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

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

Protocol

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

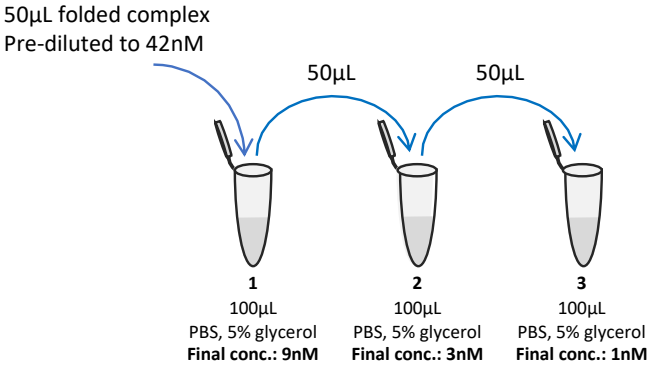
2. In the easyYmer folding set-up, the highest achievable concentration of the folded complex is 500nM.

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 give75µL 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 to the first tube. Make three subsequent serial 3-fold dilutions (50µL in 100µL dilution buffer), according to the figure below.



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: 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 beads solution 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: Dilutions of positive control; HLA with a known peptide binder
N1-3: Dilutions of negative control; HLA without peptide
S1- S8: Dilutions of the samples (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.

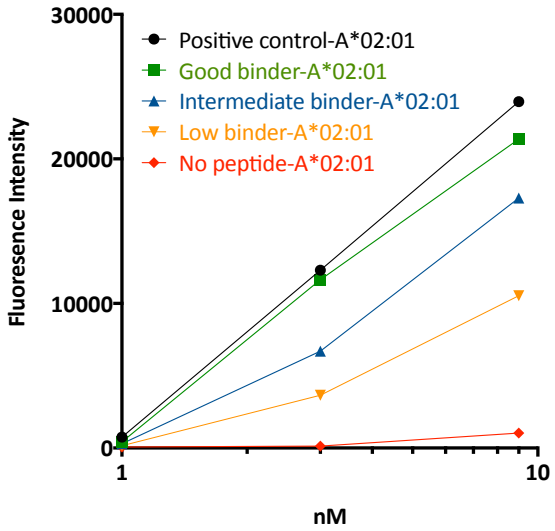
19. Wash by adding 150µl FACS buffer. Spin the plate at 700g for 3min and flip out the supernatant.

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.