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QuickSwitch™ Quant HLA-A*02:01 Peptide Screening Kit

APPLICATION

The QuickSwitch™ HLA-A*02:01 Peptide Screening Kit utilizes a patented technique for exchanging up to 93 peptides on an MHC class I monomer. Components for quantifying the extent of peptide exchange by flow cytometry are included. The primary intended application of this assay is to screen, in a high throughput fashion, peptides that stably bind to the MHC complex. Usage of this kit is particularly indicated for validating neoantigen sequences obtained by exome high throughput sequencing and prescreened with epitope prediction algorithms.

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™ HLA-A*02:01 Peptide Screening Assay is based on the capacity of MHC class I molecules to exchange peptides.

PRINCIPLE

The kit contains two modules: 1) Biotinylated MHC class I monomer units folded with an irrelevant exchangeable peptide, along with a proprietary Peptide Exchange Factor, for the generation of monomers loaded with specific peptides of interest and 2) a flow cytometry-based sandwich immunoassay containing streptavidin-conjugated magnetic beads to capture MHC class I monomers and a FITC-labeled antibody recognizing the Exiting Peptide. This assay allows for determination of the capacity of test peptides to bind to MHC by quantifying the displacement of the original peptide. Peptide exchanged MHC molecules can be used in multiple applications including identification of antigen-specific $CD8^+$ T cells after tetramerization with fluorochrome-conjugated streptavidin (Note 1).

KIT COMPONENTS

QSM QuickSwitch™ HLA-A*02:01 Monomer
Biotinylated MHC class I monomer at 50 µg/mL, in a buffered solution with added protein stabilizers and ≤0.09 % sodium azide (1,000 µL x 3 amber vials with amber cap). Store at -80°C protected from light.

QSM Peptide Exchange Factor
The proprietary Peptide Exchange Factor is in aqueous solution (125 µL x 1 clear vial with green cap). Store at ≤ -20°C.

QSM Streptavidin Magnetic Capture Beads
Magnetic beads conjugated with streptavidin for binding biotinylated Monomers in a buffered solution with added protein stabilizers and ≤0.09% sodium azide (1,500 µL x 2 clear vials with red cap). Store at 2-8°C.

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

QSM Reference Peptide 0.5 mM
Peptide dissolved in a water/DMSO mixture at a 0.5 mM concentration (20 µL x 1 vial with black cap). Store at ≤ -20°C.

QSM Assay Buffer (10x)
Buffered solution with added protein stabilizers and ≤ 0.09 % sodium azide (1,800 µL x 4 vials with natural cap). Store at 2-8°C.

STORAGE CONDITIONS

Kit components in the box (Code: TS-7300-SF) store frozen: Monomer at -80°C, and Reference Peptide and Peptide Exchange Factor at ≤ -20°C.
Kit components in the box (Code: TS-7300-SR) store at 2-8°C: Streptavidin 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. The 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.
3. When the 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.

PROCEDURE

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

MATERIALS REQUIRED BUT NOT SUPPLIED

- Flow cytometer with plate loader
- Plate shaker (Labline model 4625 or equivalent)
- Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
- Magnetic tray for microplate (Dexter LifesepTM 96F or equivalent)
- Vortex
- Calibrated adjustable precision single channel micropipettes (for volumes between 1 µL and 1000 µL) with disposable tips
- Calibrated adjustable precision multichannel micropipette (for volumes between 5 µL and 200-300 µL) with disposable tips
- Calibrated adjustable precision multichannel micropipette (for volumes between 0.5 µL and 10 µL) with disposable tips (Sartorius Biohit Picus 735321 or equivalent)
- Round or preferentially conical bottom microplates for capture assays (Brand 781601 or equivalent)
- V well shaped polypropylene 96 well PCR microplates with silicone mats for peptide storage and peptide exchange (MidSci AVT2619 and VWR 10011-002 or equivalent)
- Microtubes
- Aluminum foil
- Distilled or purified water
- DMSO
- Peptides for new specificity tetramers
- 100 mL bottle or beaker for diluting the QSM Peptide Exchange Assay Buffer (10x)

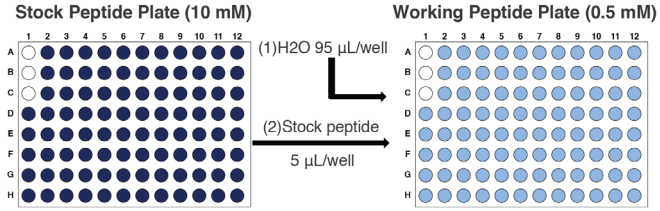
TEST PROCEDURE

A. Generation of New Specificity Monomer Using Peptide Exchange

Carefully read this protocol before performing an assay (Note 2). Bring all the reagents to room temperature prior to start and centrifuge briefly to collect liquids at the bottom of vials or 96 well storage plates.

Step 1 (Preparation of the Peptide Plate)

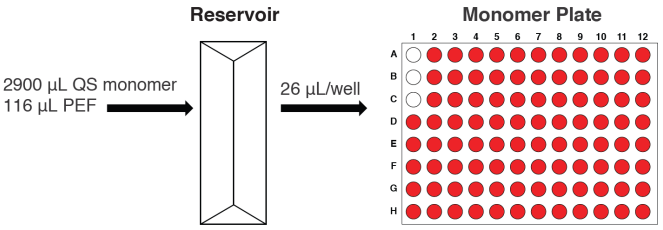
1. Prepare two V well shaped 96 well PCR microplates with silicone mats.
2. Dissolve lyophilized peptides to be assayed in DMSO to a 10 mM stock solution and dispense them to a 96 well PCR plate except wells A1, B1 and C1 (Notes 3, 4).
3. Dispense 95 µL distilled water to a second 96 well PCR plate to all wells except wells A1, B1 and C1.
4. Transfer 5 µL of peptide from the stock peptide plate to counterpart wells of the working peptide plate with a multichannel pipette to obtain a 0.5 mM concentration. (note: this corresponds roughly to a 0.5 mg/mL concentration for a 9 amino acid peptide).
5. Leave wells A1, B1, C1 empty.



Step 2 (Preparation of the Monomer Plate)

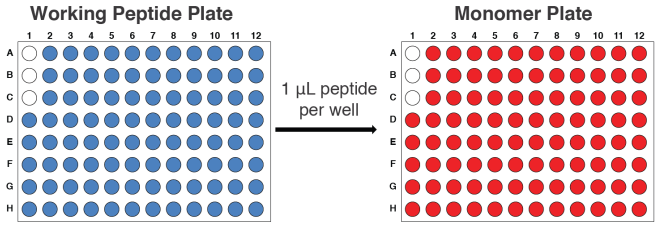
Each test well will receive 25 µL of QuickSwitch™ (QS) monomer, 1 µL of peptide exchange factor and 1 µL of 0.5 mM test peptide.

1. Spin monomer vials briefly to bring liquids down.
2. Pipet 2900 µL of QS monomer into a reservoir.
3. Add 116 µL of Peptide exchange factor and mix gently by swirling.
4. Dispense 26 µL of QS monomer to 93 wells of the 96 well plate with the multichannel pipet as described below. Make sure that dispensed volumes are equal. This corresponds to 25 µL monomer and 1 µL peptide exchange factor per well.
5. Leave wells A1, B1, C1 empty.

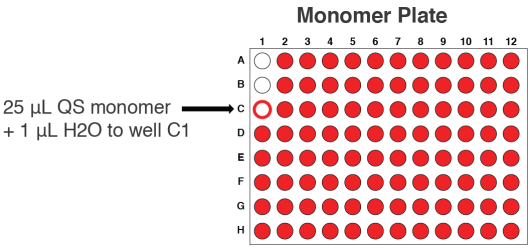


Step 3 (Setting up peptide exchange reaction)

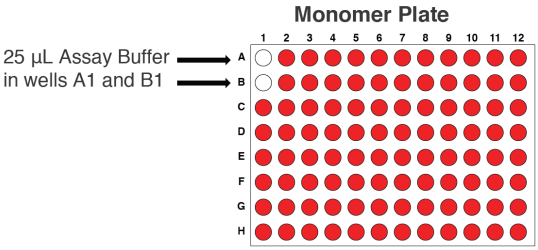
1. With a 0.5-10 µL multichannel pipette transfer 1 µL from peptide plate wells to the monomer microplate (Note 5). Make sure that equal volumes are pipetted.



2. Pipet 25 µL of HLA-A*02:01 QS monomer from the original monomer vial (without Peptide Exchange Factor) and dispense it to well C1.
   Add 1 µL of water to well C1 of the monomer plate.



3. Pipet 25 µL of Assay Buffer in wells A1 and B1 of the monomer plate



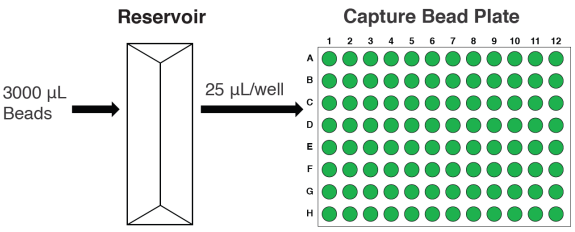
4. Perform a quick spin of the monomer plate in order to bring mixtures down.
5. Cover monomer plate with lid and let sit for 4 hours at RT, protected from light.

B. Quantification of Peptide Exchange using Flow Cytometric Sandwich Immunoassay

Capture Assay

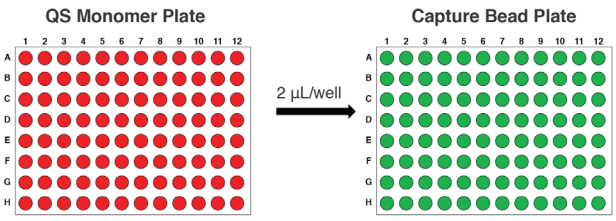
Step 1 (Dispensing capture beads)

1. Vortex and sonicate vials of streptavidin conjugated magnetic beads.
2. Dispense 3000 µL of magnetic capture beads into a reservoir.
3. Prepare a conical well plate with coverslip.
4. Dispense 25 µL beads per well in the wells indicated below.



Step 2 (Monomer capture)

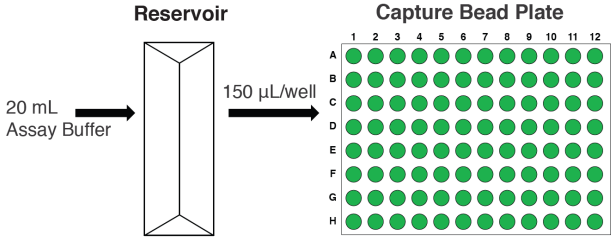
1. Transfer 2 µL from each well of the monomer plate to corresponding wells of the capture bead plate using a multichannel pipette.



2. Shake Capture Bead Plate for 30 min at 550 rpm, covered with lid and protected from light.

Step 3 (Rinse)

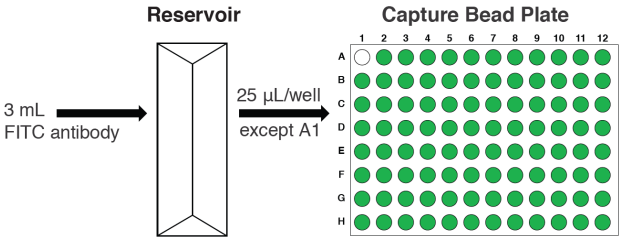
1. Dilute 10 x Assay Buffer by pipetting 63 mL distilled water in a bottle and adding 7 mL of 10 x Assay Buffer.
2. Pipet 20 mL of diluted Assay Buffer in a reservoir. With a multichannel pipette dispense 150 µL of Assay Buffer in all wells of the capture bead plate.



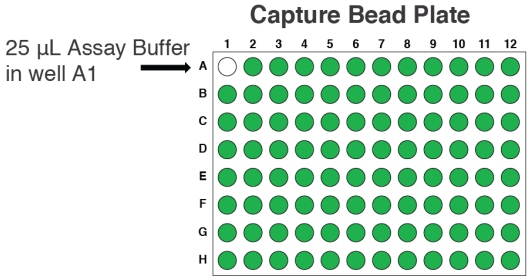
3. Place Capture Bead Plate on a plate magnet for 5-10 min (Note 6).
4. Flick the plate while holding it tightly in contact with the magnet.

Step 4 (Staining with Exiting Peptide antibody-FITC)

1. 1. Transfer the content of 2 vials of Exiting Peptide Antibody-FITC to a reservoir.
2. With the multichannel pipette dispense 25 µL antibody to all wells of the capture bead plate except well A1.



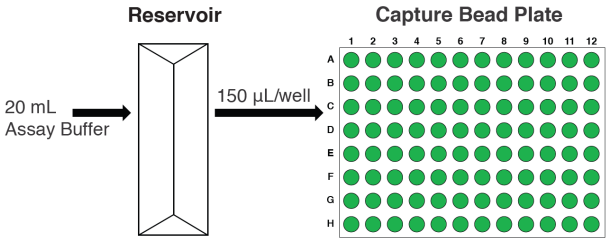
3. Dispense 25 µL of Assay Buffer in well A1.



4. Shake Capture Bead Plate for 45 min at 550 rpm, covered with lid and protected from light.

Step 5 (Rinse).

1. Pipet 20 mL of Assay Buffer in a reservoir.
2. With a multichannel pipette dispense 150 µL of Assay Buffer in all wells of the capture bead plate.



3. Place plate on a plate magnet for 5-10 min.
4. Flick the plate while holding it tightly in contact with the magnet.

Step 6 (Bead resuspension for flow acquisition)

1. Pipet 25 mL of Assay Buffer in a reservoir.
2. With a multichannel pipette resuspend beads by pipetting 200 µL of Assay Buffer in all wells of the capture bead plate.

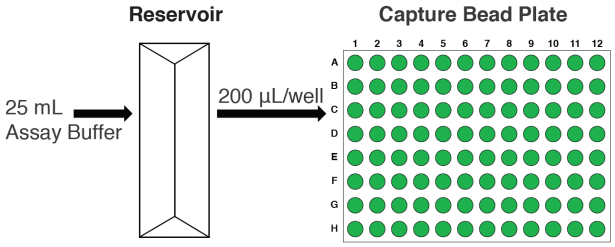
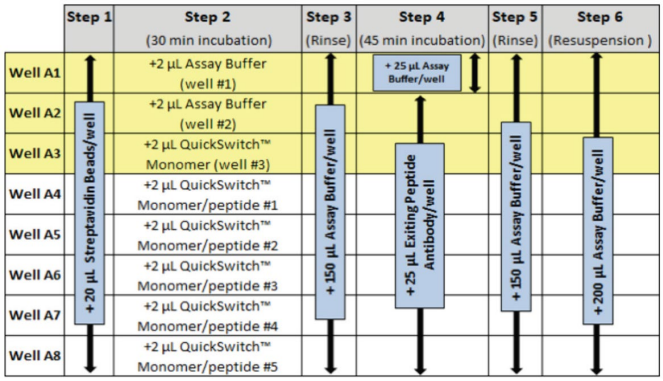


Fig. 1 summarizes the different steps of a capture assay in which five peptide-exchanged monomers are tested. The grey-filled wells are dedicated to controls that must be included in every assay.



Flow cytometer 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 doublets and aggregates (Fig. 2)

Fig.2

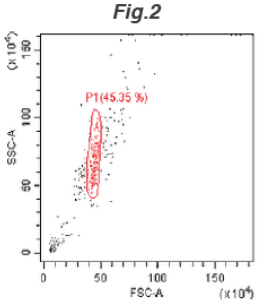
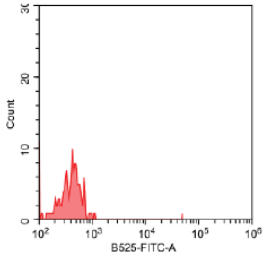


Fig. 3



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

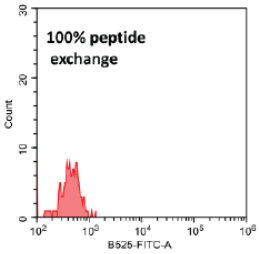
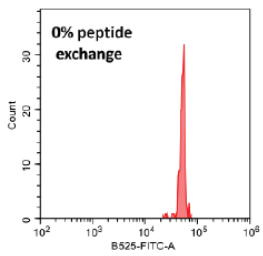


Fig. 5



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).
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 newly loaded peptide on the MHC molecules (Fig. 6).

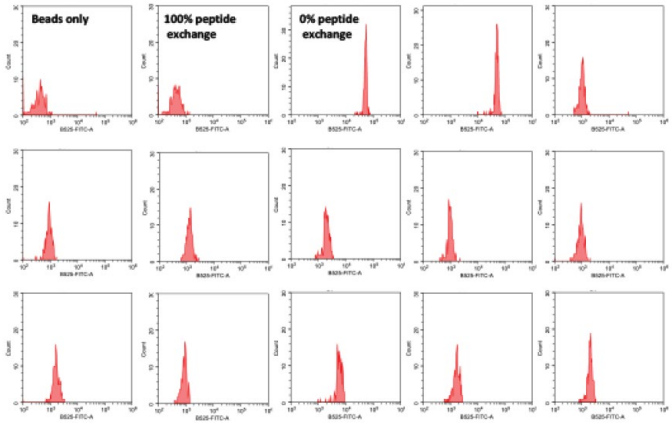


Fig. 6. Flow analysis of various peptide exchanged monomers generated with peptides of different affinities.
The measured MF_{IFITC} associated to the different peaks are intermediary between MFI values obtained with bead controls #2 and #3.

Data analysis

1. Collect the different MFI and calculate the different peptide exchanges using the spreadsheet from the MBL website. The QuickSwitch™ Calculator on the 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*02:01 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

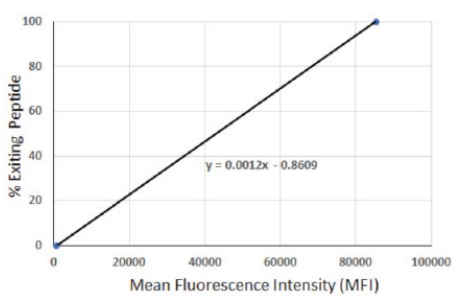
3. Enter the MFI obtained with the different tests (2nd column) to obtain the percentages of peptide exchange. Note that the calculator provides results only for MFI values below control #3. Higher values will return a "FALSE" response, as indicated in row E.

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 using the HLA-A*02:01 QuickSwitch™ Monomer (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-A*02:01 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

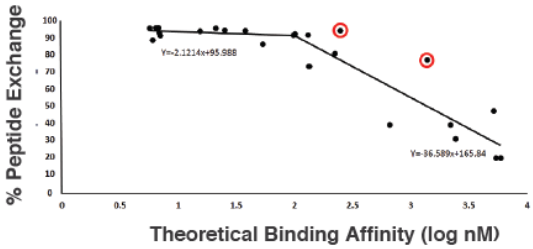
USE OF THE REFERENCE PEPTIDE

The Reference Peptide included in the kit serves as a positive control for peptide exchange of the QuickSwitch™ Monomer. Typically the high affinity binding HLA-A*02:01 Reference Peptide undergoes a > 90% exchange when used at a final 20 µM solution. Note that this peptide is irrelevant and absent from mammals, parasites, viruses or bacteria.

PEPTIDE EXCHANGE RATES ARE CORRELATED WITH PEPTIDE BINDING AFFINITIES TO HLA-A*02:01

Theoretical binding affinities of 28 peptides were plotted against their exchange rates as assessed by QuickSwitch™ (average of 3 independent experiments) (Note 7). Fig. 8 below indicates that 90 to 100% peptide exchange rates correspond to 0-100 nM theoretical binding affinities to HLA-A*02:01. A 75-85% peptide exchange rate corresponds to a 150-300 nM peptide binding affinity.

Fig. 8



Thus a rough estimate of peptide binding affinities can be deduced from their exchange rates on the HLA-A*02:01 QuickSwitch™ monomer. Note the presence of outliers indicating that QuickSwitch™ detects binders that might have been rejected by epitope prediction algorithms (red circles). This underscores that QuickSwitch™ is an important tool that complements and validates in silico screens data.

LIMITATION

Do not mix components from other lots and kits (such as QuickSwitch™ tetramer kits) as compositions and concentrations of components are different from kit to kit. In particular, Peptide Exchange Factor cannot be interchanged between kits due to potential incompatibility issues.

NOTES

1. The QuickSwitch™ HLA-A*02:01 Peptide Screening Kit has been devised exclusively for measuring peptide binding to HLA-A*02:01. Peptides screened with this kit can be used with the MBL QuickSwitch Tetramer peptide exchange kits for generating functional PE, APC or BV421 conjugated tetramers.
   (https://ruo.mbl.co.jp/bio/sch/?kw=quickswitch)
2. The assay setup presented here can be modified at will as long as ratios of peptide exchange factor, test peptide and monomers are unchanged and control samples are always included. For example, control wells and peptides can be assayed as duplicates or triplicates.
3. Most peptides are soluble in DMSO. However, some highly basic or acidic peptides may precipitate in DMSO and would require alternative buffers.
4. Peptides can be purchased already dispensed in 96 well plates as lyophilized 1-mg samples per well. Dissolving 1 mg peptide in 100 µL DMSO results in a 10 mg/mL peptide final concentration which can be approximated to a 10 mM solution since a 9 amino acid peptide has a molecular mass close to 1 kDa.
5. 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 will increase the percentage of peptide exchange of low affinity peptides but may trigger monomer aggregation in some cases.
6. This assay uses a magnet to pellet the 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 bead loss in some cases.
7. The peptide binding affinities to HLA-A*02:01 were obtained from the IEDB neural network-based alignment algorithm.

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.

RELATED PRODUCTS

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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 monomer or Exiting Peptide Antibody are too dilute or absent	Make sure that the correct volumes and dilutions of MHC monomer and Exiting Peptide Antibody are used.
	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.