

Generation of peptide MHC class I monomers and multimers through ligand exchange.

This unit presents a set of linked protocols that can be used to create large collections of recombinant MHC class I molecules loaded with peptides of interest (Fig. 1). The collections of peptide-MHC (pMHC) complexes that can be produced by this method are useful for two major purposes. First, the measurement of MHC binding affinity of a set of potential MHC ligands. Second, the production of large collections of MHC multimers to measure antigen-specific T cell responses by MHC multimer flow cytometry .

The first basic protocol describes the expression and purification of recombinant MHC class I heavy and light chains. The second basic protocol describes the refolding and biotinylation of MHC class I complexes that are occupied with a conditional ligand. The third and central basic protocol describes how such MHC class I complexes that are occupied with conditional ligands can be utilized to produce pMHC complexes of interest in UV-induced peptide exchange reactions. The fourth basic protocol describes an ELISA-based strategy to measure the binding of peptides of interest to ‘empty’ MHC complexes generated by UV exposure. The fifth basic protocol describes how MHC complexes generated by ligand exchange can be used to produce MHC multimers for T cell staining. Three support protocols are included that describe the production of conditional ligands, the production of recombinant biotin ligase, and the measurement of biotinylation efficiency.

The technology that is described in this unit has been developed for the human MHC alleles HLA-A*0101, -A*0201, -A*0301, -A*1101 and -B*0702 (Toebes et al., 2006; Rodenko et al., 2006; Bakker et al., 2008) and the mouse alleles H-2D^b, K^b and L^d (Toebes et al., 2006; Frickel et al., 2008; Grotenbreg et al., 2008), and it should be possible to extend it to other MHC alleles. HLA molecules that are loaded with UV sensitive peptide are also available through commercial sources (see background information), providing an alternative to some of the protocols described in this Unit.

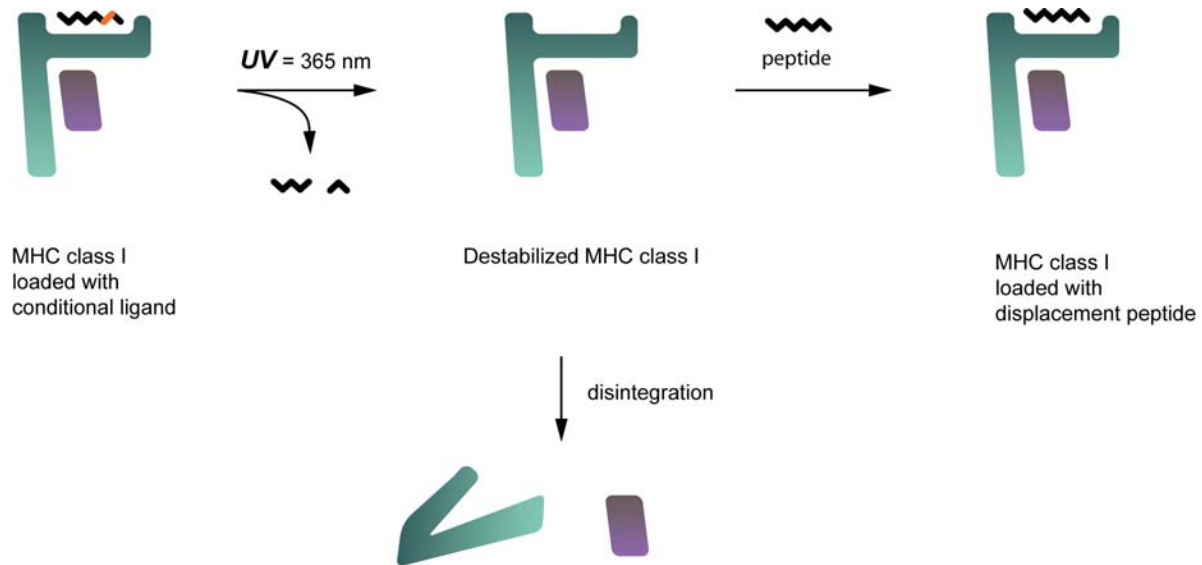


Figure 1. Basic strategy of UV mediated peptide exchange. MHC class I molecules refolded with an allele-specific conditional ligand are irradiated with UV light (365 nm). The photolabile peptide ligand cleaves to afford two fragments with strongly reduced affinity that dissociate from the MHC binding groove. The resulting empty MHC class I complex has a short half-life at 37° C if not stabilized by the binding of a peptide ligand. Displacement of the conditional ligand with an epitope of choice allows the generation of MHC complexes in high-throughput fashion.

BASIC PROTOCOL 1

BACTERIAL EXPRESSION AND PURIFICATION OF HLA CLASS I HEAVY CHAINS AND HUMAN β 2M

A soluble version of the MHC class I heavy chain containing a COOH-terminal biotinylation sequence (biotag) accepted by the Bir A biotin ligase and β 2m are both produced by expression in *E coli* and are isolated as inclusion bodies. The procedure detailed below for HLA-A*0201 is based on that first described by Garboczi et al. (Garboczi et al., 1992) and can be used for different human and mouse MHC class I alleles.

Materials:

- LB medium
- LB agar plates + antibiotics
- Bacterial strain BL21 (DE3) pLysS (cat. #: 69451, Novagen)
- Carbenicillin (cat. #: C1389, Sigma-Aldrich)
- Chloramphenicol (cat. #: C0378, Sigma-Aldrich)
- 1-*S*-Isopropyl- β -(D)-thiogalactopyranoside (IPTG) (cat #: I5502, Sigma-Aldrich)
- Lysozyme (cat. #: L6876, Sigma-Aldrich)
- MnCl₂ (cat. #: 244589, Aldrich)
- MgCl₂ (cat. #: 449172, Aldrich)
- sucrose (cat. #: 84099, Fluka)
- deoxycholic acid (cat. #: D5670, Sigma-Aldrich)
- Nonidet P 40 Substitute (cat. #: 74385, Fluka)
- Triton X-100 (cat. #: T9284, Sigma-Aldrich)
- DNase I (cat. #: 104 159, Roche) 10 mg/mL stock in 50 % glycerol, 150 mM NaCl, stored at -20 °C.
- Lysis buffer: 50 mM Tris-HCl pH 8, 25 % sucrose, 1 mM EDTA
- Detergent buffer: 0.2 M NaCl, 1 % deoxycholic acid, 1 % NP40,
20 mM Tris-HCl pH 7.5, 2 mM EDTA
- Wash buffer: 0.5 % Triton X-100, 1 mM EDTA
- Expression constructs: MHC class I heavy chain and β 2m:
The cDNA sequence encoding amino acid residue 1-100 of human β 2m is cloned in pET3a. The cDNA sequence encoding the MHC class I heavy chain without the signal peptide, transmembrane domain and cytoplasmic domain (amino acid residue 1-276 for HLA-A*0201) is cloned into pMBio, a derivative of pET3a that encodes an in-frame COOH terminal peptide tag (GSGGSGGSAGGGLNDIFEAQKIEW) that serves as a substrate for BirA biotin ligase. Vectors are available upon request for academic purposes.
- Incubator/orbital shaker

- Centrifuge
- 250 mL – 1 L buckets
- Corex tubes (Cat. #: 1-8445, Gentaur)
- 0.8 mm needle/syringe
- Spectrophotometer (for measuring OD at 600 nm)
- Plastic cuvettes (Cat. #: 67.742, Sarstedt)
- Dry ice/EtOH
- Waterbath
- Sarstedt polypropylene microtubes 1.5 mL with cap (cat. #: 72692, Sarstedt)

1 MHC class I heavy chains and β 2m are expressed separately in the bacterial strain B121 (DE3) pLysS. Transform bacteria according to supplier's protocol and grow on Luria-Bertani (LB) plates in the presence of 50 μ g/ml carbenicillin and 34 μ g/mL chloramphenicol.

Plates can be stored at 4 °C for up to 4 days.

- 2 For every liter of bacterial culture, inoculate one colony in 10 ml LB plus antibiotics until OD_{600 nm} is approx. 0.8 (37 °C, approx. 6 hrs). Use fresh colonies, *i.e.* from plates generated at most a few days before. Place inoculate at 4 °C overnight.
- 3 After overnight storage of inoculate, dilute culture (1/100) in 1 L LB plus antibiotics and divide evenly over two 2 L Erlenmeyer flasks.

This protocol describes a 1 L expression, which may be scaled up or down accordingly.

- 4 Grow bacteria at 37 °C to a density of OD₆₀₀ = 0.6 (this takes 3 to 4 hrs, check regularly, starting after 2 hrs). At this stage, a 0.5 mL sample can be taken for analysis by SDS-PAGE.
- 5 Add 200 μ L of a 1 M IPTG solution in H₂O (0.4 mM final concentration) to each 2 L Erlenmeyer containing 500 mL bacterial culture to induce protein expression.
- 6 Grow cells for approximately 4 h at 37 °C in an orbital shaker. At this stage, a 0.5 mL sample may be taken for analysis by SDS-PAGE.
- 7 Harvest the cells by centrifugation at 4 °C, 4,000 g for 15-20 min. Remove all medium.

- 8** Pool pellets in 50 mL LB, divide over two 50 mL falcon tubes and spin at 4 °C, 4,000 g for 30 min. Remove all medium. Typically 1 L of bacterial culture yields 5 g of cells.

Cell pellets can be stored at –20 °C (for 6 months at least), or can be used directly to isolate inclusion bodies.

- 9** Weigh the cell pellet (the whiter the pellet the better the yield of protein will be).
10 Add 8 ml of lysis buffer to approximately 5 g of cells (expected yield from 1 L of bacterial culture) and resuspend well!

Failure to resuspend well will result in a significantly reduced protein yield.

- 11** Add 20 mg lysozyme dissolved in 2 mL lysis buffer.
12 Incubate for 30 min on ice or (preferably) tumble in cold room for 30 min.
13 Add 100 µl (10 mM final conc.) of a stock of 1 M MgCl₂; 100 µl (1 mM final conc.) of a stock of 100 mM MnCl₂; and 50 µl (50 µg/ mL final conc.) of a 10 mg/mL DNase stock in 50 % glycerol, 150 mM NaCl, stored at -20 °C.
14 Incubate for 30 min on ice or, preferably, tumble in cold room for 30 min.
15 Add 20 mL of detergent buffer.
16 Freeze in dry ice/EtOH until solid is white (approximately 15 min).

Solid can be stored at –80 °C for up to 6 weeks.

- 17** Leave at RT for 5 min to avoid breaking of the tubes. Place in a 37 °C bath until just thawed.
18 Centrifuge lysate in corex tubes for 20 min, 14,000 g at 4 °C.
19 Discard the pale orange supernatant.
20 Wash the pellet with wash buffer. First add 1 or a few mL, vortex and resuspend by carefully drawing through an 0.8 mm needle/syringe. This will take quite some time and effort. After resuspending the pellet, add wash buffer to 30 mL.
21 Repeat steps 18-20 twice.
22 Centrifuge as in step 18, discard supernatant and take up the pellet in 20 mL of wash buffer. Distribute over 1.5 mL polypropylene Sarstedt tubes (1 mL/tube).
23 Spin for 5 min at 16,000 g in a microfuge and remove supernatant

Inclusion bodies can be stored at –80 °C for at least 1 year.

BASIC PROTOCOL 2

REFOLDING, BIOTINYLATION AND PURIFICATION OF MHC CLASS I COMPLEXES WITH CONDITIONAL LIGANDS

The refolding of HLA A*0201 complexes occupied with conditional ligand is largely based on the procedures established by Garboczi et al. (Garboczi et al., 1992). The conditional ligand used for HLA-A*0201 is the sequence KILGFV**FJV**, in which J indicates the UV sensitive amino acid (See Support protocol 1). Conditional ligands for a series of other MHC class I alleles developed by us and others are listed in Table 1.

HLA A*0101	STAPG J LEY	Bakker et al., 2008
HLA A*0201	KILGFV FJV	Toebes et al., 2006
HLA A*0301	RIYR J GATR	Bakker et al., 2008
HLA B*0702	AARG J TLAM	Bakker et al., 2008
HLA A*1101	RVFA J SFIK	Bakker et al., 2008
H2-D ^b	ASNEN J ETM	Toebes et al., 2006
H2-K ^b	FAPGNY J AL	Grotenbreg et al, 2008
H2-L ^d	YPNVNIH J F	Frickel et al., 2008

Table 1 Conditional ligands described to date.

Extended list: Grotenbreg, Reker-Hadrup & Schumacher labs available upon request

Materials:

- urea (cat. #: U5128, Sigma-Aldrich)
- Protease inhibitor cocktail, tablets (cat. #: 11836145001, Roche)
- EDTA-free protease inhibitor cocktail, tablets (cat. #: 11 873 580 001, Roche)
- Denaturing buffer: 8 M urea, 100 mM Tris-HCl pH 8
- L-Arginine HCl (cat. #: A5131, Sigma-Aldrich)
- EDTA (cat. #: E9884, Sigma-Aldrich)
- Reduced glutathione (cat. #: G6529, Sigma-Aldrich)
- Oxidized glutathione (cat. #: G4626, Sigma-Aldrich)
- D-Biotin (cat. #: B4501, Sigma-Aldrich)
- ATP (cat. #: A2383, Sigma-Aldrich)
- Biotin ligase. We utilize biotin ligase obtained by *E coli* expression (see Support protocol 2), biotin ligase is also available from Avidity.
- Ligase buffer (10X): 50 mM MgCl₂, 0.2 M Tris pH 7.5
- Glycerol 87% AR (cat. #: 071105, Bio-lab Ltd)
- Quartz cuvettes
- Sarstedt polypropylene microtubes 1.5 mL with cap (cat. #: 72692, Sarstedt)
The use of polypropylene tubes minimizes sticking of peptide or protein to the tube.
- Amicon ultrafiltration membrane (M_w cut-off 30 kDa) for pressure filtration.
Optional: filter size may be adjusted, depending on the volume of the refolding
- Amicon Ultra-4 PLTK Ultracel-PL Membrane, 30KDa (cat. #: UFC803024)
- Amicon Ultra-15 PLTK Ultracel-PL Membrane, 30KDa (cat. #: UFC903008)
- HPLC or FPLC system with a gel-filtration column, e.g. a Phenomenex Biosep SEC S-3000 column, 300x21.2 mm (cat. #: OOH-2146-PO, Phenomenex). Running buffer is PBS pH=7.4 For analytical runs: BioSep-SEC S-3000 column 300x7.8 mm (cat. #: 00H-2146-K0, Phenomenex)
- Centrifuge
- 0.45 μM low protein binding filters, size depending on refolding volume
- PMSF (cat. #: P7626, Sigma-Aldrich)

- PE-streptavidin solution, 1 mg/mL (cat. #: S866, Molecular Probes) or APC-streptavidin solution, 1 mg/mL (cat. #: S868, Molecular Probes)

The quality of APC- or PE-streptavidin conjugates is an essential factor for the formation of MHC tetramers that stain brightly, and presumably depends on the stoichiometric composition of the fluorochrome-streptavidin conjugates (Bakker and Schumacher, 2005).

Reagent	Amount	Final concentration
1 M Tris-HCl pH 8	5 ml	100 mM Tris-HCl pH 8
L-Arginine HCl	4.2 g	400 mM L-arginine.HCl
100 mM reduced glutathione	2.5 ml	5 mM reduced glutathione
50 mM oxidized glutathione	0.5 ml	0.5 mM oxidized glutathione
0.5 M EDTA	0.2 ml	2 mM EDTA
Protease inhibitor cocktail	1 tablet	Not applicable
H ₂ O (milliQ)	41.8 ml	Not applicable

Dilution buffer must be prepared fresh.

1. Prepare fresh, cold denaturation buffer.
2. Dissolve approx. 0.20 μ mol MHC class I heavy chain and approx. 0.4 μ mol β 2m in separate tubes using 1 mL denaturing buffer for each.
3. Spin down remaining aggregates for 2 min at 16,000 g.
4. Take off supernatant and place in fresh 1.5 mL polypropylene Sarstedt tubes.
5. Measure protein concentration in a spectrophotometer at 280 nm, by diluting 1/100 in denaturing buffer: 6 μ L protein with 594 μ L denaturing buffer.

ϵ (280 nm) of HLA A*0201 (1-276 pMBio) with biotag = 80,750 $M^{-1}cm^{-1}$.

ϵ (280 nm) of β 2m (1-100) = 19,180 $M^{-1}cm^{-1}$.

For example: if OD HLA A*0201 = 0.24 cm^{-1} , then the protein concentration of the sample is $[(0.24 \text{ cm}^{-1} * 100) / 80,750 \text{ M}^{-1}cm^{-1}] * 10^6 = 297.2 \mu M$.

To each 50 ml refolding, an aliquot of HLA class I heavy chain corresponding to a 1 μ M final concentration is added on 3 consecutive days (resulting in an MHC class I heavy chain concentration of 3 μ M on day 3). Thus, in this example, a total of: 3 *

$1\mu\text{M} * 50\text{ mL} / 297.2\ \mu\text{M} * 1000 = 505\ \mu\text{L}$ of the dissolved HLA-A*0201 heavy chain is required to perform a 50 mL refolding. This is added in three steps of 168 μL .

The concentration of $\beta 2\text{m}$ is calculated in the same fashion. For each refolding an aliquot of $\beta 2\text{m}$ corresponding to 2 μM final concentration is added on 3 consecutive days (resulting in a $\beta 2\text{m}$ concentration of 6 μM on day 3).

HLA class I heavy chain and $\beta 2\text{m}$ fractions to be added on day 2 and 3 should be stored at -20°C in denaturation buffer until use.

6. Set up the refolding reaction by adding the following components to cold refolding buffer in the critical order indicated below. Leave the tube slowly stirring in a cold room (approximately 10°C) for a total of three days. Keep dark.
 - a) Conditional ligand dissolved in 100 % DMSO (50 mM stock), to a final concentration 60 μM .
 - b) $\beta 2\text{m}$, to a final concentration of 2 μM .
 - c) HLA class I heavy chain, add dropwise, to a final concentration of 1 μM ,
 - d) PMSF (100 mM stock in isopropanol), to a final concentration 1 mM.
PMSF is very unstable in aqueous solution.
 - e) Stir O/N
 - f) Repeat addition of $\beta 2\text{m}$ and HLA class I heavy chain
 - g) Repeat addition of PMSF
 - h) Stir O/N
 - i) Repeat addition of $\beta 2\text{m}$ and HLA class I heavy chain
 - j) Repeat addition of PMSF
 - k) Stir for 48hrs
7. Wash an Amicon ultrafiltration membrane (M_w cut-off 30 kDa) with milliQ.
8. Spin refolding reaction at 4,000g to sediment aggregates, and filter supernatant through a 0.45 μM low protein binding filter.
9. Concentrate the refolding reaction to approximately 6 mL by nitrogen flow concentration over the Amicon ultrafiltration membrane.

Alternatively, Amicon Ultra-15 PLTK Ultracel-PL Membrane, 30KDa filters may be used. The desired end volume depends on the HPLC or FPLC system used for subsequent purification.

10. Rinse filter twice with 1 mL of PBS and combine with the 6 mL sample to give 8 mL in total.

11. Filter sample through a .45 μ m filter and purify by gel-filtration HPLC or FPLC, e.g. using two 4 mL injections on or a Biosep 3000 Phenomenex column with a 5 mL loop. Collect relevant fractions (approximately 16 mL) and keep the sample on ice.

Following purification, the sample is generally used immediately for biotinylation. The use of MHC class I complexes stored in glycerol is not recommended as glycerol affects biotinylation efficiency. However, MHC class I complexes occupied by conditional ligand may be stored by snap-freezing in liquid nitrogen without the addition of glycerol.

To obtain a higher yield: refolding of larger volumes is possible using the same ratios. After refolding samples are concentrated with the Viva Flow, MWCO 30.000 (VF20p2, Sartorius) the arginine buffer is exchanged to 20 mM Tris-HCl pH=8 and anion exchange is performed on Poros 50HQ material (1-2559-06, Applied Biosystems) The biotinylation can be performed immediately after purification , taking into account you have more monomers to biotiylate.

12. To biotinylate the biotag of the HLA class I heavy chain, add 1 volume of freshly prepared biotinylation solution to 1 volume of pMHC class I monomer, using 0.03 μ M ligase for 1 μ M of MHC class I.

Reagent	Amount
D-biotin (5 mM dissolved in 100 mM phosphate buffer, pH 7.5)	0.04 vol
ATP (0.5 M dissolved in 1 M Tris pH 9.5)	0.04 vol
BirA his-tagged biotin ligase (6.6 μ M)	0.01 vol
10x ligase buffer	0.2 vol
EDTA-free protease inhibitor cocktail (25x: 1 tablet in 2 mL H ₂ O	0.08 vol

or exchange buffer)

H₂O (MilliQ)

0.63 vol

Prepared fresh and ensure that ATP is dissolved in Tris buffer.

Biotin ligase is inhibited by high concentrations of NaCl and Tris. See also the Avidity website: <http://www.avidity.com/tech-protein.html>.

- 13.** Incubate 1hr at 37 °C or overnight at 25 °C.
- 14.** Wash an Ultra-15 PLTK Ultracel-PL Membrane (M_w cut-off 30 kDa) by placing 10 mL milliQ H₂O on the filter and centrifugation at 4 °C, 4,000 g for 10 min.
- 15.** Discard H₂O, add sample and concentrate the sample on the membrane filter to a volume of 3 mL by centrifugation at 4 °C, 4,000 g.
- 16.** Recover concentrated sample, rinse the filter once with 500 μ L PBS, and add to concentrated sample.
- 17.** Purify by gel-filtration HPLC or FPLC and collect the biotinylated HLA class I molecules (approx. 8 mL with the preparative gel-filtration column).
- 18.** Wash an Ultra-4 PLTK Ultracel-PL Membrane (M_w cut-off 30 kDa) by placing 4 mL milliQ H₂O on the filter and centrifugation at 4 °C, 4,000 g for 5 min.
- 19.** Discard H₂O, add sample (2 times 4 mL) and concentrate the sample on the membrane filter to a volume of 400 μ L.
- 20.** Recover concentrated sample, rinse the filter once with 50 μ L PBS, and add to concentrated sample.

From a 50 ml refolding, a final concentration of approximately 32 μ M (1.5 mg/mL) is obtained, depending on the refolding efficiency.

- 21.** Add 100 μ L glycerol (16 % final concentration) and divide into 11 x 50 μ L aliquots in 1.5 mL Sarstedt tubes.

Biotinylated MHC class I molecules (M_w 47 kDa) with a final concentration of approximately 1 mg/mL can be stored at -20 °C for up to 1 year.

Biotinylation reactions performed according to the protocol above should give complete or near-complete biotinylation of the tagged MHC class I heavy chain. It is nevertheless recommended to verify the degree of biotinylation for every batch of MHC class I before further use. See Support protocol 3.

BASIC PROTOCOL 3

UV MEDIATED PEPTIDE EXCHANGE

UV-mediated cleavage of the conditional ligand is time dependent. With the set-up described below, peptide cleavage is detected after 1 min and is essentially complete after approximately 15 min. A 30 to 60 min incubation time is normally used to ensure optimal exchange of the conditional ligand with the peptide of interest. Protein concentration may influence the rate of UV-mediated cleavage, as both the nitrophenyl moiety and the reaction product absorb long wavelength UV light. In addition, path length may affect the reaction speed. Empty, peptide receptive MHC molecules that are formed upon UV exposure can be rescued by performing the UV-mediated cleavage in the presence of an MHC ligand of interest. In most experiments, a 100 fold molar excess of peptide over MHC is used. UV-induced peptide exchange is routinely performed using 25 µg/mL of UV sensitive MHC class I complexes. However, peptide exchange reactions have been performed with MHC class I concentrations up to 100-200 µg/mL.

Materials:

- 96 well plates (cat. #: 651201 polypropylene microplate 96 well V sharp, Greiner Bio-one)

Use polypropylene material to avoid sticking of peptide or protein to the plate.

- UV-lamp 366 nm CAMAG UV Cabinet 3 (cat. #: 022.9070, CAMAG) fitted with UV Lamp long-wave UV, 366 nm, 2x8 W (cat. #: 022.9115, CAMAG) or Uvitec tube light, with 2x15W, 365 nm blacklight blue tubes (Model - LI215BLB sizes LxWxH 505x140x117 mm)

The use of short wavelength (254 nm) or broad-band UV lamps is detrimental to the MHC complex and should be avoided at all times.

- Centrifuge with rotor for microtiter plates.

1. In a 96 well plate, add the following reagents to each well:

Reagent	Amount	Final concentration
PBS	100 μ L	Not applicable
10x Exchange peptide (500 μ M in PBS)	12.5 μ L	50 μ M
10x UV-sensitive MHC class I molecules (250 μ g/mL; \sim 5 μ M)	12.5 μ L	25 μ g/mL (approx. 0.5 μ M)

In some cases it may be preferable to use a more concentrated peptide stock, dissolved in DMSO. See background information.

2. Place the 96-well plate under a UV lamp (366nm) for 1 hr, with a distance between the UV lamp and sample of approximately 5 cm.

Temperature of the solution may rise to approximately 30 $^{\circ}$ C, avoid overheating.

3. Spin the plate at 3,300g for 5 minutes. Transfer 100 μ L of supernatant (keep the plate at an angle to avoid transferring any pellet) to a new 96 well plate for downstream applications.

Resulting MHC complexes may be used to determine exchange efficiency by MHC ELISA (Basic protocol 4), or to produce MHC multimers for T cell detection (Basic protocol 5).

BASIC PROTOCOL 4

MEASURING PEPTIDE-MEDIATED MHC STABILIZATION BY HLA ELISA

This protocol describes an ELISA that measures the concentration of correctly folded HLA class I molecules. This assay can be used to verify the success of routine peptide exchange reactions, i.e. to ensure that the HLA ligand of choice indeed rescued the HLA class I complex, but can also be used for high-throughput screening of potential MHC ligands. Depending on the number of epitopes that is screened, the assay can be performed both in a 96 (described below) or a 384 well format. A 96 well-based assay is also available commercially (see Background information).

The HLA ELISA (described here for HLA-A*0201) measures the concentration of HLA class I complexes in the range of 0.5 to 10 nM. It is recommended to include a standard MHC titration curve, to ensure that the obtained absorbance values are within the linear range of the assay. UV-irradiation of the UV-sensitive HLA A*0201-KILGFVVFJV complex leads to a reduction of the absorbance signal of approximately 70-80% and an estimated reduction in concentration of folded HLA of 85%. This reduction is comparable to the reduction in HLA concentration as detected by gel-filtration chromatography. It is important to note that this assay will generally underestimate the extent of peptide cleavage, which is usually complete (see anticipated results). Inclusion of a high affinity HLA A*0201 restricted ligand in UV-mediated exchange reactions leads to recovery of 80-100% of the original ELISA signal. Lower rescue is observed using peptides with a moderate to low affinity for HLA A*0201.

Materials:

- Nunc immuno plate 96 wells (cat. #: 439454, F96 CERT Maxisorp, Nunc)
- Absorbance plate reader
- Coating solution: 2 µg/mL streptavidin (cat. #: S888, Invitrogen, Molecular probes) in PBS
- Wash buffer: 0.05% Tween 20 (cat. #: P1379, Sigma-Aldrich) in PBS
- Blocking buffer: 2% BSA (cat. #: A-7030, Sigma-Aldrich) in PBS
- Horseradish peroxidase (HRP)-conjugated anti-human β2m antibody
We have used directly conjugated rabbit anti-human β2m antibody from DakoCytomation (cat. #: P0174), but alternatives may be used.
- Developing reagent:
 - 10x substrate buffer
 - 50x 2',2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) solution
 - 100x H₂O₂ solution
- Stop buffer
We have used custom-produced developing reagent and stop buffer from Sanquin Reagents. Alternatives are available but have not been tested.

1. In a 96 well plate, add 100 μL of 2 $\mu\text{g}/\text{mL}$ streptavidin in PBS per well.
2. Incubate for 2 hr at 37 $^{\circ}\text{C}$ or overnight at RT. Cover the plate to avoid evaporation and contamination.
3. Wash 4 times with wash buffer, 300 μL per well, discarding the wash buffer after each wash.
4. Add blocking buffer, 300 μL per well.
5. Incubate for 30 min at RT. Cover the plate with a lid.
6. Dilute HLA class I samples to a final concentration of 5nM. Prepare 250 μL of each HLA class I complex to allow analysis in duplicate (e.g. a 100-fold dilution of 2.5 μL of the exchange reactions described in Basic protocol 3).
7. Tip out blocking buffer. Add 100 μL per well of diluted HLA class I solutions from experimental samples or of standard HLA class I dilutions to obtain a standard-titration curve.

*To generate a standard titration-curve: Add dilutions of a stock of HLA class I in a concentration range of 0.08 to 40 nM. Refolded HLA A*0201– GILGFVFTL (influenza A Matrix 1₍₅₈₋₆₆₎ epitope) may for instance be used as a standard HLA class I complex, but any stable HLA class I complex should suffice. Concentration of this standard HLA class I complex is determined by absorbance at 280 nm.*

8. Incubate the plate for 1 h at 37 $^{\circ}\text{C}$. Cover the plate with a lid.
9. Wash 4 times with wash buffer, 300 μL per well, discarding the wash buffer after each wash. Carefully dry plates by tipping out excess liquid on a tissue.
10. Add HRP conjugated anti- β 2m antibody solution (1 $\mu\text{g}/\text{mL}$ in blocking buffer), 100 μL per well.
11. Incubate the plate for 1 h at 37 $^{\circ}\text{C}$. Cover the plate with a lid.
12. Wash 4 times with wash buffer, 300 μL per well.
13. Prepare 10 mL ABTS coloring solution for each 96 well plate by mixing:
 - 8.7 mL H_2O
 - 1 mL 10x substrate buffer
 - 200 μL 50x ABTS solution
 - 100 μL 100x H_2O_2 solution (3%)
14. Add 100 μL of ABTS coloring solution per well.

15. Incubate for 10-15 min at RT. Monitor color development by eye.
16. Block the reaction by adding 50 μ L of stop buffer per well.
17. Measure absorbance at 405 nm in a plate reader.

A 384-well ELISA may also be used to measure HLA class I stabilization by added ligands. In our hands the assay is somewhat more sensitive in this format (possibly a reflection of a different dynamic range of the plate reader used) and pHLA class I complexes should be diluted to a final concentration of 1.6 nM. All reaction steps are performed using 25 μ L/well, blocking and washing steps using 100 μ L/well, and stop buffer is added in 12.5 μ L/well.

BASIC PROTOCOL 5

MULTIMERIZATION OF MHC CLASS I MOLECULES

MHC class I complexes may be complexed with fluorophore-labeled streptavidin to form MHC class I tetramers for T cell analysis. Commonly used fluorophores include allophycocyanin and phycoerythrin, and the formation of MHC multimers with these conjugates is described below. However, streptavidin-coated quantum dots may also be used to prepare MHC multimers for T cell detection.

We strongly recommend to first determine the efficiency of biotinylation of the UV-sensitive HLA class I batch, as described in Support protocol 3. In case biotinylation is found to be complete or near complete, multimerization of peptide-MHC class I complexes obtained by UV-induced exchange is performed as described below.

Materials:

- PE-streptavidin solution 1 mg/mL (cat. #: S866, Molecular Probes) or APC-streptavidin solution 1 mg/mL (cat. #: S868, Molecular Probes)

The source of APC- or PE-streptavidin is an essential factor determining in the quality of formed MHC tetramers, and presumably depends on the stoichiometric composition of the fluorochrome-streptavidin conjugates (Bakker and Schumacher, 2005). Do not use FITC conjugates.

- Microtiter plates with exchanged MHC class I complexes prepared according to Basic protocol 3, step 3, containing 25 µg/mL of pMHC in 100 µL /well. This corresponds to 2.5 µg or 0.05 nmol MHC class I per well.
 1. Generate dilutions of 27 µg/mL of streptavidin-PE in PBS, or of 14.6 µg/mL of streptavidin-APC in PBS, preparing 100 µL for each well of MHC class I.
 2. Add streptavidin-PE or –APC to MHC class I by four sequential additions of 25 µL with 10 minute intervals.

With four binding sites per streptavidin molecule, 0.0125 nmol fluorophore-streptavidin conjugate is needed to bind all MHC class I, assuming 100% recovery following ligand exchange. For streptavidin-PE (Mw 300 kDa), this corresponds to 3.75 µg, for streptavidin-APC (Mw 164 kDa) this corresponds to 2.05 µg. To ensure optimal MHC tetramer formation even for peptides that do not fully stabilize MHC class I, and to correct for loss of MHC class I upon centrifugation/transfer, only 70% of this amount is added to each well.

MHC tetramers can be used directly for T cell staining or can be stored at –20 °C. In the latter case, BSA and glycerol are added to a final concentration of 0.5 % and 16 %, respectively. Typically, 2-4 µL of MHC tetramer is used for the staining of approx 10⁶ cells in 50 µL FACS buffer.

SUPPORT PROTOCOL 1

SYNTHESIS OF “J” AND CONDITIONAL LIGANDS

This support protocol describes the synthesis of Fmoc protected UV-sensitive amino acid J, 3-amino-2-(2-nitrophenyl) propionic acid, and the subsequent generation of UV-sensitive peptides using this building block. It is noted that the UV-sensitive building block described here is also available commercially (see background information). UV-sensitive peptides that have successfully been used for various human and mouse MHC class I alleles are described in Table 1. The photocleavage reaction of a 3-amino-2-(2-nitrophenyl) propionic acid modified influenza M1 epitope is depicted in Fig. 2.

Materials:

- 3-Amino-3-(2-nitro)phenyl-propionic acid (cat. #: B22176, Lancaster)
 - Fluorenylmethylchloroformate (Fmoc-chloride) (cat. #: 160512, Sigma-Aldrich)
 - Dioxane (cat. #: 103115, Merck)
 - 2M HCL
 - 10% Sodium carbonate
- 1 Dissolve fluorenylmethylchloroformate (4.0 g, 15.5 mmol, 1.2 equivalents) in dioxane (60 mL) in a 100 mL addition funnel.
 - 2 To a 500 mL round-bottom flask add 3-amino-(2-nitro)phenyl propionic acid (3 g, 14.3 mmol), dioxane (60 mL) and 10 % (w/w) aqueous sodium carbonate (60 mL). Place the flask in an ice-bath and stir vigorously.
 - 3 Add the Fmoc solution dropwise to the cooled suspension over 15 min. Allow the reaction mixture to warm up to room temperature and leave stirring overnight, covering the reaction vessel with aluminum foil.
 - 4 Pour the reaction mixture into 600 mL of water and extract twice with Et₂O (2 x 200 mL) to remove Fmoc residues.

- 5 Cool the aqueous layer in an ice bath and adjust the pH to 1 by the addition of 2 M aqueous HCl.
- 6 Filter off the resulting precipitate and dry under high vacuum overnight, covering the flask with aluminum foil, to obtain *N*-fluorenylmethyloxycarbonyl 3-amino-3-(2-nitro)phenyl-propionic acid as a white solid (5.5 g, 12.7 mmol, 86 % yield).

N-fluorenylmethyloxycarbonyl 3-amino-3-(2-nitro)phenyl-propionic acid can be stored in the dark at -20°C for years.

- 7 Prepare the conditional peptide by standard automated Fmoc-peptide synthesis methodology (Wellings and Atherton, 1997) using the UV-labile building block Fmoc-3-amino-(2-nitro)phenyl propionic acid.

During handling, the conditional peptide should be kept away from direct long wavelength UV light (e.g. sunlight). It is stable on the bench exposed to artificial light, but we advise to keep stocks in the dark.

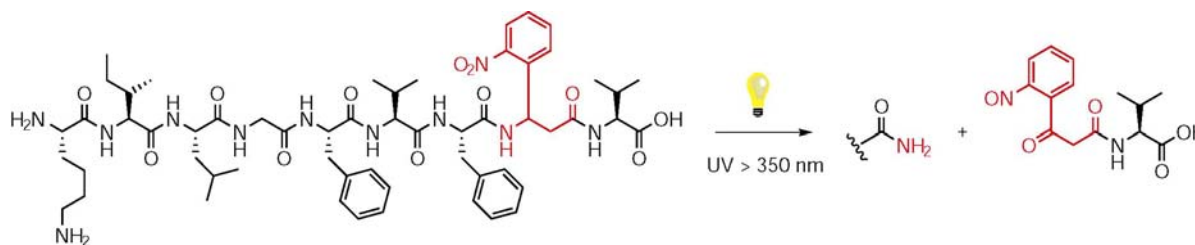


Figure 2. Conditional peptide ligand. The photolabile peptide KILGFVFJV is based on the HLA A*0201 restricted influenza Matrix 1₍₅₈₋₆₆₎ epitope GILGFVFTL. In this sequence, threonine at position 8 is replaced with an *o*-nitrophenylamino acid residue termed J, glycine-1 is replaced with lysine to increase solubility and anchor residue leucine-9 is replaced with valine to increase HLA affinity. Upon irradiation with long wavelength UV light, the *o*-nitrobenzyl moiety rearranges under cleavage of the amino-benzylic bond. This results in the formation of two fragments: a carboxamido terminal 7-mer and an *o*-nitrosoacetophenone containing fragment.

SUPPORT PROTOCOL 2

Expression and purification of biotin ligase

Materials:

- Incubator/orbital shaker
- Centrifuge
- 250 mL – 1 L buckets
- Spectrophotometer (for measuring OD at 600 nm and 280 nm)
- Plastic cuvettes
- Quartz cuvette
- Sonicator
- LB medium
- LB agar plates + antibiotics
- Expression construct: BirA biotin ligase cloned in pET21B vector (amino acids 1-321, in frame with a His-tag). M_w 36.4 kDa
- Bacterial strain BL21 (DE3) pLysS (cat. #: 69451, Novagen)
- Carbenicillin (cat. #: C1389, Sigma-Aldrich)
- Chloramphenicol (cat. #: C0378, Sigma-Aldrich)
- Imidazole (cat. #: I5513, Sigma-Aldrich)
- 1-*S*-Isopropyl- β -(D)-thiogalactopyranoside (IPTG) (cat #: 11 411 446001, Roche)
- Talon Co^{2+} -resin (cat. #8901-2, Clontech)

- 1 Transform biotin ligase expression plasmid into BL21 (DE3) pLysS. Plate on LB plates containing 50 $\mu\text{g}/\text{mL}$ carbenicillin and 34 $\mu\text{g}/\text{mL}$ chloramphenicol.
- 2 Pick colony and grow a 10 mL culture (LB + antibiotics) for approx. 6 hrs.
- 3 Store culture at 4 °C overnight.
- 4 Next day, inoculate 1 L with 10 mL culture and grow at 37 °C until $\text{OD}_{600\text{nm}} = 0.5$. Allow the culture to cool down to 30 °C before proceeding with the next step.

- 5 Add IPTG (0.4 mM final concentration) and grow culture overnight at 30 °C
- 6 Spin at 4 °C, 4,000 rpm for 15-20 min.
- 7 Resuspend pellet in 80 mL 20 mM Tris pH 8, 100 mM NaCl, divide over two 50 mL falcon tubes and sonicate (4 times 10 sec). Minimize sonication time, as prolonged sonication may destroy protein functionality.
- 8 Spin samples at 12,000g for 10 min at 4 °C to pellet all insoluble material.
- 9 From here Clontech protocol B for Co²⁺-batch/gravity column purification is followed:
- 10 Prepare 2 mL of Co²⁺ beads, which is sufficient for approx. 5 mg of protein.
- 11 Use sonicated supernatant for Co²⁺-batch/gravity column purification Clontech protocol B step 8 page 30.
- 12 Wash the resin with 10 bed volumes of wash buffer (page 31, step 12-14) and repeat this step.
- 13 Prepare a column (page 31, step 17).
- 14 Wash with 5 bed volumes of wash buffer.
- 15 Wash the protein by adding 3 bed volumes of 5 mM imidazole, 20 mM Tris pH 8, 100 mM NaCl. Collect 1 mL fractions and analyze by SDS-PAGE.
- 16 Repeat step 15 with 10 mM imidazole, 20 mM Tris pH 8, 100 mM NaCl. Collect 1 mL fractions and analyze by SDS-PAGE.
- 17 Elute the protein by adding 6 bed volumes of 50 mM imidazole/ 20 mM Tris pH 8, 100 mM NaCl. Collect 1 mL fractions and analyze by SDS-PAGE. Most protein will be in fraction 1 and 2.
- 18 Add β-mercaptoethanol to 5 mM and glycerol to 5 %. Measure OD_{280nm} (ε = 47,440) and make 20 μL aliquots of 100 μM. M_w 36.4 kDa. One aliquot is sufficient for the biotinylation of 3 μM MHC class I heavy chain in 20 mL.
- 19 Store the protein at -20 °C.

The expected yield of BirA enzyme from 1 L of bacterial culture is 2 mL of a 100 μM solution.

- 20 Optionally, the activity of the purified BirA enzyme can be tested using the following assay. To a 1.5 mL Sarstedt polypropylene microtube add:

- x μL BirA substrate (peptide GGGLNDIFEAQKIEWH, M_w 1813) to a 50

- μM final concentration.
- 50 μL 10X ligase buffer (200 mM Tris pH 7.5, 50 mM MgCl₂)
 - y μL purified BirA (approx. 2 μM BirA is needed to modify 50 μM peptide)
 - 10 μL biotin (5 mM stock)
 - 10 μL ATP (500 mM stock)
 - z μL H₂O
- 500 μL total volume

21 Incubate overnight at RT.

22 Add TFA to samples to 0.1 % final concentration. Analyze by reverse phase HPLC (*e.g.* Waters Delta-Pak, C18, 100 Å, 15 μm, 3.9 X 300 mm). As a reference, analyze peptide without BirA exposure. Run a 60 minute gradient: H₂O: CH₃CN (80:20), 0.1% TFA to H₂O:CH₃CN (65:35), 0.1 % TFA.

SUPPORT PROTOCOL 3

DETERMINATION OF BIOTINYLATION EFFICIENCY

The degree of biotinylation of MHC class I molecules can be determined by incubation of biotinylated MHC class I molecules with streptavidin and subsequent analysis of samples by gel-filtration chromatography. In case of full biotinylation, all MHC class I molecules should be bound to streptavidin when an excess of streptavidin is used (more than one streptavidin molecule per four MHC class I molecules), resulting in a shift in retention time of streptavidin and the complete disappearance of the monomeric MHC class I peak in gel-filtration chromatography (Fig. 3).

Materials:

- PE-streptavidin solution 1 mg/mL (cat. #: S866, Molecular Probes) or APC-streptavidin solution 1 mg/mL (cat. #: S868, Molecular Probes)
- 10x UV-sensitive MHC monomer (250 μg/mL; ~5 μM)

- PBS
- Sarstedt polypropylene microtubes 1.5 mL with cap (cat. #: 72692, Sarstedt)

1 To determine the optimal ratio of streptavidin conjugate and HLA class I complex, prepare the following 4 samples in Sarstedt 1.5 mL tubes

Sample 1:

Reagent	Amount
PBS	90 μ L
10x UV-sensitive HLA class I monomer (250 μ g/mL; \sim 5 μ M)	10 μ L

Sample 2:

Reagent	Amount
PBS	89 μ L
PE-streptavidin solution (1 mg/mL)	1 μ L
10x UV-sensitive HLA class I monomer (250 μ g/mL; \sim 5 μ M)	10 μ L

Sample 3:

Reagent	Amount
PBS	88 μ L
PE-streptavidin solution (1 mg/mL)	2 μ L
10x UV-sensitive HLA class I monomer (250 μ g/mL; \sim 5 μ M)	10 μ L

Sample 4:

Reagent	Amount
PBS	86 μ L
PE-streptavidin solution (1 mg/mL)	4 μ L
10x UV-sensitive HLA class I monomer (250 μ g/mL; \sim 5 μ M)	10 μ L

2 Analyze each sample by gel-filtration chromatography. Typically, we inject the full 100 μ L per sample for HPLC analysis. Aim for a high “multimer” peak, while leaving some residual HLA class I monomer present (Fig. 3).

This analysis will reveal the fraction of HLA class I that is maximally bound by streptavidin and this number can be used to calculate the optimal ratio of HLA class I and streptavidin

conjugate, that gives high order multimers, while leaving little residual monomeric HLA class I present. It is noted that an excess of fluorochrome-streptavidin over HLA class I results in the formation of HLA class I multimers with a lower valency and the binding of such lower order multimers to antigen-specific T cells is poor. In addition, a failure to saturate the available biotin-binding sites can lead to spurious background signal in case biotinylated antibodies are used for co-staining.

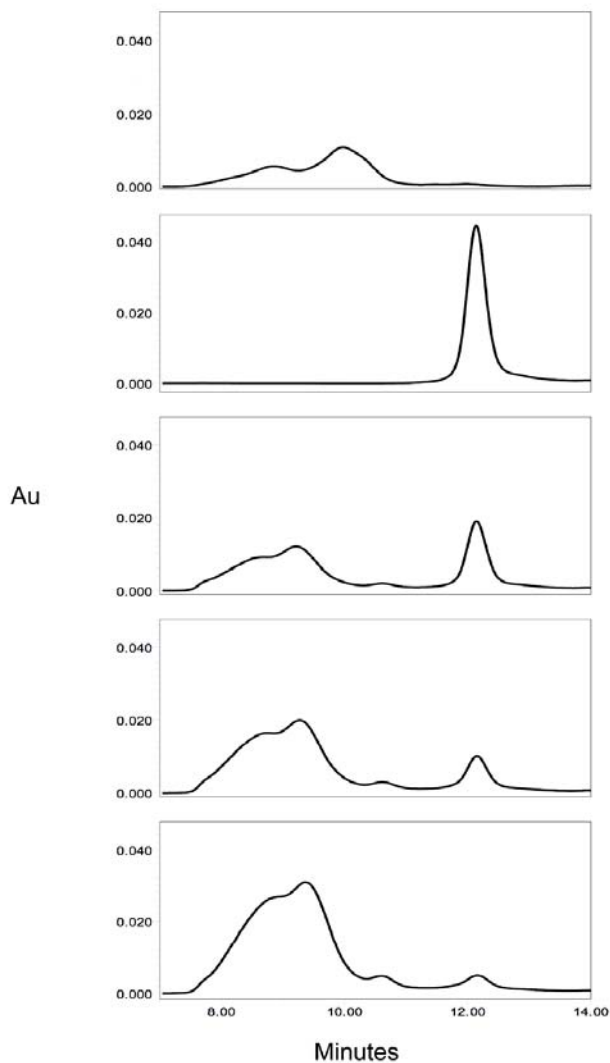


Figure 3. Typical HPLC profile of an MHC streptavidin-PE titration. In this titration, sample 3 (panel IV) contains the optimal amount of streptavidin-PE, giving high order multimers, while leaving little residual monomeric MHC class I present. Note that a further doubling of the amount of streptavidin (panel V) leads to only a small decrease in free MHC class I, indicating that under this condition not all biotin binding sites are saturated

SUPPORT PROTOCOL 4

Refolding of mouse MHC class I heavy and light chain and peptide (100 ml)

1. Dissolve inclusion bodies in 1 ml 8M urea/ 20 mM Tris-HCl, pH =8 by repeated pipetting (for a 100 ml refolding reaction, this takes about three cups of heavy chain and 1 cup of light chain inclusion bodies), spin at 14K to remove insoluble contaminants.
2. Determine protein concentration by measuring OD280nm (of a 1:100 dilution) in 8M urea/ 20 mM Tris.
 - ϵ H-2DbHC = 89,600 H-2Kb = 79,800
 - ϵ β_2 M = 18,200(M_w H-2DbHC = 34,990, H-2Kb = 34,739, β_2 M = 11,679)
3. Dissolve 8 mg of peptide in 20 mM Tris pH=8 (M_w peptide approx. 1000 -> gives you an 8 mM solution).
4. Refolding is performed with a final concentration of 10 μ M of HC, 10 μ M of β_2 M and 80 μ M of peptide (molar ratio of HC: β_2 M: peptide of 1: 1: 8).
 - a. This is a fairly large excess of peptide, depending on the affinity of the peptide, less may suffice.
 - b. A slight excess of β_2 M over HC doesn't hurt (and is clearly preferable over the reverse).
5. Calculate the volume of heavy chain, and light chain that is needed for a 100 ml refolding. Add these volumes, plus the 1 ml peptide solution to 90 ml 4 M urea, 20 mM Tris pH = 8, adjust volume with 4 M urea, 20 mM Tris pH = 8 to 100 ml.
6. Add 1 tablet of protease inhibitors (Roche MB tablets without EDTA).
7. Transfer the combined protein solution into dialysis tubing (spectrapor MWCO 500)
8. Dialyze against dialysis buffer (10 mM Potassium Phosphate pH=7.4) at 4° C without stirring. Use approx. 20x the volume of the refolding reaction.
9. Replace dialysis buffer after approx. 24 hrs, dialyse for another 24 hrs.
10. Concentrate sample in a centriplus (Amicon) to a volume of several ml or less (depending on the loop size of your chromatography system and the size of your column).

COMMENTARY

Background information

The recognition of defined antigen-MHC complexes by antigen-specific T cells forms the molecular basis of T cell immunity. Exploiting the central role of this interaction, Altman and colleagues demonstrated that fluorescently labeled recombinant MHC tetramers can be utilized to detect antigen-specific T cells by flow cytometry in a landmark paper in 1996 (Altman et al., 1996). Since this first description, MHC tetramers and other types of MHC multimers have become a core tool to monitor the development of disease- and therapy-induced antigen-specific T cell responses in both humans and in animal model systems.

This unit describes a set of protocols that transform classical MHC multimer technology into a high-throughput platform, allowing one to produce large collections of MHC class I molecules charged with different peptides. This technology is based on the development of conditional MHC ligands that can be triggered to self-destruct while being in the MHC-bound state.

UV-sensitive MHC class I ligands to be used for peptide exchange are preferentially produced by replacing one of the T cell receptor-exposed amino acids in a known or predicted MHC ligand by a non-natural amino acid that contains a 2-nitrophenyl side chain. Because of ease of synthesis, the conditional ligands used to date have all been based on the UV-sensitive building block J. It is noted that the introduction of this β -amino acid does alter the structure of the peptide in the peptide binding groove and, depending on the site at which the UV-sensitive amino acid is introduced, this may affect the affinity of the peptide for the MHC groove. Nevertheless for all alleles tested, UV-sensitive peptides that bind with high affinity have been identified. In addition, an α -amino acid containing a nitrophenyl sidechain has also been developed (Rodenko et al., 2006).

The protocols contained within this Unit describe both the production of UV-sensitive MHC class I molecules and the use of these molecules to produce MHC multimers or to measure MHC class I binding of potential ligands. It is noted that recombinant MHC class I molecules charged with UV-sensitive peptides are also available from Sanquin

(<http://www.sanquin.nl/Sanquin-eng/Pelimers.nsf/All/More-Than-Mhc-Tetramers.html>) in an ELISA kit format.

Multimerized forms of pMHC complexes obtained by UV-mediated peptide exchange can be used to stain antigen-specific T cells with the same selectivity as conventional pMHC multimers (e.g. Toebes et al., 2006, Bakker et al., 2008). T cell staining may be performed with pMHC multimers conjugated to the classical fluorophores phycoerythrin and allophycocyanin (Basic protocol 5). In addition, in view of the high complexity of T cell responses and the possibility of creating large arrays of pMHC complexes by MHC exchange, strategies that allow the parallel measurement of a large number of different T cell responses in a single sample will likely become of value (Reker-Hadrup et al., unpublished information).

A main advantage of the peptide exchange technology as compared to classical MHC tetramer production is the feasibility of generating very large collections of reagents of interest. In addition, because ligand exchange allows the direct use of peptide-MHC multimers that are generated in a 1 hr ligand exchange procedure without further purification steps, the technology is also well-suited to study immune responses against low affinity ligands such as cancer- or autoimmune disease-associated epitopes (e.g. the unmodified MART-I peptide; Toebes et al., 2006).

As detailed in Basic protocol 4, MHC class I molecules charged with UV-sensitive ligands can not only be used to produce sets of MHC multimers, but can also be utilized to measure the ability of newly added ligands to stabilize MHC class I molecules in an ELISA-based strategy. A second approach to measure the binding of newly added ligands, which is based on fluorescence polarization, has also been developed and is described in Rodenko et al, 2006. Setting up the latter assay is somewhat more involved. However, this assay may be more suited in cases where highly quantitative analyses are required.

Critical Parameters and Troubleshooting

During handling, the conditional peptide should be kept away from direct long wave UV light (*e.g.* sunlight). It is stable on the bench exposed to artificial light, but we advise to keep stocks in the dark.

Ligand exchange reactions may be less efficient when using peptides that have a very poor solubility in water. In that case it may be preferable to add ligands from stocks in DMSO, and ligand exchange reactions have been shown to proceed normally in the presence of up to 10% DMSO. Solubility issues should also be reduced when performing ligand exchange reactions at lower concentrations of MHC and peptide. In case DMSO is included in ligand exchange reactions, do make sure that the DMSO is sufficiently diluted when using the generated MHC complexes for T cell staining.

It is important to note that while the peptide fragments generated by UV exposure have a very low affinity for MHC class I as compared to conventional MHC class I ligands, some residual affinity for the peptide binding groove does exist. Consequently, the UV-induced peptide fragments can still result in some stabilization of the MHC class I complex, in particular in case ligand exchange reactions are performed at high concentrations. Structural studies have shown that the UV-induced peptide fragments are replaced by a newly added peptide ligand. Finally, the ratio of fluorophore-streptavidin conjugate to MHC has a strong effect on the quality of the resulting tetramers. An excess of fluorophore-streptavidin over MHC results in the formation of MHC multimers with a lower valency. This affects the avidity of the T cell-pMHC multimer interaction and thereby results in poor T cell staining and higher background.

Anticipated Results

Production of HLA class I molecules charged with UV-sensitive peptide is a routine process that should deliver approximately 0.6 mg of HLA from a 50 mL refolding, but yields may vary somewhat for different HLA alleles. Inclusion of a medium or high affinity MHC class I ligand in a peptide exchange reaction should result in a complete or near complete rescue of MHC class I upon UV exposure, with the possible exception of extremely hydrophobic

peptides (see troubleshooting). Rescue may be lower when using extremely low affinity peptides, and in that case it may be useful to keep samples cold to avoid MHC disassembly. When measuring ligand exchange by ELISA, the extent of peptide cleavage will be underestimated, as the peptide fragments that are generated can still stabilize MHC class I to some extent, in particular when present at higher concentrations. Importantly, structural studies have demonstrated that the addition of a new peptide ligand leads to the displacement of these peptide fragments from the peptide binding groove (Celie et al., unpublished). Multimerized forms of pMHC complexes obtained by UV-mediated peptide exchange can be used to stain antigen-specific T cells with the same selectivity as conventional pMHC multimers.

Time Considerations

The time required to produce a batch of refolded MHC class I occupied with UV sensitive ligand (Basic protocol 1 and 2) is fairly significant, but not different from that required to produce a batch of MHC class I occupied with any other peptide ligand. The actual ligand exchange process (Basic protocol 3) is extremely straightforward, allowing one to produce collections of hundreds of different peptide-MHC complexes in a timespan of hours. The time required for the measurement of MHC class I stabilization by ELISA (Basic protocol 4) is restricted to several hours, and formation of MHC multimers (Basic protocol 5) can likewise be completed within hours.

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The first description of conditional MHC class I complexes and the use of such complexes to monitor antigen-specific T cell responses in a high-throughput fashion.

Contributed by

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