easYmer

Monomer/Tetramer production protocol

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

The easYmer kit contains a peptide receptive preparation of HLA, a folding buffer, a positive control peptide for HLA-complex folding. The exact identities of these 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 kirt 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 small-scale production of many monomers in parallel

Generation of small-scale peptide-HLA complexes

1. Work on ice
2. Dilute the positive control peptide and the peptide(s) of choice to 100µM in ddH_2O.

- Peptide 1mM stock: 3 µl of peptide stock + 27 µl ddH_2O (positive control)

3. Make 90 µL peptide-HLA complex. This should be enough to validate folding and produce tetramers sufficient for at least 5 tests.
4. Make a smaller volume of a positive and negative folding controls, the included peptide and no peptide, respectively.
5. Per well, add the following reagents in a 96-well U-bottom plate:

Reagents	5 tests	Positive Control	Negative Control
ddH_2O	57 µL	15 µL	16 µL
Peptide (100µM)	3 µL	1 µL	
FoldingBuffer (X6)	15 µL	4 µL	4 µL
easYmer	15 µL	4 µL	4 µL
Total volume	90 µL	24 µL	24 µL

6. Mix by pipetting up and down. Assure a thorough mixing – be careful not to form bubbles.

   Tip: to avoid mixing of samples make an empty well spacing between samples.

7. Seal the plates with Sealing Tape and incubate at 18°C for 48h.
8. When using optimal binding peptides, 90µL 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

1. Transfer 60 µL of each sample into a new 96-well U-bottom plate.
2. To tetramerize 60 µL of 500nM complexes use the equivalent of 2.4 µL of a 0.2 mg/ml Streptavidin-fluorophore.

   It is recommended to use either of the following:

   - *1µL Streptavidin-PE (BD; Cat# 554061; 0.5 mg/ml),*
   - *4.8µL Streptavidin-APC (BD; Cat#554067; 0.1 mg/ml),*
   - *4.8µL Streptavidin-BV421 (BD; Cat# 563259; 0.1 mg/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

One tetramer enough for 50 tests

1. Work on ice
2. Dilute the peptide to 100 µM in ddH_2O.
3. Make 540 µL peptide-HLA complex (this should 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	323 µL	15 µL	16 µL
Peptide (100µM)	17 µL	1 µL	
Folding Buffer (X6)	85 µL	4 µL	4 µL
easYmer	85 µL	4 µL	4 µL
Total volume	510 µL	24 µL	24 µL

6. Mix by vortexing the tube.
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)*
   - *40µL Streptavidin-APC (BD; Cat#554067; 0.1mg/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*".