CD8 antibody.
	Incorrect compensation and/or voltages set too low	Set voltages so that negative control is on scale, in the first decade. 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).

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