Protocol for preparation and loading of easYmers® MHC I-peptide monomer onto U-Load Dextramer®

Background

easYmers® powered by immunAware is a formulation of peptide-receptive MHC I monomer, which can be used to generate specific MHC-peptide monomer by loading your peptide of choice. The easYmers® MHC I-peptide monomer can easily be loaded onto fluorescently labeled U-Load Dextramer® and used to detect antigen-specific $CD8^+$ T cells using flow cytometry. Optionally, the peptide-loaded easYmers® can be stored frozen at -20°C for later use. The easYmers® technology is highly flexible and suitable for screening a single epitope in many samples or screening a large number of different epitopes in parallel. The easYmers® technology also allows evaluation of peptide binding to MHC I by assaying proper refolding of peptide-loaded easYmers® monomer.

Materials required

The materials listed here are required for preparation of MHC I-peptide monomer and U-Load Dextramer® MHC I.

easYmers®
easYmers® loading buffer
easYmers® positive control peptide
U-Load Dextramer®
U-Load Dextramer® dilution buffer

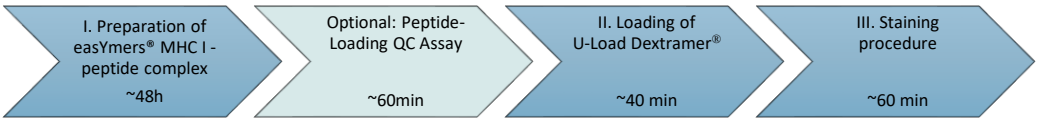
Materials required (not provided)

The materials listed here are required for preparation of MHC I-peptide monomer and U-Load Dextramer® MHC I and for the flow cytometry-based assay for evaluation of proper folding of easYmers® MHC I-peptide monomer.

Peptide of choice
DMSO (e.g., Sigma cat.# D2650)
Dilution buffer (PBS, 5% glycerol)
FACS buffer (PBS, 1% BSA (or FCS), 0.01% NaN_3)
Streptavidin-coated beads (Spherotech cat.# SVP-60-5)
Anti-human β_2m BBM.1-PE (Santa Cruz cat.# sc-13565 PE)
PBS = Phosphate-buffered saline pH 7.4

Protocol steps and timing

Experimental workflow using the easYmers® and U-Load Dextramer® and estimated time to complete each step.



I. Preparation of easYmers® MHC I-peptide monomer

1. Reconstitute your peptides of interest according to the manufacturer's instructions.
2. Dilute Peptide (easYmers® control peptide or peptide of interest) to 100 µM in ddH_2O. Keep on ice from this step on.
3. To prepare easYmers® MHC I-peptide monomer, mix the reagents in Table A1 for Human MHCI or Table A2 for Murine MHCI according to the listed sequence in a 1.5 mL tube or 96-well U-bottom plate. The listed amounts will be enough to make 10, 20, or 50 tests of U-Load Dextramer® MHC I.

   Optional: To evaluate the peptide loading efficiency make a smaller volume of the easYmers® positive and the negative control (no peptide), i.e., easYmers® loaded with the included easYmers® positive control peptide or no peptide as listed in Table A.

Table A1

Reagents	**10 tests**	**20 tests**	**50 tests**	**Positive Control**	**Negative control**
ddH_2O	26.5 µL	53 µL	132.5 µL	2.5 µL	3 µL
Peptide (100 µM)	3.5 µL	7 µL	17.5 µL	0.5 µL	-
easYmers® Loading Buffer	10 µL	20 µL	50 µL	3 µL	3 µL
easYmers® (3 µM)	20 µL	40 µL	100 µL	3 µL	3 µL
Total volume of easYmers® MHC I-peptide monomer (1 µM)	**60 µL**	**120 µL**	**300 µL**	**9 µL**	**9 µL**

Table A2

Reagents	**10 tests**	**20 tests**	**50 tests**	**Positive Control**	**Negative control**
PBS pH 7.4	36.5 µL	73 µL	182.5 µL	5.5 µL	6 µL
Peptide (100 µM)	3.5 µL	7 µL	17.5 µL	0.5 µL	-
easYmers® (3 µM)	20 µL	40 µL	100 µL	3 µL	3 µL
Total volume of easYmers® MHC I-peptide monomer (1 µM)	**60 µL**	**120 µL**	**300 µL**	**9 µL**	**9 µL**

4. Mix by pipetting gently – *be careful not to form bubbles.*
5. Briefly centrifuge to collect all materials in the bottom of the tube and incubate at 18°C for 48 h.
6. Briefly centrifuge to collect all material in the bottom of the tube. 1 µM folded easYmers® MHC I-peptide monomer are now ready for loading onto U-Load Dextramer® or can be stored at -20°C for long-term storage.

7. Proceed to page 4 to evaluate peptide-loading efficiency or continue to load onto U-Load Dextramer®.

II. Loading of U-Load Dextramer®

8. To load the easYmers® MHC I-peptide monomer onto U-Load Dextramer®, mix the reagents in Table B in a 1.5 mL tube:
U-Load Dextramer® APC require different volume of reagents. See Procedural notes.

Table B

Reagents	10 tests	20 tests	50 tests
easYmers® MHC I-peptide monomer (1 µM)	57 µL	114 µL	285 µL
U-Load Dextramer® (PE/FITC)	20 µL	40 µL	100 µL
incubate for 30 min at RT in the dark			
U-Load Dextramer® Dilution Buffer	23 µL	46 µL	115 µL
Total volume U-Load Dextramer® MHC I	**100 µL**	**200 µL**	**500 µL**

9. Store the fluorescent U-Load Dextramer® MHC I reagents at 2-8°C in the dark until use.

III. Staining procedure

To analyze antigen-specific $CD8^+$ T cells in blood using flow cytometry for one or more specificities using U-Load Dextramer® MHC I, see www.immudex.com/Protocols/mhc-dextramer-staining-protocol

Procedural notes

1. Protocol step 8: To assemble the peptide-loaded easYmers® with U-Load Dextramer® APC, mix the reagents in Table C in a 1.5 mL tube:

Table C

Reagents	10 tests	20 tests	50 tests
easYmers® MHC I-peptide monomer (1 µM)	38 µL	76 µL	190 µL
U-Load Dextramer® (APC)	20 µL	40 µL	100 µL
incubate for 30 min at RT in the dark			
U-Load Dextramer® Dilution Buffer	42 µL	84 µL	210 µL
Total volume U-Load Dextramer® MHC I	**100 µL**	**200 µL**	**500 µL**

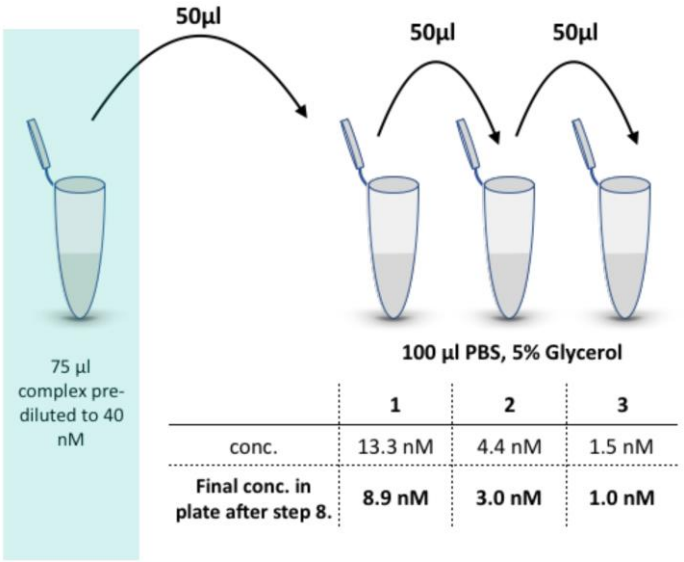
Optional: Flow cytometry-based quality control assay for determination of peptide loading efficiency

Background

After easYmers® MHC I-peptide monomerization (step 6 in the protocol), the relative peptide loading efficiency can be determined by comparing your peptide of interest to the negative and positive loading controls using this assay. The negative loading control is empty easYmers® (no peptide). The positive loading control peptide is specific to and provided with the easYmers® you purchase. If this is your first time testing a particular easYmers® MHC I-peptide combination, this assay is highly recommended.

Procedure: evaluation of easYmers® MHC I-peptide monomer formation

1. Prepare a sufficient volume of dilution buffer (PBS, 5% glycerol).
2. To determine the efficiency of the easYmers® MHC I-peptide folding take 3 µL of the prepared easYmers® MHC I-peptide monomer (1 µM) and dilute to 500 nM by adding 3 µL of dilution buffer.
3. Dilute each of the easYmers® MHC I-peptide monomer to give 75 µL of a 40 nM solution (e.g., for a 500 nM monomer: 6 µL folded monomer in 69 µL dilution buffer).
4. For all samples and positive and negative loading controls, transfer 50 µL of this pre-dilution (prepared in step 3) to the first tube. Make three subsequent serial 3-fold dilutions (50 µL in 100 µL dilution buffer), according to the figure below.



	1	2	3
conc.	13.3 nM	4.4 nM	1.5 nM
Final conc. in plate after step 8.	**8.9 nM**	**3.0 nM**	**1.0 nM**

5. Transfer 40 µL of each of these dilutions to the wells in a U-bottom shape 96-well plate, as suggested below. Also, prepare a background well (BLANK): 40 µL of dilution buffer (no beads or antibody will be added to this well).

6. Prepare a sufficient volume of a 45-fold dilution of the streptavidin-coated beads in dilution buffer. Transfer 20 µL of the diluted bead suspension to each well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK		P-1		S1-1		S3-1		S5-1		S7-1	
B			P-2		S1-2		S3-2		S5-2		S7-2	
C			P-3		S1-3		S3-3		S5-3		S7-3	
D												
E			N-1		S2-1		S4-1		S6-1		S8-1	
F			N-2		S2-2		S4-2		S6-2		S8-2	
G			N-3		S2-3		S4-3		S6-3		S8-3	
H												

BLANK : No complex

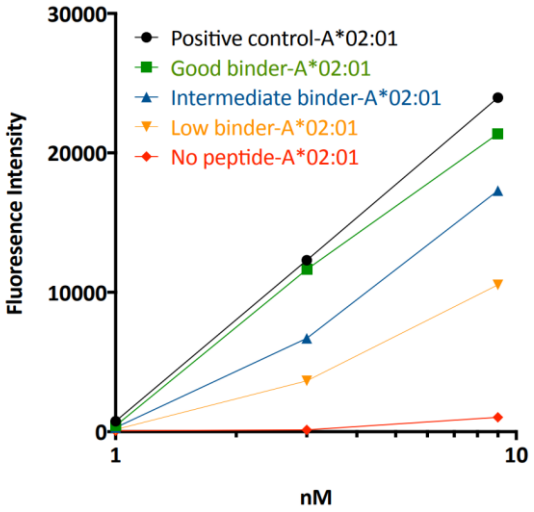
P1-3 : Positive control dilutions (HLA with know peptide

N1-3 : Negative control dilutions (HLA without peptide)

S1-S8 : Sample dilutions (complexes to evaluate)

7. Mix well and seal the plates with sealing tape to avoid well to well contamination.
8. Incubate the plate on a rocking table at 37°C for 1 h.
9. Remove the sealing tape and wash by adding 160 µL FACS buffer.
10. Spin the plate at 700 x g for 3 min and discard the supernatant.
11. Resuspend the beads in 200 µL FACS buffer.
12. Spin the plate at 700 x g for 3 min and discard the supernatant.
13. Wash two more times by repeating step 10 and 12.
14. During the above washing steps, prepare a 200-fold dilution of the PE-labeled anti-human β2m monoclonal antibody BBM.1 in FACS buffer.
15. Resuspend the beads in 50 µL antibody solution per well.
16. Incubate the plate for 30 min at 4°C.
17. Wash by adding 150 µL FACS buffer. Spin the plate at 700 x g for 3 min and discard the supernatant.
18. Resuspend the beads in 200 µL FACS buffer. Spin the plate at 700 x g for 3 min and discard the supernatant.
19. Wash two more times by repeating step 17 and 18.
20. Resuspend the beads in 200 µL FACS buffer and analyze on a Flow cytometer.

Example of the Flow cytometry-based assay:



Flow cytometry-based detection of four different peptide-HLA-A*0201 monomers. MHC I-peptide monomer of A*0201 and 4 different peptides, and a negative control (no peptide), were folded. CMV pp65 495-503 (NLVPMVATV) a known HLA-A*0201 restricted epitope was used as positive control. The three other peptides are based on their A*0201 binding stability categorized as good binder (T½ 6.5 h), intermediate binder (T½ 3.5 h), and low binder (T½ 0.7 h). Three dilutions of the folded monomer were analyzed in the flow cytometry-based assay. The X-axis gives the monomer concentration if complete folding is achieved.