easYmer

Monomer/Tetramer production protocol

immunAware

Assay background

The easYmer kit features a highly active formulation of HLA class I (HLA-I) molecules, which can be used to generate specific peptide-HLA class I monomers of your choice in your own laboratory. These monomers can easily be tetramerized with fluorophore conjugated streptavidin and used to stain antigen specific T cells for analysis in flow cytometric assays. Optionally, the monomers can be stored frozen for later use. The easYmer technology is highly flexible and suitable for screening of a single epitope in a large number of samples, as well as for screening of a large number of different epitopes in parallel.

easYmer kit content

easYmer, a peptide receptive preparation of HLA class I (conc.: 3µM). Store at -20°C.

Folding buffer: 0.3M Tris/Maleate with 0.2%Pluronic F68 (6x concentrate).

Positive control peptide for HLA-complex folding: 20nmole lyophilized peptide.
For use dissolve in 20 µL DMSO (1mM), and store at -20°C.

The exact identities of the components are given in the product specification sheet

Additional materials and equipment required

Fluorophore-conjugated streptavidin e.g.: *Streptavidin-PE (BD; Cat# 554061)*; *Streptavidin-APC (BD; Cat#554067)*; *Streptavidin-BV421 (BD; Cat# 563259)*

DMSO (e.g. Sigma cat# D2650)

ddH_2O

Sealing Tape (Thermo Scientific Nunc™ cat# 236366)

FACS buffer: PBS with 1%BSA (or FCS) and 0.1% NaN_3.

Vortex mixer

96-well U-bottom plate

Centrifuge with a plate rotor

Flow cytometer

Recommendations

The easYmer kit cannot generate monomers unless the underlying peptide-MHC-I interaction is a productive one, i.e. that it is of reasonable affinity and stability. We recommend that you use predictors (e.g. one from the NetMHC series) to assist you in the design of suitable monomers. We strongly recommend that you experimentally validate the interaction(s) of the peptide-MHC-I interaction(s) of your choice. To this end, the easYme kit includes a simple protocol, *Flow cytometry-based assay of peptide-HLA-I complex formation*, which allows you to examine proper peptide-MHC class I interaction.

Protocol for HLA-I monomer production

Use the easYmers to produce several monomers in small sample sizes or a few monomers in a larger sample size. A calculation spreadsheet for the setup protocol "Support spreadsheet (easYmer setup)" can be found at our website www.immunaware.com under Resources. The spreadsheet calculates the setup reagent volumes at various sample size, surplus handling volumes, and peptide concentrations.

Generation of small-scale peptide-HLA complex – 5 tests:

1. Work on ice
2. Make 72 µL peptide-HLA complex. This will be enough to validate folding and produce tetramers sufficient for at least 5 tests.
3. To evaluate folding, make a smaller volume of a positive and negative control, the included peptide and no peptide, respectively.
4. Dilute the positive control peptide and the peptide(s) of choice to 75µM in ddH_2O.
5. Make a smaller volume of a positive and negative folding controls, the included peptide and no peptide, respectively.
6. Mix the following reagents in the listed sequence. The reaction can be setup in Eppendorf tubes or in a 96-well U-bottom plate:

Reagents	5 Tests	Positive Control	Negative Control
ddH_2O	45 µL	11 µL	12 µL
Peptide (75µM)	3 µL	1 µL	
FoldingBuffer (X6)	12 µL	3 µL	3 µL
easYmer	12 µL	3 µL	3 µL
Total volume	72 µL	18 µL	18 µL

7. Assure a thorough mixing. Vortex Eppendorf tubes, and plate setup by pipetting up and down – be careful not to form bubbles.

   Tip: to avoid mixing of samples in the plate, make an empty well spacing between samples.
8. Seal the plates with Sealing Tape and incubate at 18°C for 48h.
9. When using optimal binding peptides, 72µL with 500nM folded monomer will be obtained.
10. To validate and evaluate the efficiency of the folding follow the protocol for "*Flow cytometry-based assay of peptide-HLA-I complex formation".*

Production of Tetramer

1. Transfer 50 µL of each sample into a new 96-well U-bottom plate or an Eppendorf tube.
2. To tetramerize 50 µL of 500nM complexes use the equivalent of 2.1 µL of a 0.2mg/ml Streptavidin-fluorophore.

   It is recommended to use either of the following:

   - *0.8µL Streptavidin-PE (BD; Cat# 554061; 0.5mg/ml),*
   - *2.1µL Streptavidin-APC (BD; Cat#554067; 0.2mg/ml),*
   - *4.2µL Streptavidin-BV421 (BD; Cat# 563259; 0.1mg/ml)*
3. Add the Streptavidin-fluorophore and mix thoroughly. Seal the plate and incubate at 4°C in the dark for at least 1h.
4. To analyze for antigen specific CD8+ T cells follow the protocol for "*HLA class I tetramer staining of human T cells".*

Protocol for large-scale production of one or a few monomers

Generation of large-scale peptide-HLA complex – 50 tests

1. Work on ice
2. Dilute the peptide to 100 µM in ddH_2O.
3. Make 540 µL peptide-HLA complex. This will be enough to validate folding and produce tetramers sufficient for at least 50 tests.
4. To evaluate folding, make a smaller volume of a positive and negative control, the included peptide and no peptide, respectively.
5. In a 1.5-2mL tube add the following reagents:

Reagents	50 tests	Positive Control	Negative Control
ddH_2O	338 µL	11 µL	12 µL
Peptide (100µM)	22 µL	1 µL	
Folding Buffer (X6)	90 µL	3 µL	3 µL
easYmer	90 µL	3 µL	3 µL
Total volume	540 µL	18 µL	18 µL

6. Vortex the tube - assure a thorough mixing.
7. Incubate at 18°C for 48h.
8. When using optimal binding peptides, 540µL with 500nM folded monomer will be obtained.
9. To validate and evaluate the efficiency of the folding follow the protocol for *Flow cytometry-based assay of peptide-HLA-I complex formation***.**

Production of Tetramer

5. The monomer production or aliquots hereof can be tetramerized.
6. Transfer 500µL of each sample into a new tube.
7. To tetramerize 500µL of the monomer complexes use the equivalent of 20µL of a 0.2mg/ml Streptavidin-fluorophore.

   It is recommended to use either of the following:
   - *8µL Streptavidin-PE (BD; Cat# 554061; 0.5mg/ml)*
   - *20µL Streptavidin-APC (BD; Cat#554067; 0.2mg/ml)*
   - *40µL Streptavidin-BV421 (BD; Cat# 563259; 0.1mg/ml)*
8. Add the Streptavidin-fluorophore volume in 3 steps:
   1. Add ⅓ of the streptavidin volume mix and incubate in the dark for 10 min. at 4°C
   2. Add ⅓ of the streptavidin volume mix and incubate in the dark for 10 min. at 4°C
   3. Add ⅓ of the streptavidin volume mix and incubate in the dark for 30 min. at 4°C
9. The tetramer is now ready for use, and can be stored at 4°C for several months.
10. To analyze for antigen specific CD8+ T cells follow the protocol for "*HLA class I tetramer staining of human T cells"*.

Assay background

This protocol is designed to evaluate the efficiency of peptide-HLA-I interaction and complex formation. The assay is based on detecting the β_2-microglobulin (β_2m) light chain subunit of recombinant HLA class I (HLA-I) complexes, where the heavy chain has been biotin tagged. These tagged complexes are subsequently captured by streptavidin coated beads, labelled with PE-conjugated anti-human β_2m, and analyzed by flow cytometry. Since peptide-HLA-I complex formation is entirely peptide dependent, bead-associated signals will only be detected if the peptide in question supports the folding of the HLA-I allotype of interest; peptides that efficiently support folding will give strong signals whereas peptides that support folding sub-optimally, or not at all, will give moderate to non-detectable signals.

Materials and equipment, you need to provide

Dilution buffer: PBS with 5% glycerol.
FACS buffer: PBS with 1%BSA (or FCS) and 0.01% NaN_3).
Streptavidin coated beads: (6-8µm beads from Spherotech Cat.: SVP-60-5)
BBM.1-PE: (anti-human β_2m) (Santa Cruz Cat# sc-13565 PE)
Sealing Tape: (Thermo Scientific Nunc™ cat.: 236366)
96-well plate: U-bottom shape
Centrifuge with a plate rotor
Rocking table
Flow cytometer

Protocol

1. The folded HLA complexes are prepared according to protocol: "HLA-I tetramer production". The folding setup should include a **positive control** *(provided with the easYmer®)*: a peptide that is known to support folding of the HLA molecule of interest, and a **negative control**: without peptide.

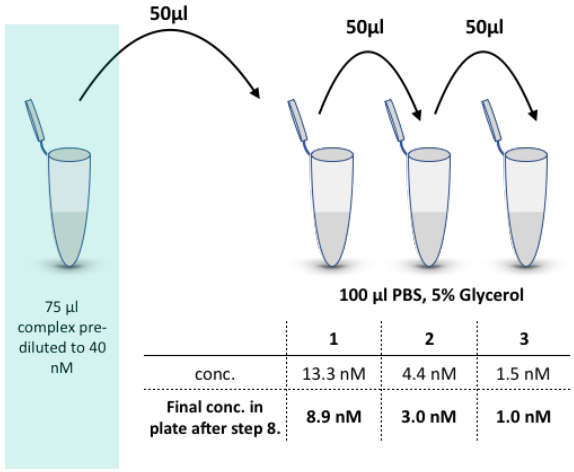
2. In the easYmer® folding set-up, the highest achievable concentration of the folded complex is 500nM.

3. After completed folding incubation the complex formation can be evaluated.

4. Prepare sufficient dilution buffer (PBS, 5% glycerol) for the whole assay.

5. Dilute each of the folded complexes to give 75µL of a 40nM solution (e.g. for a 500nM complex: 6µL folded complex in 69µL dilution buffer.

6. For all samples and **positive and negative controls**, transfer 50µL of this pre-dilution *(prepared in 5.)* to the first tube. Make three subsequent serial 3-fold dilutions (50µL in 100µL 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**

7. Transfer 40µL of each these dilutions to the wells in a U-bottom shape 96-well plate, as suggested below. Also prepare a background well (BLANK): transfer 40µL of dilution buffer.

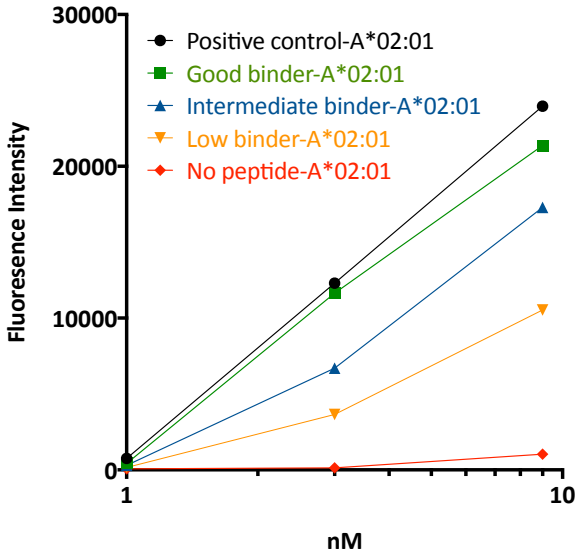
8. Prepare a sufficient volume of a 45-fold dilution of the streptavidin coated beads (6-8um; SVP-60-5) in dilution buffer. Transfer 20µL of the diluted bead suspension to each well.

	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)

9. Mix well and seal the plates with Sealing Tape to avoid well to well contamination.
10. Incubate the plate on a rocking table at 37°C for 1h.
11. Remove the Sealing Tape and wash by adding 160µl FACS buffer.
12. Spin the plate at 700g for 3min and flip out the supernatant.
13. Resuspend the beads in 200µl FACS buffer.
14. Spin the plate at 700g for 3min and flip out the supernatant.
15. Wash two more times by repeating step 13. and 14.
16. During the above washing steps, prepare a 200-fold dilution of the PE-labeled anti-human β_2m monoclonal antibody BBM.1 in FACS buffer
17. Resuspend the beads in 50µL antibody solution per well.
18. Incubate the plate for 30 min. at 4°C.
19. Wash by adding 150µl FACS buffer. Spin the plate at 700g for 3min and flip out the supernatant.
20. Resuspend the beads in 200µl FACS buffer. Spin the plate at 700g for 3min and flip out the supernatant.
21. Wash two more times by repeating step 19. and 20.
22. Resuspend the beads in 200µl 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 complexes.
Complexes of A*02:01 and 4 different peptides, and a negative control (No Peptide), were folded. CMV pp65 $_{495\text{-}503}$ (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.

Materials and equipment

Fluorophore-labeled HLA class I tetramer(s)
Fluorophore-labeled antibodies against phenotypic markers (CD3, CD8, and other optional markers)
96-well U-bottom plate
FACS buffer: PBS with 1%BSA (or FCS) and 0.1% NaN_3.
Centrifuge with a plate rotor
Flow cytometer

Recommendations

Since, the staining intensity can vary between tetramer specificities, the tetramer concentration should be titrated the first time a specific tetramer is used.
Note, it can be some advantages to stain for the same tetramer specificity with two different fluorochrome labels. It gives a more accurate definition of the tetramer positive population. It also allows for analysis of more than one T cell specificity in the same cell sample. Using various fluorochrome labeled tetramers each specificity can be defined by its unique two fluorochrome combination.

HLA class I tetramer staining of human T cells

1. Prepare the cells of interest. For PBMC use 1-2*10^6, for cell lines use 2-4*10^5
2. Transfer the cells to a 96-well U-bottom plate: Adjust the volume to 200µl with FACS buffer
3. Spin the plate at 700g for 3min. - flip out the supernatant in one smooth move.
4. Tetramer staining: Dilute the tetramer to 30nM in FACS buffer and resuspend the pellet in 40µl of this dilution, and incubate in the dark at RT for 20 min.
5. Wash once in cold FACS buffer.
6. Spin the plate at 700g for 3min. - flip out the supernatant in one smooth move.
7. Co-stain with surface antibodies (CD8, CD3, other phenotype markers) prepare the antibody cocktail based on optimal staining concentration of each reagent.
8. Incubate in the dark at 4°C for 30 min.
9. Wash twice in cold FACS buffer.
10. Resuspend in FACS buffer and analyze in a Flow Cytometer.