U-Load Dextramer® assembly protocol (MHC I)

Products

U-Load Dextramer® Kit MHC I (Cat. No. U-LXXX)
U-Load Dextramer® (Cat. No. U-LDEX)
easYmers® (Cat. No. U-LXXM)

Background

easYmers® designed by immunAware are peptide-receptive MHC I molecules that can be loaded with the peptide of your interest to generate custom peptide-MHC complexes. These complexes can then be easily attached to fluorescently labeled U-Load Dextramer® for multimerization and subsequent detection of antigen-specific $CD8^+$ T cells by flow cytometry. Proper refolding of peptide-loaded easYmers® can be evaluated in a QC assay.

Materials required*

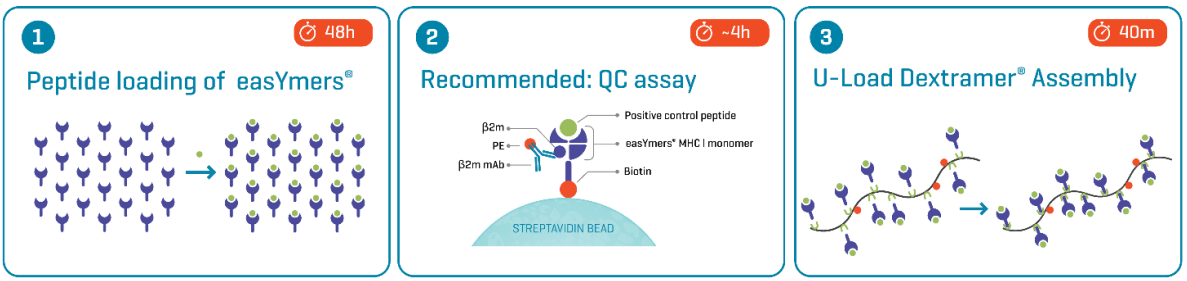
- easYmers® (Cat. No. U-LXXM) which includes
  - easYmers® loading buffer (IR5107) (only needed for human easYmers®)
  - easYmers® Positive Control Peptide (Cat. No. DRXXXX)
- U-Load Dextramer® (Cat. No. U-LDEX) which includes
  - U-Load Dextramer® dilution buffer (Cat. No: ULBUF)

U-Load Dextramer® Kit MHC I (Cat. No. U-LXXX) includes all reagents necessary for assembly

Materials required (not provided)

- Peptide of choice
- Peptide solvent (e.g., ddH_20, PBS, DMSO)
- Dilution buffer (PBS, 5% glycerol)
- Flow cytometry buffer (for peptide loading QC) (PBS, 1% BSA (or FCS), 0.01% NaN_3)
- 6-8 µm streptavidin-coated beads (Spherotech Cat. No.: SVP-60-5)
- Anti-human β_2m BBM.1-PE (e.g., Santa Cruz Cat. No.: sc-13565 PE; 200 µg/ml) - can be used with human and mouse easYmers®
- PBS (Phosphate-buffered saline) pH 7.4
- 1.5 mL Protein LoBind Eppendorf tubes (e.g., Eppendorf Cat. No. 022431081) or equivalent.
- Falcon tissue culture plate, 96 well, U bottom with low evaporation lid (e.g., BD Cat. No. 353077)

Timing



Procedure

I. Peptide loading of easYmers® (MHC I monomer)

1. Determine the number of tests needed; for flow cytometry 1 test is equivalent to the reagent volume needed to stain 1-3 x 10^6 PBMCs or 2-5 x 10^4 clonal cells.
2. Reconstitute your peptides of interest according to the manufacturer's instructions. The supplied positive control peptide should be reconstituted in 20ul DMSO.
3. Dilute peptide (easYmers® control peptide or peptide of interest) to 100 µM in ddH_2O. Keep on ice from this step on.

   Load the easYmers® with the peptides of interest by mixing the reagents in Table A1 (**Human** easYmers®) or Table A2 (**Mouse** easYmers®) in a 1.5 mL tube or 96-well U-bottom plate according to the desired number of tests. Mix by pipetting gently. Recommended: To evaluate peptide loading efficiency in a QC assay (see page 4) please include the positive/negative control tests as listed in the table below. Use the easYmers® control peptide for the positive control test.

   Table A1

Human easYmers®	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 **loaded monomers** (1 µM)	**60 µL**	**120 µL**	**300 µL**	**9 µL**	**9 µL**

   Table A2

Mouse easYmers®	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 **loaded monomers** (1 µM)	**60 µL**	**120 µL**	**300 µL**	**9 µL**	**9 µL**

4. Briefly spin down to collect all materials in the bottom of the tube and incubate at 18°C for 48 h.
5. Briefly spin down to collect all material in the bottom of the tube. 1 µM folded easYmers® MHC I-peptide monomers are now ready for further use or for long-term storage at -20°C.
6. Proceed to page 4 to evaluate peptide-loading efficiency (recommended) and/or continue to load onto U-Load Dextramer®.

II. Assembly of U-Load Dextramer®

7. Assemble U-Load Dextramer® with the loaded monomers according to the order specified in the tables below (for U-Load Dextramer® **BV421**, **FITC**, or **PE** use Table B1; for U-Load Dextramer® **APC** use Table B2). Incubate the loaded monomers and U-Load Dextramer® for 30 min at RT **before** adding U-Load Dextramer® Dilution Buffer. This dilution step yields 32 nM of assembled U-Load Dextramer® MHC I (final Dextramer concentration for flow cytometry staining for use in the MHC Dextramer® Staining Protocol.

Table B1

Reagents	**10 tests**	**20 tests**	**50 tests**
Loaded monomers (1 µM)	57 µL	114 µL	285 µL
U-Load Dextramer® **(BV421/FITC/PE)**	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 (32nM)**	**100 µL**	**200 µL**	**500 µL**

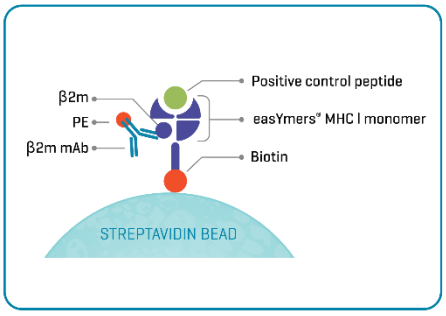
Table B2

Reagents	**10 tests**	**20 tests**	**50 tests**
Loaded monomers (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 (32 nM)**	**100 µL**	**200 µL**	**500 µL**

8. Continue with flow cytometry staining (MHC Dextramer® Staining Protocol) or store the fluorescent U-Load Dextramer® MHC I reagents at 2-8°C in the dark until use.

Recommended: Quality control assay to determine relative peptide loading efficiency

Background



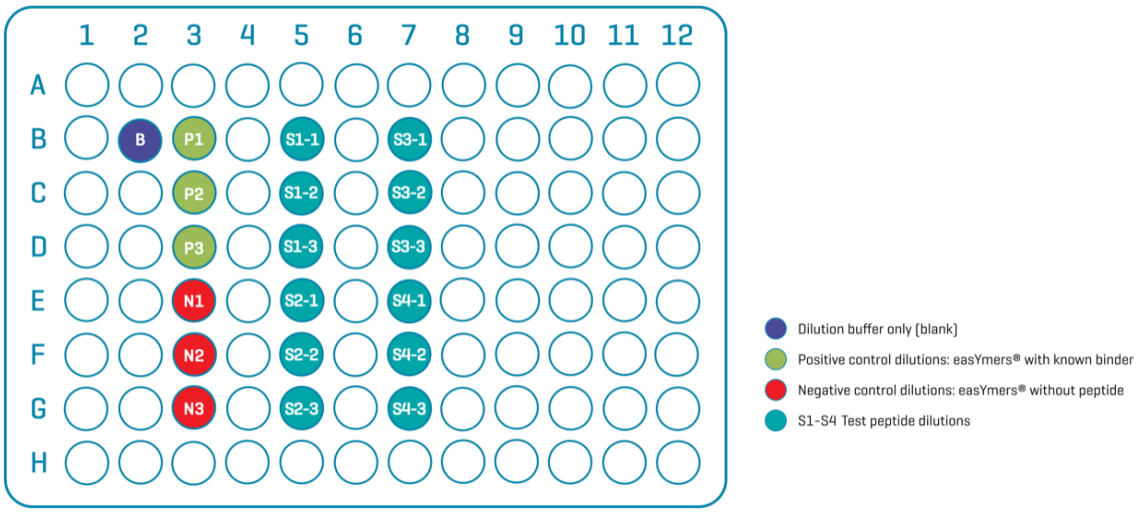
It is highly recommended to determine relative peptide loading efficiency of your peptide of interest compared to a positive loading control. The following binding assay is a semi-quantitative, bead-based assay designed to evaluate proper folding of a peptide-MHC (pMHC) complex. Biotin-tagged pMHC complexes are captured by streptavidin beads, labelled with PE anti-human β2m, and analyzed by flow cytometry. Peptides conducive to stable complex folding will yield good bead-associated signals whereas weak binders will exhibit low signals.

Procedure

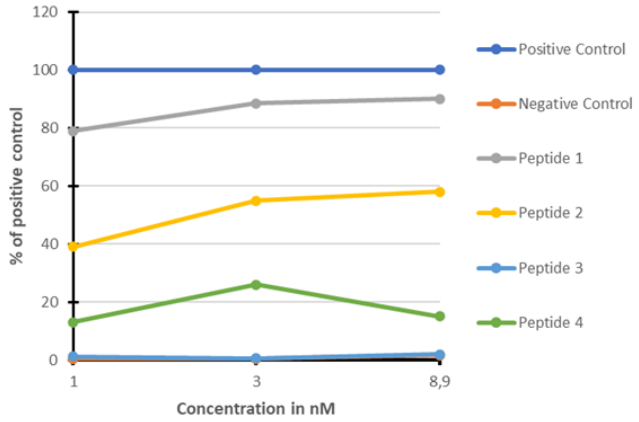
1. Prepare a sufficient volume of dilution buffer (PBS, 5% glycerol).
2. Dilute 3 µL loaded easYmers® from step 5 (all samples and positive/negative controls) in 72 µL dilution buffer which yields a 40 nM solution. Use this to prepare a set of serial dilutions according to the table below:

Dilution #	Dilution Buffer	Loaded easYmers®	Concentration	Final concentration (after step 4)
1	100 µL	50 µL of 40 nM pre-dilution	13.3 nM	8.9 nM
2	100 µL	50 µL of Dilution #1	4.4 nM	3.0 nM
3	100 µL	50 µL of Dilution #2	1.5 nM	1.0 nM

3. Transfer 40 µL of each dilution to a 96-well U-bottom plate (see plate layout below). Include a background well (BLANK) which consists of 40 µL dilution buffer only (no beads or antibody will be added to this well).



4. 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.
5. Mix well and seal the plates with sealing tape to avoid well to well contamination.
6. Incubate the plate on a rocking table at 37°C for 1 h.
7. Remove the sealing tape and wash by adding 160 µL flow cytometry buffer. Spin the plate at 700 x g for 3 min and discard the supernatant.
8. Resuspend the beads in 200 µL flow cytometry buffer. Spin the plate at 700 x g for 3 min and discard the supernatant. Repeat twice more for a total of three washes.
9. During the washing steps above, prepare a 200-fold dilution of the PE-labeled anti-human β2m monoclonal antibody BBM.1 in flow cytometry buffer.
10. Resuspend the beads in 50 µL antibody solution per well.
11. Incubate the plate for 30 min at 4°C.
12. Wash by adding 150 µL flow cytometry buffer. Spin the plate at 700 x g for 3 min and discard the supernatant.
13. Resuspend the beads in 200 µL flow cytometry buffer. Spin the plate at 700 x g for 3 min and discard the supernatant. Repeat twice more for a total of three washes.
14. Resuspend the beads in 200 µL flow cytometry buffer and analyze on a flow cytometer, acquiring at least 1000 singlets.



Sample data shown as % of positive control which is set to 100%.
Peptide 1 shows high peptide loading efficiency compared to the control whereas peptide 3 demonstrates low binding. Determining loading efficiency will be based on an arbitrary threshold set by the user.

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