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

Background easYmers® powered by immunAware is a formulation of peptide-receptive MHC I monomer, which can be used to generate specific MHC I-peptide monomers by loading your peptide of choice. The easYmers® MHC I-peptide monomer can easily be loaded onto U-Load dCODE Dextramer® and used for characterization and quantification of antigen-specific T cells in a cell sample by next-generation sequencing (NGS) or single-cell multi-omics. U-Load dCODE Dextramer® is a DNA barcode labeled Dextramer® with a unique DNA barcode for each specificity. In addition, U-Load dCODE Dextramer® is labeled with PE for cell-sorting purposes. U-Load dCODE Dextramer® comes with DNA barcodes applicable for different applications:

- U-Load dCODE Dextramer® (HiT) - for epitope discovery, neoantigen screening, designed for multiplexing using PCR and NGS

- U-Load dCODE Dextramer® (RiO) or U-Load dCODE Dextramer® (10x) for the detection of antigen-specific CD8+ or CD4+ T cells with additional information of gene expression, surface marker expression, and full-length TCR sequence by single-cell multi-omics using the BD Rhapsody™ Single-Cell Analysis System or the 10x Chromium™ Single Cell Gene Expression platform.

The easYmers® and U-Load dCODE Dextramer® technologies are highly flexible and suitable for screening single epitopes in many samples or screening of large numbers of different epitopes in parallel. The easYmers® technology also allows the evaluation of peptide binding to MHC I by assaying proper refolding of peptide-loaded monomer.

Optimized for U-Load dCODE Dextramer® (HiT, RiO, 10x) - Gold/Explore easYmers® MHC I monomers

Materials Provided The materials listed here are required for preparation of easYmers® peptide-MHC (pMHC) monomer and U-Load Dextramer® MHC I.

easYmers®
easYmers® loading buffer
easYmers® positive control peptide
U-Load dCODE Dextramer® (HiT, RiO, 10x)
U-Load dCODE Dextramer® dilution buffer

Materials Required (not provided) The materials listed here are required for preparation of easYmers® pMHC I and U-Load dCODE Dextramer® MHC I and for the flow cytometry-based assay for evaluation of proper folding of easYmers® pMHC I monomer.

Peptide of choice
DMSO (e.g., Sigma cat.# D2650)
Dilution buffer (PBS, 5% glycerol)
FACS buffer (PBS, 1% BSA (or FCS), 0.01% NaN3)
Streptavidin-coated beads (Spherotech cat.# SVP-60-5)
Anti-human β2m BBM.1-PE (Santa Cruz cat.# sc-13565 PE)

Procedure, steps and timing

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

| I. Preparation of easYmers® pMHC I complex ~48 hours | Optional: Loading QC Assay ~60 min. | II. Loading of U-Load dCODE Dextramer® ~40 min. | III. Staining procedure ~60 min. |

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 ddH2O. Keep on ice from this step on.
3. To prepare easYmers® MHC I-peptide monomer, mix the reagents in Table A for human easYmers® alleles or Table B for murine alleles 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 dCODE 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 A Human

Reagents	10 tests	20 tests	50 tests	Positive Control	Negative Control
ddH$_2$O	3 µL	6 µL	15 µL	2.5 µL	3 µL
Peptide (100 µM)	2 µL	4 µL	10 µL	0.5 µL	-
easYmers® Loading Buffer	5 µL	10 µL	25 µL	3 µL	3 µL
easYmers® (3 µM)	20 µL	40 µL	100 µL	3 µL	3 µL
Total Volume of easYmers® pMHC I monomer (2 µM)	**30 µL**	**60 µL**	**150 µL**	**9 µL**	**9 µL**

Table B Murine

Reagents	10 tests	20 tests	50 tests	Positive Control	Negative Control
PBS, pH 7.4	8 µL	16 µL	40 µL	5.5 µL	6 µL
Peptide (100 µM)	2 µL	4 µL	10 µL	0.5 µL	-
easYmers® (3 µM)	20 µL	40 µL	100 µL	3 µL	3 µL
Total Volume of easYmers® pMHC I monomer (2 µM)	**30 µL**	**60 µL**	**150 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. 2 µM folded pMHC I monomer are now ready for loading onto U-Load dCODE Dextramer® backbone 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 dCODE Dextramer®.

II. Loading of U-Load dCODE Dextramer® MHC I

1. To load the easYmers® MHC I-peptide monomer onto U-Load dCODE Dextramer®, mix the reagents in Table C in a 1.5 mL tube:

Table C

Reagents	10 tests	20 tests	50 tests
easYmers® pMHC I monomer (2 µM)	27 µL	54 µL	135 µL
U-Load dCODE Dextramer® (PE)	12 µL	24 µL	60 µL
Incubate for 30 min at RT in the dark			
U-Load dCODE Dextramer® dilution Buffer	11 µL	22 µL	55 µL
Total volume dCODE Dextramer® MHC I	**50 µL**	**100 µL**	**250 µL**

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

III. Staining Procedures & Sequencing Workflows

For U-Load dCODE Dextramer® (HiT): See www.immudex.com/Protocols/HiT
For U-Load dCODE Dextramer® (RiO): See www.immudex.com/Protocols/RiO
For U-Load dCODE Dextramer® (10x): See www.immudex.com/Protocols/10x

Technical Support

For additional Tips & Tricks, FAQs and protocols, please visit https://www.immudex.com/resources/ or contact our support team at customer@immudex.com
Telephone: +45 3110 9292 (Denmark)

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® pMHC I 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
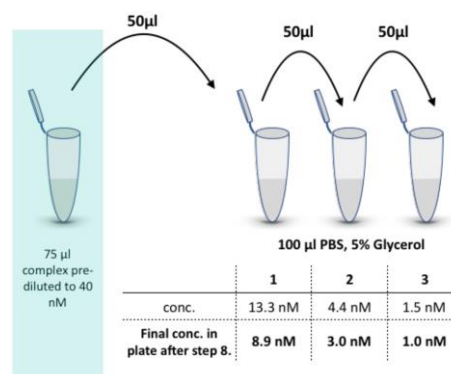
5. Transfer 40 µL of each of these dilutions to the wells in a U-bottom shape 96-well plate, as suggested below. Also, prepare a background well (BLANK): 40 µL of dilution buffer (no beads or antibody will be added to this well).
6. Prepare a sufficient volume of a 45-fold dilution of the streptavidin 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			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		S9-3	
H												

Blank: Dilution buffer, no MHC complexes
**P1-3** Positive control dilutions (MHC with known peptide)
N1-3 Negative control dilutions (MHC without peptide)
S1-8 Sample dilutions (MHC 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 labelled 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:
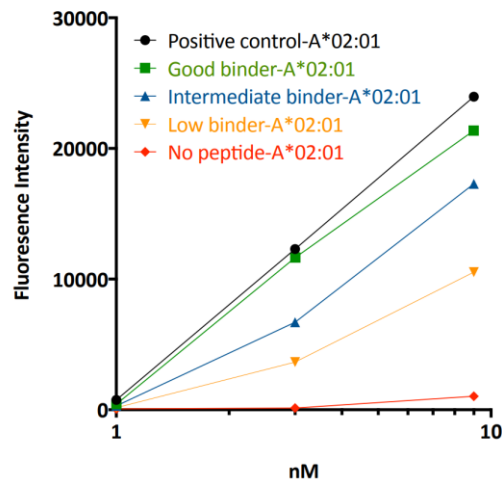
Example of flow cytometry-based assessment of 4 different peptide-HLA-A*02:01 complexes.
Complexes of A*02:01 with 4 different peptides including the positive control HLA-A*02:01-restricted peptide CMV pp65 495-503 (NLVPMVATV), and 1 negative control (no peptide) were folded. The three other peptides were categorized as good binder, intermediate binder, and low binder based on their A*02:01 binding stability. Three dilutions of the folded complexes were analysed in the flow cytometry-based assay. The X-axis shows the complex concentration if complete folding is achieved.