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QuickSwitch™ Quant HLA-A*02:01 Monomer Kit

APPLICATION

The QuickSwitch™ Quant Monomer Kit utilizes a patented technique for exchanging up to ten peptides on an MHC Class I Monomer. Components for quantifying the extent of peptide exchange by flow cytometry are included in this kit. New specificity monomers obtained by peptide exchange can be used for multiple applications that include generation of tetramers or higher order multimers, stimulation and isolation of antigen-specific $CD8^+$ T cells.

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™ MHC Class I Quant Monomer 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 to determine the capacity of test peptides to bind to MHC by quantifying the displacement of the original peptide. Peptide exchanged monomers are suitable for identification of antigen-specific $CD8^+$ T cells and can be used in multiple applications (Note 1).

KIT COMPONENTS

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 (500 µL x 1 amber vial with amber cap). Store at -80°C.

Peptide Exchange Factor
The proprietary Peptide Exchange Factor is in aqueous solution (13 µL x 1 clear vial with green cap). Store at ≤ -20°C.

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

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 (500 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.

STORAGE CONDITIONS

Kit components in the box (Code: TS-7300-MF) store frozen: Monomer at -80°C, and Reference Peptide and Peptide Exchange Factor at ≤ -20°C.
Kit components in the box (Code: TS-7300-MR) are stored 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. 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 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.

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 monomers

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 Monomer Using Peptide Exchange

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

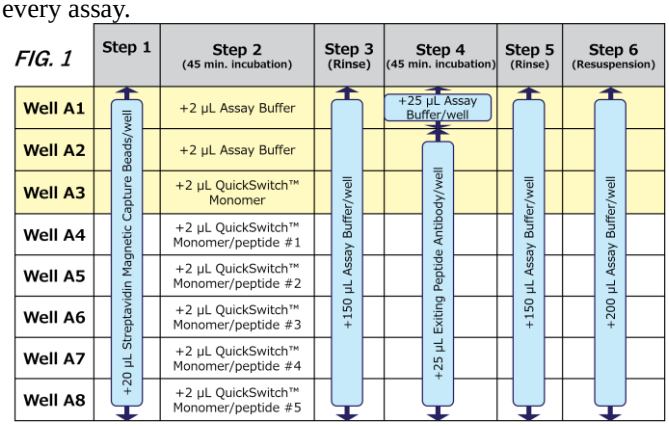
1. Dissolve each lyophilized peptide to be assayed in DMSO to a 10 mM solution (~10 mg/mL for a 9 amino acid peptide). (Note 3). Aliquots of this peptide solution can be further diluted in water to the desired concentration. For high affinity peptides, a 1 mM stock solution is a reasonable starting concentration for the assay. For lower affinity peptides, a higher concentration may be necessary, but may cause monomer aggregation particularly with hydrophobic peptides.
2. Pipet 50 µL of QuickSwitch™ Monomer into a microtube or well of round- or conical-bottom 96 well microtiter plate.
3. Add 1 µL of Peptide Exchange Factor from green capped vial and mix gently with pipetting.
4. Add 1 µL of peptide and mix gently with pipetting. (Note 4)
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. Monomers are now ready for use in quantitation (see section B) and/or staining assays after tetramerization. (Note 6). Monomers generated with the Reference Peptide are used as a positive control for exchange quantitation (see Section B).
8. Peptide exchanged monomers can be kept at 2-8°C for short period of time and frozen at < -20°C for long time storage. 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 monomer 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 monomers 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 Streptavidin Magnetic Capture Beads for essential controls.
2. Pipet 20 µL Streptavidin Magnetic Capture Beads to additional wells for each peptide-exchanged Monomer to test.

Step 2 (Monomer Capture).

1. Pipet 2 µL 1x Assay Buffer in wells #1 and #2.
2. Pipet 2 µL QuickSwitch™ Monomer in well #3.
3. In well #4, pipet 2 µL taken from the first peptide exchange microtube or well of 96 well microtiter plate. Repeat for each additional peptide exchanged monomer 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.
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. Pipet 25 µL of 1x Exiting Peptide Antibody in all wells, except well #1.
2. Pipet 25 µL of 1x Assay Buffer in well #1.
3. 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

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

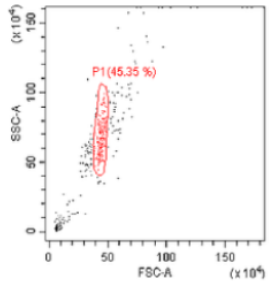
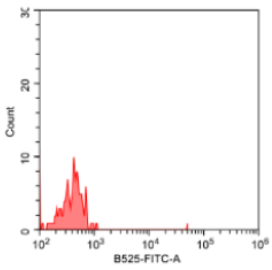


FIG. 3



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

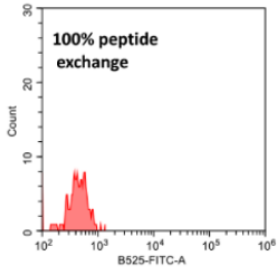
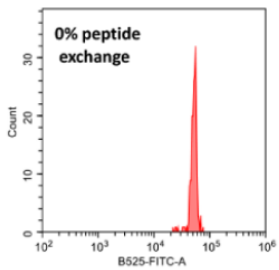
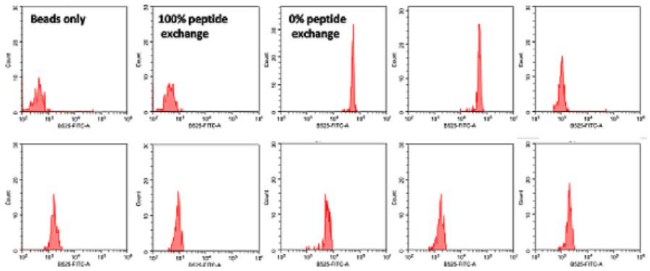


FIG. 5



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.

FIG. 6



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

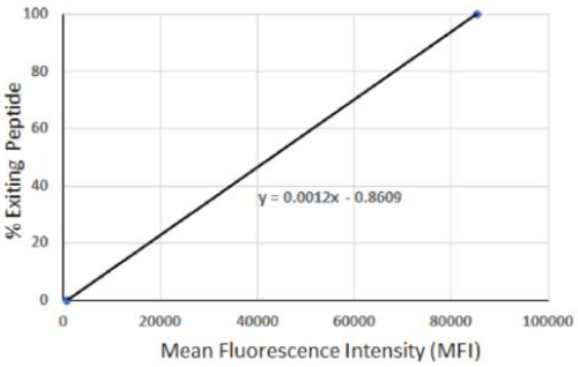
Table 2

Peptide Sample	QuickSwitch™ Tetramer 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,

Fig. 7



   as shown in the example below using the HLA-A*02:01 QuickSwitch™ Monomer (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**

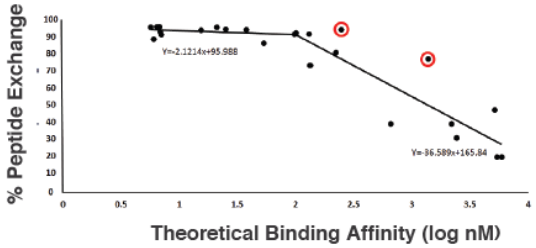
USE OF THE REFERENCE PEPTIDE

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

Fig. 8



corresponds to a 150-300 nM peptide binding affinity.

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 different lots and types of kits (QuickSwitch™ tetramer kits). Composition and concentration of components vary from kit to kit. In particular, Peptide Exchange Factor cannot be interchanged between kits due to potential incompatibility issues.

NOTES

1. The QuickSwitch™ Quant Monomer Kit has been devised for two purposes: first for measuring peptide binding to HLA-A*02:01 and second for creating new specificity monomers that can be used in various applications that include staining, activation or isolation of T cells.
2. 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 however because this could result in monomer aggregation and/or bead clumping and bead loss in some cases.
3. Most peptides are soluble in DMSO. However, some highly basic or acidic peptides may precipitate in DMSO and would require alternative buffers. 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.

4. 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.
5. The sequence of the HLA-A*02:01 reference peptide is irrelevant. MHC tetramers built with monomers generated with this peptide can therefore be used as negative controls in T cell stainings.
6. MHC class I monomers have an average MW of 50 kDa and since the concentration of biotinylated QuickSwitch™ monomers is 50 µg/mL, a 500 µL monomer volume contains 0.5 nanomole. Streptavidin has four biotin binding sites, thus 0.125 nanomole streptavidin will be required for tetramerizing 0.5 nanomole or 500 µL of QuickSwitch™ monomer[3].
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.
3. Altman J. D., Davis M. M. 2016. MHC-peptide tetramers to visualize antigen-specific T cells. Curr. Protoc. Immunol. 115, 17.3.1-17.3.44

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TROUBLESHOOTING PEPTIDE EXCHANGE AND CAPTURE ASSAY

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.
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 immediately 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 correct volumes and dilutions of MHC tetramer and Exiting Peptide Antibody are used.
	Incorrect compensation and/or voltages set too low.	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.

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