Protocol for Preparation of MHC I-peptide monomer and fluorescent U-Load Dextramer® MHC I

Background

easYmer® are highly active formulations of MHC I molecules, which can be used to generate specific MHC-peptide monomers by loading your own peptide of choice. The MHC-peptide monomers can easily be dextramerized with fluorescently labeled U-Load Dextramer® and used to detect antigen-specific $CD8^+$ T cells in blood using flow cytometry. Optionally, the peptide-loaded monomers can be stored frozen at -20°C for later use. The easYmer® technology is highly flexible and suitable for screening of single epitopes in many samples or screening of large numbers of different epitopes in parallel.

The easYmer® technology also allow evaluation of peptide binding to MHC I by assaying proper refolding of peptide loaded complexes.

Materials required

easYmer®
U-Load Dextramer®
Peptide
DMSO (e.g., Sigma cat# D2650)
ddH_2O

Preparation of MHC-peptide monomer

1. Dilute Peptide (easYmer® Control Peptide or peptide of choice) to 100 µM in ddH_2O. Keep on ice from this step.
2. To prepare easYmer® MHC-peptide monomer for dextramerization, mix the reagents in Table A 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.
3. *Optional: To evaluate folding of peptide with MHC make a smaller volume of a positive and negative control, i.e., easYmer® loaded with the included control peptide and no peptide as listed in Table A.*

Table A

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	-
easYmer® Loading Buffer	10 µL	20 µL	50 µL	3 µL	3 µL
easYmer® (3 µM)	20 µL	40 µL	100 µL	3 µL	3 µL
Total volume	**60 µL**	**120 µL**	**300 µL**	**9 µL**	**9 µL**

4. Mix by pipetting gently – *be careful not to form bubbles.*
5. Make a short clearing spin to collect all materials in the bottom of the tube and incubate at 18°C for 48 h.
6. Make a short clearing spin to collect all material in the bottom of the tube. 1000 nM folded MHC-peptide monomer is now ready for further processing or placed at -20°C for long-term storage.

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7. Proceed to step 8 to evaluate peptide binding or continue to dextramerization starting at step 9.
8. To determine the efficiency of the easYmer® MHC-peptide folding take 3 µL of the prepared MHC-peptide monomer (1000 nM) and dilute to 500 nM by adding 3 µL of Loading buffer. Then follow the "Protocol for Flow cytometry-based assay of peptide- easYmer® MHC I complex formation" (page 3).

Preparation of U-Load Dextramer® MHC I

9. To dextramerize the peptide-loaded easYmer® MHC monomers (MHC-peptide monomer), mix the reagents in Table B in a 1.5 mL tube:
Dextramerization with U-Load Dextramer® APC require different volume of reagents. See Procedural notes.

Table B

Reagents	**10 tests**	**20 tests**	**50 tests**
MHC-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**

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

Flow analysis

To analyze antigen-specific $CD8^+$ T cells in blood using flow cytometry follow the "General staining procedure MHC Dextramer® – PBMC's". (https://www.immudex.com/resources/protocols/).

Procedural notes

1. Protocol step 9: To dextramerize the peptide-loaded easYmer® MHC monomers (MHC-peptide monomer) using 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**
MHC-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**

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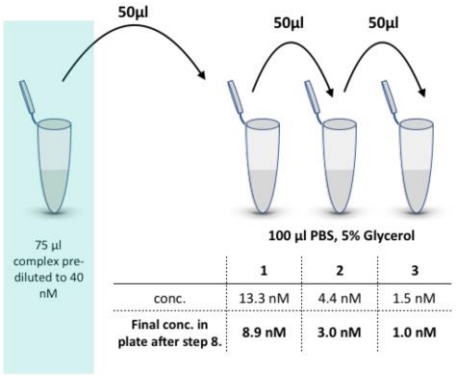
Flow cytometry-based assay of peptide-MHC I complex formation

Background

After completed peptide loading of easYmer® monomer (step 8 in the protocol "Preparation of MHC I-peptide monomer and fluorescent U-Load Dextramer® MHC I"), proper folding of MHC-peptide monomers can be evaluated using the assay described in this procedure.

Procedure: evaluation of peptide-MHC I complex formation

1. The MHC-peptide monomers to be assayed are prepared according to protocol: "Preparation of MHC I-peptide monomer and fluorescent U-Load Dextramer® MHC I". The peptide loading setup should include a positive control (provided with the easYmer®): a peptide that is known to support folding of the MHC molecule of interest, and a negative control: without peptide.
2. Prepare sufficient dilution buffer (PBS, 5% glycerol) for the whole assay.
3. Dilute each of the MHC-peptide monomers to give 75 µL of a 40 nM solution (e.g., for a 500 nM complex: 6 µL folded complex in 69 µL dilution buffer).
4. For all samples and positive and negative 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): transfer 40 µL of dilution buffer
6. Prepare a sufficient volume of a 45-fold dilution of the streptavidin-coated beads (6-8um; SVP-60-5) in dilution buffer. Transfer 20 µL of the diluted bead suspension to each well.

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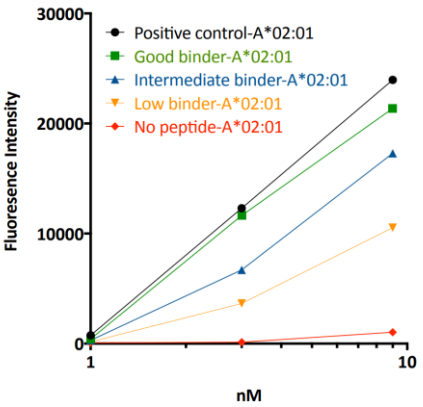
	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK		P-1		S1-1		S3-1		S5-1		S7-1	
B			P-2		S1-2		S3-2		S5-2		S7-2	
C			P-3		S1-3		S3-3		S5-3		S7-3	
D												
E			N-1		S2-1		S4-1		S6-1		S8-1	
F			N-2		S2-2		S4-2		S6-2		S8-2	
G			N-3		S2-3		S4-3		S6-3		S8-3	
H												

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 1h.
9. Remove the Sealing Tape and wash by adding 160 µL FACS buffer.
10. Spin the plate at 700xg for 3 min and flip out the supernatant.
11. Resuspend the beads in 200 µL FACS buffer.
12. Spin the plate at 700xg for 3 min and flip out the supernatant.
13. Wash two more times by repeating step 11. 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.

    Wash by adding 150 µL FACS buffer. Spin the plate at 700g for 3 min and flip out the supernatant.
17. Resuspend the beads in 200 µL FACS buffer. Spin the plate at 700xg for 3 min and flip out the supernatant.
18. Wash two more times by repeating step 17. and 18.
19. 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 4 different peptide-HLA-A*02:01 monomers. MHC-peptide monomers of A*02:01 and 4 different peptides, and a negative control (No Peptide), were folded. CMV pp65 495-503 (NLVPMVATV) a known HLA-A*02:01 restricted epitope was used as positive control. The three other peptides are based on their A*02:01 binding stability categorized as good binder (T½ 6.5h), intermediate binder (T½ 3.5h), and low binder (T½ 0.7h). Three dilutions of the folded complexes were analysed in the flow cytometry-based assay. The X-axis gives the complex concentration if complete folding is achieved.

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