# Rapid generation of new specificity MHC tetramers for the detection of antigen-specific T cells using a novel peptide exchange tetramer kit that allows for quantification of peptide exchange


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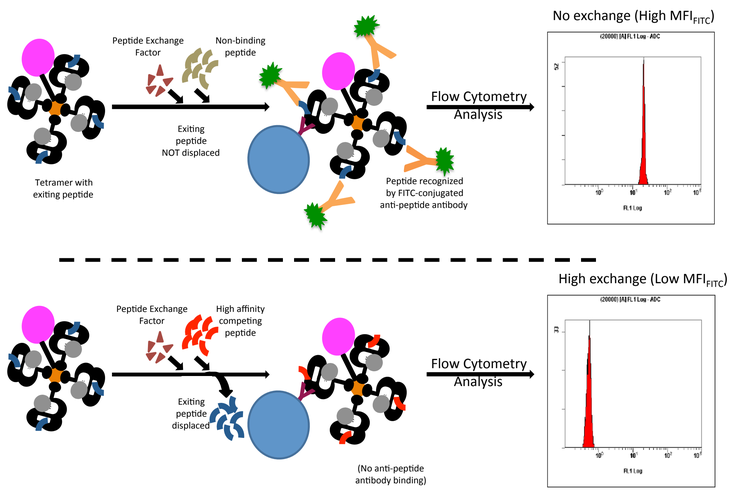
## INTRODUCTION

- 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.
- Using this principle of peptide exchange, we have developed a kit for the generation of new specificity MHC tetramers, whereby a peptide of interest and a proprietary peptide exchange factor is incubated with a fluorescently labeled "QuickSwitch™" tetramer containing a special exiting peptide. While alternate methodologies rely on UV cleavage of exiting peptide on monomeric MHC complexes and a subsequent lengthy tetramerization procedure, the QuickSwitch™ Tetramer Kit produces tetramers ready for cell staining to detect antigen-specific T cells in just four hours.
- The efficiency of peptide exchange can be quantified using a novel flow cytometry-based sandwich immunoassay, QuickSwitch™ Quant, using magnetic beads conjugated with an anti-HLA-A,B,C antibody for tetramer capture and a FITC conjugated antibody reacting against the exiting peptide.

## QuickSwitch™ Quant Assay




## METHODS

- New specificity tetramers were generated by room temperature incubation of 50 µl QuickSwitch™ tetramer, 1 µl of Peptide Exchange Factor, and 1 µl of 1 mM peptide (20 µM final) per well of a round bottom 96-well plate.
- Peptide exchange was quantified on the tetramer using a flow cytometric immunoassay using anti-HLA-A,B,C coated magnetic capture beads and a FITC labeled antibody detecting the QuickSwitch exiting peptide.
- Resulting tetramers with high percentage of peptide exchange were used in staining assays for flow cytometry at various concentrations, based on MHC monomer content.

**QuickSwitch Quant Assay Setup**

| | Step 1 | Step 2 (45 min incubation) | Step 3 (Rinse) | Step 4 (45 min incubation) | Step 5 (Rinse) | Step 6 (Resuspend) |
|---|---|---|---|---|---|---|
| Well A1 | + 20 µL Magnetic Capture Beads/well | +5 µL QuickSwitch™ Tetramer (well #1) | + 150 µL Assay Buffer/well | + 25 µL Assay Buffer/well + 25 µL diluted Exiting Peptide Antibody/well | + 150 µL Assay Buffer/well | + 200 µL Assay Buffer/well |
| Well A2 | | +5 µL Assay Buffer (well #2) | | | | |
| Well A3 | | +5 µL QuickSwitch™ Tetramer (well #3) | | | | |
| Well A4 | | +5 µL QuickSwitch™ Tetramer/peptide A | | | | |
| Well A5 | | +5 µL QuickSwitch™ Tetramer/peptide B | | | | |
| Well A6 | | +5 µL QuickSwitch™ Tetramer/peptide C | | | | |
| Well A7 | | +5 µL QuickSwitch™ Tetramer/peptide D | | | | |
| Well A8 | | +5 µL QuickSwitch™ Tetramer/peptide E | | | | |

- A peptide exchange calculation sheet downloadable from the MBL International website is used to readily calculate peptide exchange rates based on MFI values

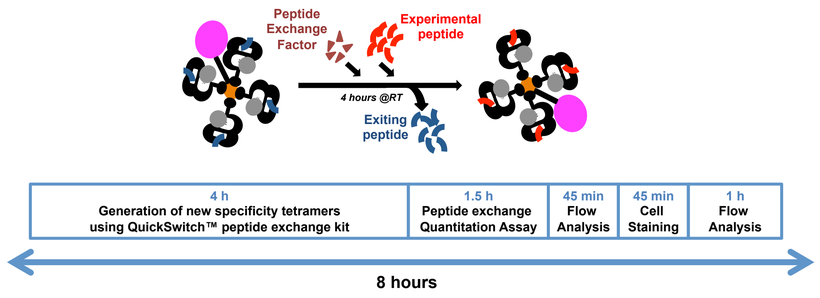
**QuickSwitch™ Quant Peptide Exchange Calculation Sheet**

ENTER VALUES FROM EXPERIMENT

| Analyzed sample | MFI$_{FITC}$ |
|---|---|
| Control #2: 0% Exiting Peptide or 100% peptide exchange | 0.48 |
| Control #3: 100% Exiting Peptide or 0% peptide exchange | 28.4 |

| Peptide Sample/Sequence | QuickSwitch MFI$_{FITC}$ after Peptide Exchange | % Peptide Exchange |
|---|---|---|
| HIV (ILKEPVHGV) | 3.03 | **87.00** |
| MART1 (ELAGIGILTV) | 2.12 | **91.73** |
| CMV (NLVPMVATV) | 2.56 | **89.44** |

## New specificity tetramer generation with QuickSwitch™



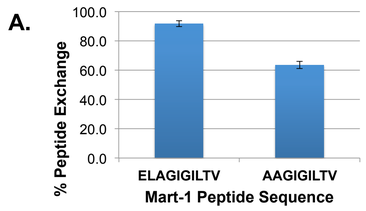

## RESULTS

### Figure 1. HLA-A*02:01 QuickSwitch™ Quant is reproducible

| Kit Fluorochrome | APC | APC | PE | PE | BV421 | PE | APC | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Operators | #1 | #2 | #3 | #3 | #4 | #4 | #4 | | | |
| Cytometer | Accuri | Accuri | FC500 | FC500 | Gallios | Gallios | Gallios | **Average** | **SD** | **N** |
| Reference peptide | 92.5 | | 91.1 | 95.3 | | | | **93.0** | **2.2** | **3** |
| HIV pol (ILKEPVHGV) | 89.0 | 88.0 | 87.3 | 92.5 | 96.9 | 92.0 | 87.0 | **90.4** | **3.6** | **7** |
| Mart-1 (ELAGIGILTV) | 90.3 | 89.4 | 90.0 | 94.2 | 98.7 | 95.6 | 91.7 | **92.8** | **3.5** | **7** |
| CMV (NLVPMVATV) | 90.4 | 90.4 | 90.0 | 94.6 | 99.0 | 93.6 | 89.4 | **92.5** | **3.5** | **7** |

- Peptide exchange reaction was performed using PE-, APC-, and BV421-labeled QuickSwitch™ tetramers by four different operators and analyzed using the QuickSwitch™ Quant assay.

### Figure 2. Peptide exchange percentage correlates with MHC binding affinity



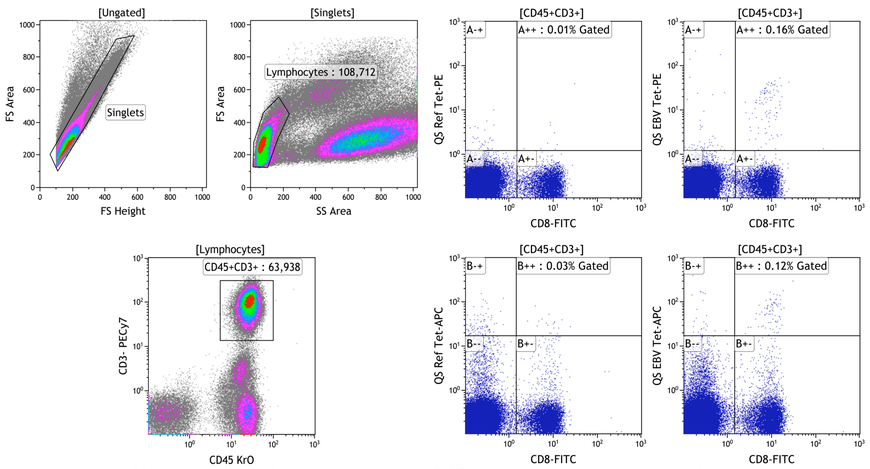

B.

| Competing peptide | Theoretical affinity (nM) (SMM) | Peptide exchange (in %) |
|---|---|---|
| ELAGIGILTV | 145.71 | 85.0558 |
| ILKEPVHGV | 115.64 | 82.94 |
| AAGIGILTV | 1429.26 | 68.902 |
| KAFSPEVIPMF | 14686.22 | 12.748 |

*IEDB database

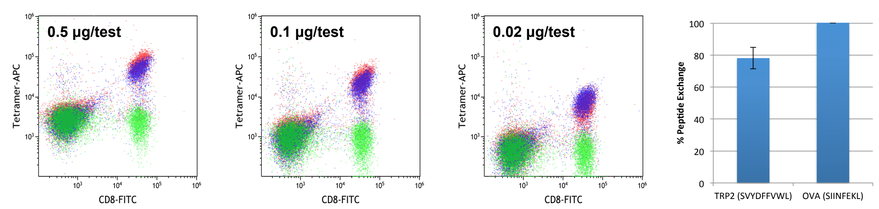
- QuickSwitch™ Quant can be used to assess peptide exchange to verify that functional tetramers have been generated prior to cell staining. HLA-A*02:01 QuickSwitch™ tetramer was incubated for 4 hours with peptides at a final of 20 µM (A) or 10 µM (B) in the presence of peptide exchange factor. Peptide exchange correlated with the theoretical peptide affinity of each peptide towards HLA-A*02:01.

### Figure 3. HLA-A*02:01 QuickSwitch™ tetramers detect rare EBV responses in blood



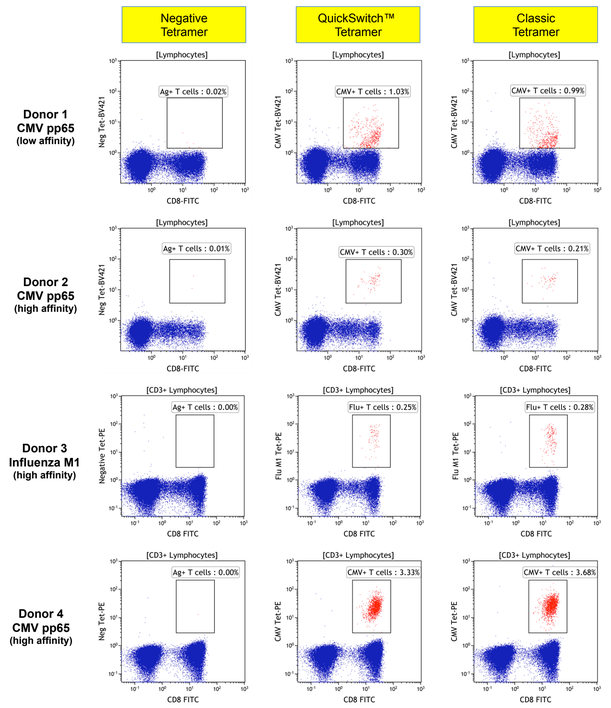

- Whole blood (200 µl) was incubated in flow tubes with 0.25 µg of tetramer along with directly labeled antibodies to CD45, CD3, and CD8 for 30 minutes at room temperature, protected from light.
- Red blood cells were lysed (VersaLyse supplemented with 0.2% PFA; 1 ml/tube) for 20 minutes/dark at room temperature. Samples were pelleted, washed with 1.5 ml staining buffer, and resuspended in 0.1% PFA.
- Approximately 100,000 lymphocyte events were acquired on a 3 laser/10 color Gallios flow cytometer and analyzed using Kaluza 1.5a.

### Figure 4. H-2 Kb QuickSwitch™ tetramers detect OVA-specific T cells in OT-I mice




- OT-I splenocytes (1.2x10$^5$/well) were incubated in flow tubes with 0.5 µg, 0.1 µg, or 0.02 µg APC-labeled tetramer along with CD8-FITC (clone KT15; 0.4 µl/test) in 100 µl final assay volume for 30 minutes at room temperature, protected from light.
- Cells were washed in 1.5 ml/tube cell staining buffer and resuspended in 0.1% PFA.
- Approximately 10,000 cell events were acquired on a 3 laser/10 color Gallios flow cytometer and analyzed using Kaluza 1.5a.
- H-2 Kb TRP2 used a negative control (#T03015; green), classically folded H-2 Kb OVA (#T03002; blue), and H-2 Kb QuickSwitch™ OVA (red) tetramer staining, as well as peptide exchange quantification, are shown.

### Figure 5. HLA-A*02:01 QuickSwitch™ tetramers detect similar percentages of low and high affinity antigen-specific responses in PBMCs as classically folded tetramers




***Donors 1 and 2***

- PBMCs (3-6x10$^5$/well) were incubated with 0.25 µg BV421-labeled tetramer along with CD8-FITC (clone RPA-T8; 1 µl/test) for 30 minutes at room temperature.
- Cells were washed in 1.5 ml/tube cell staining buffer and resuspended in 0.1% PFA.
- Approximately 25,000 lymphocyte events were acquired on a 3 laser/10 color Gallios flow cytometer and analyzed using Kaluza 1.5a.

***Donors 3 and 4***

- PBMCs (5x10$^5$/well) were incubated with 1 µg PE-labeled tetramer along with anti CD3-PC5 and anti CD8-FITC (clone SFCI21Thy2D3; diluted 1/20) for 30 minutes at room temperature, protected from light.
- Cells were washed in 1.5 ml/tube cell staining buffer and resuspended in 0.1% PFA.
- Approximately 20,000 CD3+ lymphocyte events were acquired on a 2 laser/4 color FC500 flow cytometer and analyzed using Kaluza 1.5a.

## SUMMARY

***Simple and Fast Protocol***

- Mix Tetramer, Peptide, and Peptide Exchange Factor
- Complete reaction in 4 hours

***Key Features***

- Peptide exchange directly on tetramers
- Ability to quantify the exchange reaction using a simple, quick, flow-based assay
- No UV treatment or tetramerization required
- Contains patented α3 mutation to reduce background seen using wild-type tetramers
- Scalable to high-throughput assay
- Can be used for screening and exploratory work
- Plan to expand to additional human and mouse alleles

## QuickSwitch™/QuickSwitch™ Quant Tetramer Kits

| µL per test | µg per test | # tests per peptide exchange | # tests per kit |
|---|---|---|---|
| 5 | 0.25 | 10 | 100 |
| 10 | 0.5 | 5 | 50 |
| 20 | 1 | 2.5 | 25 |

***Note: Quant reaction uses 5 µL per exchange***

## REFERENCES


Reaper DR, Cresswell P. Regulation of MHC class I assembly and peptide binding. Annu Rev Cell Dev Biol 2008, 24:343-368.

Mayerhofer PU, Tampé R. Antigen translocation machineries in adaptive immunity and viral immune evasion. J Mol Biol 2015, 427(5):1102-1118.