Flex-T™ HLA Class I ELISA Protocol

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Abstract

The HLA class I ELISA is an enzyme immunoassay based on the detection of β2-microglobulin subunit of HLA class I complexes, after capturing the complex through the conjugated biotin. To this end, biotinylated HLA class I complex is first captured in streptavidin coated microtiter wells. Subsequently, HRP-conjugated anti-human β2-microglobulin is added to detect intact HLA class I complexes. Only intact HLA class I complexes are recognized. Peptides with high affinity binding will be clearly detected by this ELISA technique, while peptides with a moderate to low binding affinity for HLA class I provide a moderate to non-detectable signal. This protocol is designed to evaluate the efficiency of peptide exchange when using the Flex-T™ system.

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Guidelines

Reagent Preparation

See Appendix 1 for recipes and recommended reagents. All reagents should be prepared or diluted immediately prior to use. Before use, bring all reagents to room temperature (18-25°C) with the exception of the HRP anti-human β2-microglobulin antibody and the ELISA positive control which have to be kept on melting ice to ensure stability. Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials. Centrifuge all vials before use (1 minute 3000xg at 4°C). Do not allow wells to stand uncovered or dry for extended periods between incubation steps.

Materials

- Coating Buffer 5X concentrate (Cat# 421701)
- Streptavidin solution at 1 mg/ml (BioLegend Cat# 280302)
- Wash Buffer 20X concentrate: PBS with 0.05% (v/v) Tween 20 (BioLegend Cat# 421601)
- Dilution Buffer 10X concentrate (1 M NaCl, 0.5M Tris, 1% BSA, 0.2% Tween 20, pH8.0. See Appendix 1)
- HRP anti-human β2-microglobulin antibody (Cat# 280303)
- Flex-T™ ELISA Positive Control (BioLegend Cat# 280301)
- Substrate Buffer, 10X concentrate (0.1 M citric acid monohydrate/tri-Sodium citrate dehydrate, pH 4.0, See Appendix 1)
- ABTS substrate solution (50X 40mM solution made from e.g. Sigma Cat#A1888, or equivalent. See Appendix 1)
- Hydrogen peroxide solution (e.g. Sigma Cat#H3410, or equivalent. See Appendix 1)

- Stop Solution (2% [w/v] oxalic acid dehydrate, e.g. Sigma Cat#247537. See Appendix 1)
- Nunc™ MaxiSorp™ ELISA Plates, Uncoated (BioLegend Cat# 423501)
- Plate sealers (BioLegend Cat# 423601)
- Deionized (DI) water

Equipment
- Incubator (37°C)
- ELISA plate shaker
- Wash bottle or automated microplate washer
- Microtiter plate reader for measuring absorbance at 414nm
- Adjustable pipettes to measure volumes ranging from 3µl to 1 ml

- Multichannel pipetting devices

Represenative Data and Appendix 1.

Plate map example for the HLA class I ELISA:

	1	2	3	4	5	6	7	8	9	10	11	12
A	H	Duplicate	P1	Duplicate	P2	Duplicate	P3	Duplicate	P4	Duplicate	P5	Duplicate
B	M		P6		P7		P8		P9		P10	
C	L		P11		P12		P13		P14		P15	
D	Blank		P16		P17		P18		P19		P20	
E	Pos		P21		P22		P23		P24		P25	
F	Neg		P26		P27		P28		P29		P30	
G	UV		P31		P32		P33		P34		P35	
H	Blank		P36		P37		P38		P39		P40	

Pos: Positive exchange control peptide (See table in appendix 1)
Neg: Negative exchange control peptide (See table in appendix 1)
UV: UV-illuminated conditional HLA complex in the absence of exchange peptide
Blank: 1X Dilution Buffer
H: HLA control H
M: HLA control M
L: HLA control L
P: peptide of choice

Protocol

Reagent Preparation

Step 1.

Dilute the 5X Coating Buffer to 1X with DI water

Reagent Preparation

Step 2.

Prepare Streptavidin solution to coat the plate:

-24µl of Streptavidin solution

-11.976 ml 1X Coating Buffer

Reagent Preparation

Step 3.

Calculate the amount of 1X Dilution Buffer required and prepare the solution by diluting the 10Xconcentrated buffer 10 times in DI water before use. The 1X Dilution Buffer can be stored for up to oneweek at 2-8°C.

Reagent Preparation

Step 4.

Prepare Wash Buffer; dilute the 20X wash buffer with DI water, at least 300ml per plate.

Reagent Preparation

Step 5.

Dilute concentrated HRP-conjugated antibody to 0.3µg/ml in 1X Dilution Buffer just before use. Prepare12ml per plate.

Reagent Preparation

Step 6.

Prepare the substrate solution approximately 10 minutes before use:
10.34ml DI water
1.2ml of Substrate Buffer 10X concentrate
240µl of ABTS stock solution
120µl of Hydrogen peroxide solution
Vortex to mix well
The substrate solution should be at room temperature (18-25°C) for optimal reproducible results.

Reagent Preparation

Step 7.

Dilute the Flex-T™ ELISA Positive Control to prepare a 2.7µM solution:
-5µl of Flex-T™ ELISA Positive Control at 0.2mg/ml
-2.9µl of 1X Dilution Buffer

Assay Procedure

Step 8.

Coat the wells of the plate with Streptavidin. Add 100µl of Streptavidin solution to all wells. Seal theplate and incubate overnight (16-18 hrs) at room temperature (18-25°C).

Assay Procedure

Step 9.

Discard the coating solution and wash the plate 3 times with at least 300µl Wash Buffer per well andblot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washesshould be performed similarly.

Assay Procedure

Step 10.

To block non-specific binding and reduce background, add 300µl of 1X Dilution Buffer to all wells.

Assay Procedure

Step 11.

Seal the plate and incubate for 30 minutes at room temperature (18-25°C).

Assay Procedure

Step 12.

Prepare three dilutions of the HLA control by serial dilution in 1X Dilution Buffer. Prepare the controlsfresh and keep them on melting ice until usage.

Assay Procedure

Step 13.

To evaluate the outcome of UV-mediated HLA peptide exchange, dilute a small aliquot of the exchange reaction mixture 300-fold in 1X Dilution Buffer (refer to the peptide exchange step in Protocol for fluorescent Flex-T™ generation and antigen specific CD8+ T cell staining). Mix thoroughly.

Assay Procedure

Step 14.

Discard the Dilution Buffer from the plate and blot the residual buffer.

Assay Procedure

Step 15.

Pipette 100µl of 1X Dilution Buffer (blank), diluted ELISA controls (H, M, L, positive, negative, and UV only), or exchange reaction mixture dilutions, into the appropriate wells (recommended in duplicate, see plate map).

Assay Procedure

Step 16.

Seal the plates and incubate for 1 hour at 37°C.

Step 17.

Discard the liquid from the wells and wash 3 times with 300µl of wash buffer per well.

Assay Procedure

Step 18.

Add 100µl of diluted HRP-conjugated antibody.

Assay Procedure

Step 19.

Seal the plates and incubate for 1 hour at 37°C.

Assay Procedure

Step 20.

Discard the liquid from the wells and wash 3 times with 300µl of wash buffer per well.

Assay Procedure

Step 21.

Add 100µl of substrate solution.

Assay Procedure

Step 22.

Incubate for 8 minutes at room temperature (18-25°C) in the dark on a plate shaker at 400-500 rpm.

Assay Procedure

Step 23.

Add 50µl of Stop Solution to all wells and read at 414nm in an ELISA reader within 30 minutes.

Step 24.

View BioLegend website for Representative Data, and Appendix 1.