



Chapter 14

Generation of Allergen-Specific Tetramers for a Murine Model of Airway Inflammation

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Abstract

The identification and analysis of allergen-specific CD4⁺ T cells is critical for understanding how these cells contribute to atopic disease and how to subvert their behavior through immune therapy. The advent of fluorescently labeled soluble tetramers of peptide:MHCII complexes (pMHCII tetramers) has provided investigators with an invaluable means to achieve this goal. Although pMHCII tetramers were first developed over two decades ago, their widespread use has been limited by the technical difficulty of generating these reagents. However, the adoption of various technical innovations from several labs over time has contributed greatly to the increased success in tetramer generation today. Here, we describe a comprehensive protocol for generating pMHCII tetramers using as an example a Derp1:I-A^b tetramer used to study allergen-specific CD4⁺ T cell responses in murine models of airway inflammation and allergic disease.

Key words Allergy, House dust mite, CD4⁺ T cell, Peptide:MHC complex, Tetramer

1 Introduction

Asthma is an inflammatory disease of the airways that affects more than 300 million people worldwide and is rapidly increasing in incidence [1]. Clinically, asthma is characterized by acute, intermittent, and recurrent episodes of inflammation that can be induced by a specific allergen. Allergen-specific CD4⁺ T cells contribute to this process by producing type 2 cytokines (e.g., IL-4, IL-5, and IL-13) and inducing B cell production of IgE. Both these responses require T cell receptor (TCR) recognition of allergen-derived peptides bound to MHCII molecules on host antigen-presenting cells. In both murine models of disease and asthmatic patients, allergen peptide:MHCII (pMHCII)-specific CD4⁺ memory T cells persist in the lungs and lymphoid organs after resolution of inflammation. Upon subsequent exposure to allergen, these cells rapidly drive asthma-induced pathology, but due to the technical challenges of identifying small populations of CD4⁺ T cells that express allergen pMHCII-specific TCRs, we are

just beginning to understand how these Th2 cells differentiate, function, and persist in health and disease.

Soluble tetramers of allergen pMHCII provide a powerful state-of-the-art means to overcome the challenges of studying allergen-specific CD4⁺ T cells. By binding selectively to TCRs with cognate antigen specificity, fluorescently labeled tetramers can be used as staining reagents to directly identify allergen-specific T cells [2]. Here we describe the generation of pMHCII tetramers that can be used to identify both circulating and lung-residing allergen-specific CD4⁺ T cells. For an allergen, we have initially focused on the Der p 1 protein (Derp1) of the house dust mite (HDM), *Dermatophagoides pteronyssinus*. Derp1 is a 30 kDa Group 1 cysteine protease that breaks down epithelial tight junctions and enhances antigen presentation by dendritic cells [3]. Several regions of Derp1 contain epitopes that are highly immunogenic in atopic individuals and certain strains of mice [4, 5]. Using MHCII binding prediction algorithms, we fine-mapped the peptide sequence of an immunodominant epitope in C57BL/6 mice and generated the corresponding I-A^b tetramer [6]. While this protocol specifically describes the generation of this particular Derp1₁₁₇₋₁₃₁:I-A^b tetramer, such tetramers can be generated for any allergen so long as a well-defined immunogenic epitope in the allergen is known.

Because epitope-specific CD4⁺ T cells are usually present at extremely low frequencies in sampled tissues [7], the use of tetramers is often coupled with magnetic cell enrichment techniques to improve the sensitivity of epitope-specific T cell detection [8, 9]. In our studies, we used tetramer-based cell enrichment in conjunction with intravascular staining and multiparameter flow cytometry to directly analyze allergen-specific T cells in the lymphoid organs, blood, and lungs of mice sensitized with HDM allergen [6]. This has become an instrumental tool in our studies of how Th2 effector and memory cell differentiation is regulated during atopic asthma.

The following protocol explains the generation of tetramers using basic laboratory techniques that should enable most immunology labs without access to sophisticated resources to take advantage of this powerful technology. While pMHCI tetramers have been widely available for decades [10], the successful generation of pMHCII tetramers has been stymied by several technical issues including the instability of soluble MHCII heterodimers, the improper folding and glycosylation of MHCII proteins when expressed in bacteria, the difficulty of loading defined peptides into the MHCII binding groove, and the propensity of purified pMHCII to aggregate and precipitate from solution [11]. To overcome these difficulties, we have incorporated into our methods a number of technical innovations developed by several different labs over

the years. Collectively, they have enabled us to achieve consistent success in the generation of most tetramer species.

Our tetramers adopt a design introduced by Teyton and colleagues in which the intracellular domains of the MHCII alpha and beta chains are replaced with acidic and basic Fos-Jun leucine zipper motifs (Fig. 1) [12]. This forces heterodimerization of the extracellular domains of these subunits and increases stability of the soluble protein. To circumvent the challenges of loading peptides into the MHC binding groove, we utilize a strategy developed by Kappler and colleagues in which the peptide antigen is genetically encoded as a fusion product covalently linked to the N-terminus of the MHCII beta chain via a flexible linker [13]. To achieve proper posttranslational processing and high production yields, these constructs are stably expressed as secreted proteins in *Drosophila* S2 cells. The C-terminus of the alpha chain contains a signal sequence for site-specific biotinylation by *E. coli* BirA biotin ligase [14], ensuring that pMHCII complexes are in the proper orientation after eventual tetramerization with streptavidin. The BirA ligase is coexpressed along with the pMHCII constructs to mediate efficient in vivo biotinylation of the pMHCII during production, thereby eliminating the need for a distinct biotinylation step [15]. To facilitate purification, the C-terminus of the beta chain contains a 6× polyhistidine sequence to enable nickel column chromatography. Once purified, biotinylated pMHCII complexes are analyzed by Western blot for quantification and quality control and then tetramerized with premium grade streptavidin conjugated to fluorochromes of choice.

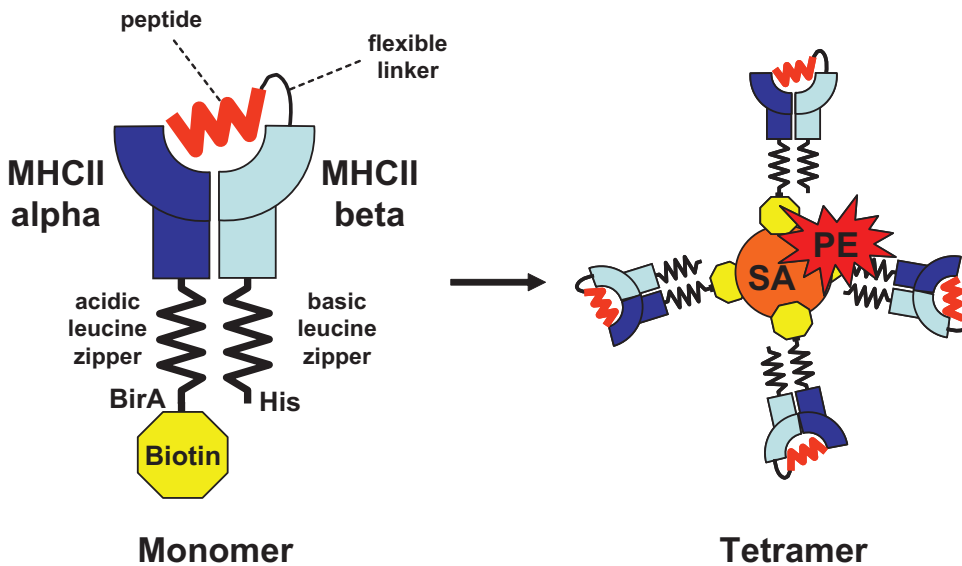


Fig. 1 Design schematic of peptide:MHCII monomer and tetramer

2 Materials

2.1 Plasmid Cloning

1. Expression plasmids (Addgene): pR I-A^b α BirA; pR I-A^b β Derp1-117; pR BirA; pCoPuro.
2. *Xma*I restriction enzyme.
3. *Spe*I restriction enzyme.
4. Low-melt agarose.
5. DNA gel extraction kit.
6. DNA ligation kit.
7. Competent cells: DH5 α , TOP10, XLI Blue, or equivalent.
8. LB agar plates.
9. LB broth.
10. Ampicillin.
11. AMP+ LB agar plates: LB agar supplemented with 100 μ g/mL ampicillin.
12. DNA miniprep kit.
13. DNA maxiprep kit.

2.2 S2 Cell Culture and Transfection

1. *Drosophila* S2 cells.
2. Complete S2 medium: Schneider's *Drosophila* Medium, 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (100 U/mL each), 50 μ g/mL gentamycin, 250 ng/mL fungizone.
3. Calcium Phosphate Transfection Kit (ThermoFisher).
4. Puromycin.
5. Complete S2 medium + Puro: Schneider's *Drosophila* Medium, 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (100 U/mL each), 50 μ g/mL gentamycin, 250 ng/mL fungizone, 5 μ g/mL puromycin.
6. DMSO.
7. Phosphate buffered saline (PBS).
8. 2 $\times$ Hanks buffered saline (HBS).
9. Serum-free medium (SFM): Express five serum-free medium, 1% penicillin/streptomycin (100 U/mL each), 50 μ g/mL gentamycin, 250 ng/mL fungizone.
10. SFM + Puro: Express five serum-free medium, 1% penicillin/streptomycin (100 U/mL each), 50 μ g/mL gentamycin, 250 ng/mL fungizone, 5 μ g/mL puromycin.
11. Pluronic F-68 (ThermoFisher).

12. 1 M Copper sulfate solution: Dissolve 12.5 g CuSO_4 (copper (II) sulfate pentahydrate) in 50 mL dH_2O , and pass through sterile 0.2 μm filter.
13. 1 mg/mL D-biotin solution: Dissolve 50 mg D-biotin in 49.5 mL dH_2O and pH to 7.0 with 1 N NaOH (this will require approximately 240 μL). Bring volume to 50 mL, and pass through sterile 0.2 μm filter.
14. 3 L spinner or 2 L shaker flasks.
15. 500 mL conical centrifuge tubes.
16. 24 cm round grade 3 filter paper (Whatman).
17. 0.45 μm bottletop vacuum filter.
18. 1 L bottle.
19. 10% sodium azide solution: 10% sodium azide in PBS.

2.3 Protein Purification

1. HisBind Purification Kit (Novagen-EMD Biosciences).
2. HisBind nickel resin (provided in HisBind Purification Kit).
3. 1 $\times$ 10 cm glass column.
4. Ring stand.
5. Clamp to attach glass column to ring stand.
6. 500 mL beaker: Waste beaker.
7. 1 L beaker: Collection beaker.
8. 425–600 μm diameter glass beads.
9. 1 $\times$ charge buffer: 50 mM NiSO_4 (provided in HisBind Purification Kit).
10. 1 $\times$ bind buffer: 500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9 (provided in HisBind Purification Kit).
11. Luer-lock stopcock.
12. Small diameter flexible tubing: 1/16 in internal diameter (I.D.) tubing (Tygon).
13. Small diameter flexible tubing: 1/8 in I.D. tubing (Tygon).
14. 10 mL syringe.
15. Hemostat (or similar clamp).
16. PBSA: PBS, 0.05% sodium azide.
17. Octyl β -D-glucopyranoside (OGP) detergent (Sigma-Aldrich).
18. 1 $\times$ elute buffer + 0.2% OGP: 1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9, 0.2% OGP.
19. 1 $\times$ strip buffer: 500 mM NaCl, 100 mM EDTA, 20 mM Tris-HCl, pH 7.9 (provided in HisBind Purification Kit).
20. Amicon Ultra-15 50 kD concentrating filter (Millipore).

21. PBSA + 0.2% OGP: PBS, 0.05% sodium azide, 0.2% OGP.
22. 0.2 μm microfuge spin filter (Corning-Costar).
23. Spectrophotometer.

2.4 Protein Purification

1. Streptavidin-R-Phycoerythrin (SA-PE) (ProZyme).
2. Streptavidin-Allophycocyanin (SA-APC) (ProZyme).
3. Tris-glycine acrylamide gel (4–15% or other concentration suitable for 50–100 kD proteins).
4. Protein molecular weight markers.
5. 4 $\times$ Native PAGE sample buffer: 200 mM Tris pH 6.8, 60% glycerol, 20 mg/mL bromophenol blue. Mix one part to three parts sample prior to gel loading.
6. 5 $\times$ Native Tris-glycine PAGE running buffer: 125 mM Tris, 1.25 M glycine. Dilute to 1 $\times$ with dH₂O before use.
7. Nitrocellulose membrane and filter paper for Western blot transfer.
8. 10 $\times$ TBS-Tween 0.1% (TBST): 200 mM Tris pH 7.6, 1.37 M NaCl, 1% Tween-20. Dilute to 1 $\times$ with dH₂O before use.
9. Streptavidin conjugated to horseradish peroxidase (HRP) or fluorescent dye.
10. Enhanced chemiluminescence (ECL) substrate.

3 Methods

3.1 Expression Plasmid Cloning (for New Antigens)

All plasmids required to generate I-A^b Derp1-117 tetramers have been deposited at Addgene. The pR I-A^b beta expression plasmid is designed with convenient *Xma*I and *Spe*I restriction sites flanking the peptide sequence to facilitate cloning of new constructs with different peptide antigens. This section describes the cloning of a new construct using complementary oligonucleotide synthesis.

1. Design complementary oligonucleotides for the new peptide insert (Fig. 2). Ideally, new peptide antigens should be designed with a minimal length including just the 9-mer MHCII binding core plus two additional N-terminal residues. No extra C-terminal residues are needed due to the presence of the linker. The ends of the oligos should extend three bases beyond the *Xma*I and *Spe*I restriction sites on each side to facilitate enzyme activity.
2. Anneal oligos. Make 100 μM stocks of each oligo in dH₂O, and mix 5 μL of each oligo in a 0.2 mL PCR tube.

3.2 *Drosophila* S2 Cell Transfection

Expression of biotinylated pMHCI in *Drosophila* S2 cells largely follows the DES *Drosophila* Expression System from ThermoFisher.

1. Using healthy S2 cells in log growth phase, set up 3×10^6 cells in 3 mL Complete S2 medium in 60 mm tissue culture dishes. Incubate overnight at 28 °C. You can place three 60 mm dishes inside a 150 mm dish to provide an additional sterile barrier (*see* **Notes 1–3**).
2. Transfect cells using the Calcium Phosphate Transfection Kit. Make sure all reagents are warmed to room temperature prior to transfection.
3. For each transfection, set up a 1.5 mL microfuge tube with the following:
 - pR I-A^b α BirA plasmid 9 μ g
 - pR I-A^b β (Derp1-117) plasmid 9 μ g
 - pR BirA plasmid 9 μ g
 - pCoPuro plasmid 1 μ g
 - CaCl₂ (added last) 240 mM (36 μ L of 2 M)
 - Sterile dH₂O needed to achieve a total volume of 300 μ L.
4. Slowly add mixture dropwise over the course of 1 min into another 1.5 mL microfuge tube containing 300 μ L 2 $\times$ HBS while vortexing. This process produces the fine precipitate that mediates transfection of DNA.
5. Incubate at room temperature for 30–40 min.
6. Slowly drop mixture into cell culture while swirling dish.
7. Incubate 1 day at 28 °C.
8. Replace Complete S2 medium. Without disturbing cells, pipet Complete S2 medium from the dish into a 15 mL centrifuge tube.
9. Carefully rinse cells with 1 mL fresh Complete S2 medium and transfer into the same 15 mL tube. Add 1 mL fresh Complete S2 medium to dish so the cells don't dry out.
10. Spin down the 15 mL tube at $400 \times g$ -force for 5 min at room temperature, and aspirate and resuspend the cell pellet with 2 mL fresh Complete S2 medium and transfer to the dish to restore volume to 3 mL.
11. Incubate 2 days at 28 °C.
12. Resuspend cells by pipetting and transfer to a T75 flask. Due to evaporation, there will only be about 2.5 mL of cells left per dish. Add 13 mL fresh Complete S2 medium + Puro. From here on, transfected cells should always be kept in puromycin-containing media.
13. Incubate 7 days at 28 °C.

14. Scale up culture for freezing and protein expression. Split cells 1:10 as follows:
 - (a) Place 0.5 mL of cells into T25 flask containing 4.5 mL of Complete S2 medium + Puro for maintenance. Split this culture 1:10 every 7 days until supernatant is successfully harvested from scale-up cultures (*see Note 4*).
 - (b) Place 1.5 mL of cells into T75 flask containing 13.5 mL Complete S2 medium + Puro for freeze-down.
 - (c) Place 5 mL into 2× T225 flasks each containing 45 mL SFM + Puro for scale-up.
15. Incubate cultures at 28 °C.
16. When the T75 freeze-down culture has reached $\sim 10^7$ cells/mL (usually about 3–5 days), transfer to a 15 mL tube, spin down, and aspirate supernatant down to ~ 2.5 mL.
17. Add 2.5 mL fresh Complete S2 medium, 0.6 mL sterile DMSO, and suspend cells. This will result in $\sim 3.0 \times 10^7$ cells/mL in 45% fresh complete S2 medium, 45% conditioned complete S2 medium, and 10% DMSO.
18. Aliquot ~ 1 mL to each of five labeled cryotubes.
19. Freeze down and transfer to liquid nitrogen for long-term storage.

3.3 pMHCII Expression

1. When the T225 SFM culture reaches $5\text{--}10 \times 10^6$ cells/mL (about 5–7 days), transfer the whole cell culture (100 mL) into a sterile 3 L spinner flask or 2 L shaker flask. Rinse remaining cells in the T225 flasks with 1.0 L prewarmed SFM + Puro and pool together in the new flask (*see Note 5*).
2. Add 10 mL Pluronic F-68 to a final concentration of 0.1% (*see Note 6*).
3. Incubate at 28 °C with constant spinning or shaking at 125 rpm. Lower speeds (~ 100 rpm) are okay too, but the cell densities may be slightly lower.
4. When the culture reaches $1.0\text{--}1.5 \times 10^7$ cells/mL (about 5–7 days), induce expression by adding 880 μL of 1 M copper sulfate solution and 2.2 mL of 1 mg/mL D-biotin solution for a final concentration of 800 μM CuSO_4 and 2 $\mu\text{g}/\text{mL}$ D-biotin.
5. Incubate 14 days at 28 °C, spinning or shaking at 125 rpm.
6. Transfer and spin down cells in 500 mL conical centrifuge tubes at $\sim 1000 \times g$ -force for 10 min in a tabletop centrifuge.
7. Transfer supernatant to new 500 mL conical tubes and spin down again to remove residual cells.

8. Filter supernatant through a piece of 24 cm round grade 3 filter paper folded into a funnel set up over a 0.45 μm bottletop vacuum filter, draining into a 1 L bottle.
9. Add 5 mL of 10% sodium azide solution to achieve a final concentration of 0.05%, and store at 4 °C. If aggregates form in the supernatant over time, filter it again before proceeding to purification.

3.4 pMHCII Purification

Soluble monomeric pMHCII complexes are purified from cell culture supernatants via nickel affinity chromatography targeting the 6 $\times$ His epitope on the C-terminus of the MHCII beta chain. This section describes a protocol adapted from the HisBind Purification Kit from Novagen-EMD Biosciences. Alternatively, purification by immunoaffinity chromatography may be used to achieve higher purity product (*see* **Note 7**).

1. Add 8 mL of well mixed HisBind nickel resin slurry to a 1 $\times$ 10 cm glass column clamped on a ring stand with a 500 mL waste beaker placed below. The resin is supplied as a 50% suspension slurry, so 4 mL of packed resin will remain in the column after the liquid portion has drained away (*see* **Note 8**).
2. Add about 1/4 inch layer of 425–600 μm diameter glass beads to the top of the resin bed to protect the top surface from disruption as fluids are added.
3. Wash the column by adding 12 mL (three column bed volumes) dH₂O and let drain.
4. Charge the column by adding 20 mL (five column bed volumes) of 1 $\times$ charge buffer and letting it drain. The color of the resin should turn from white to blue.
5. Add 12 mL (three column bed volumes) of 1 $\times$ bind buffer to the column and let it drain. The blue color will fade a little, but overall the column should still be blue.
6. Affix a closed Luer-lock stopcock to the bottom of the column. Add a few mL of culture supernatant to the top of the resin, at first by slowly running it down the inside of the column to prevent disruption of the resin. Secure the cap on top of the column.
7. Place the bottle containing the culture supernatant on a shelf a few feet above the top of the column. Set up a length of small diameter flexible tubing that will connect the bottle of culture supernatant to the cap on top of the column. Rather than sticking the tubing directly into the bottle of supernatant, you can connect the end to a 1 mL pipet (with cotton removed) and then insert the pipet into the bottle. This will allow for better draining of the bottle. We recommend using 1/16 in I.D. tubing with short lengths (~2 in) of 1/8 in I.D. tubing at

each end to act as couplings for attachment to the pipet at one end and the nipple on the column cap at the other end. Make sure that the lowest point in the tubing loop hangs below the bottom of the column. This safety loop will prevent the column from running dry after the bottle has emptied.

8. Prime the tubing by using a 10 mL syringe to draw fluid from the bottle through the entire length of tubing, and then clamp the tubing about an inch from the end with a hemostat. Remove the syringe, and attach the end of the tubing to the nipple on the column cap. Reattach the column cap to the top of the column.
9. Unclamp the tubing and open the stopcock. The fluid should start dripping from the bottom of the column. Use the stopcock to adjust the flow rate to about 2 mL per minute. Let the column drip overnight, collecting the flow-through fraction in a new 1 L bottle. The safety loop should prevent the resin from drying out once the supernatant bottle has emptied.
10. Elevate the tubing so that the remaining supernatant passes through the column. Close the stopcock, and remove the cap and tubing from the column. Transfer the column on the ring stand to a bench in the lab. The column should be bluish green with some hint of brown impurities mixed in.
11. Wash the column by adding 8 mL (two column bed volumes) PBSA, collecting the flow-through into a 15 mL tube for backup.
12. Add 16 mL (four column bed volumes) of 1× elute buffer +0.2% OGP to the top of the column. Collect eluted sample into a new 15 mL tube.
13. Regenerate the column by adding 12 mL (three column bed volumes) 1× strip buffer and let it drain. The resin should change in color from blue to white. Close the stopcock, and add another 2 mL to the top of the column. Replace the cap on the column, and store the column at 4 °C.
14. If the sample contains precipitated material, pass through a 0.2 µm spin filter before moving on.
15. Add the entire sample (~16 mL) to the upper chamber of an Amicon Ultra-15 50 kD concentrating filter and spin at ~1500 × *g*-force until the volume is reduced to ~1 mL. To prevent overspinning, start out conservatively with a 5 min spin and then adjust subsequent spin times accordingly.
16. Discard the flow-through in the lower chamber and add 15 mL PBSA + 0.2% OGP to the upper chamber. Spin down to ~1 mL again.
17. Repeat twice for a total of three buffer exchanges which will reduce the imidazole concentration to well below 1 mM.

18. Transfer sample to a 0.2 μm microfuge spin filter and spin at $>16,000 \times g$ for 1 min to remove any aggregates.
19. Measure the OD280 on spectrophotometer (try a 1:10 dilution first) of the sample, and calculate the pMHCII sample concentration.

concentration = OD280 $\times$ dilution factor/extinction coefficient (final units in mg/mL)

The extinction coefficient for pMHCII is $\sim 1.0 \text{ cm}^{-1}(\text{mg/mL})^{-1}$ (i.e., an OD280 of 1.0 corresponds to a concentration of 1.0 mg/mL).

3.5 Titration of Biotinylated pMHCII

Because the sample still contains a significant amount of impurities and the biotinylation process is not 100% efficient, your concentration based on the OD280 reading will be an overestimate of the true concentration of biotinylated pMHCII. The following titration assay will empirically determine how much of your pMHCII sample should be mixed with streptavidin-fluorochrome conjugates (SA-PE or SA-APC) to create fully armed tetramers (*see Notes 9 and 10*).

1. Prepare 35 μL of pMHCII sample at 200 $\mu\text{g/mL}$ in PBSA.
2. Prepare a twofold dilution series starting with 2 μM SA-PE or SA-APC. Start with 30 μL of 2 μM , and mix 15 μL of this stock with 15 μL of PBSA to make a 30 μL of 1 μM dilution. Repeat to make a 1:2 serial dilution series in PBSA extending down to 125 nM.
3. Add 5 μL of the 200 $\mu\text{g/mL}$ pMHCII stock (1 μg total) to each tube and then 5 μL of each of the SA-PE (or SA-APC) dilutions as shown in Table 1. Add 5 μL PBSA to the first and last samples to bring the volume of all samples to 10 μL .
4. Incubate for 30 min at room temperature.
5. Run the samples on a 4–15% PAGE gel under non-denaturing conditions. Load molecular weight markers or pMHCII from a previous prep in a separate lane for reference.
6. Transfer the proteins to a nitrocellulose membrane.
7. Analyze biotinylated pMHCII by western blotting with a streptavidin probe and enhanced chemiluminescence (ECL) or fluorescence based detection.
8. Lane #1 should show a single strong band at about 80 kD, corresponding to biotinylated pMHCII (Fig. 3). Lane #2, which contains the maximum dose of SA-PE, should present no band because all of the biotinylated pMHCII should be bound to SA-PE. The 80 kD band will eventually reappear as the SA-PE concentration falls and can no longer occupy all of

Table 1
Streptavidin titration of biotinylated pMHC

Tube	Total amount of pMHC	Volume of pMHC to be added	Total amount of SA-PE	Volume of SA-PE to be added
1	1 μ g	5 μ L of 200 μ g/mL	None	0
2	1 μ g	5 μ L of 200 μ g/mL	10 pmol	5 μ L of 2 μ M
3	1 μ g	5 μ L of 200 μ g/mL	5 pmol	5 μ L of 1 μ M
4	1 μ g	5 μ L of 200 μ g/mL	2.5 pmol	5 μ L of 500 nM
5	1 μ g	5 μ L of 200 μ g/mL	1.25 pmol	5 μ L of 250 nM
6	1 μ g	5 μ L of 200 μ g/mL	0.625 pmol	5 μ L of 125 nM
7	None	0	10 pmol	5 μ L of 1 μ M

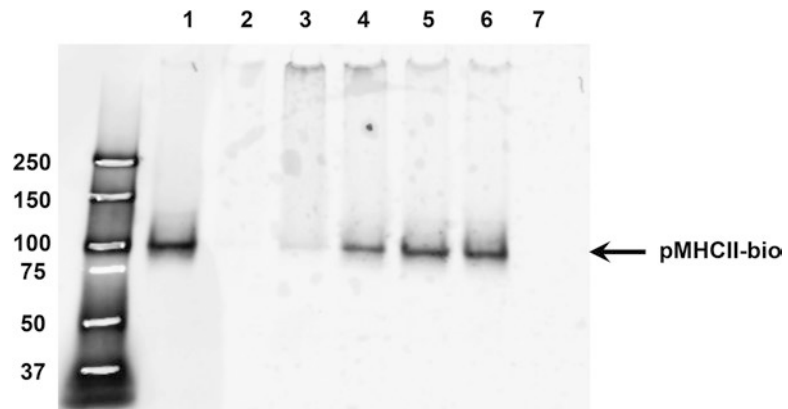


Fig. 3 Representative Western blot of biotinylated pMHC monomer following titration with streptavidin. Titrated mixtures of pMHC monomer and SA-PE from Table 1 were blotted with a streptavidin-HRP probe and visualized by enhanced chemiluminescence. The indicated band represents free biotinylated pMHC that was not saturated by SA-PE

biotinylated pMHCII. The SA-PE amount where the biotinylated pMHCII band just reappears defines the saturation point. If no saturation point is observed, then repeat the assay making adjustments to the amounts of pMHCII loaded in each lane. Be conservative with your assumptions so that you always error on the side of underestimating the true concentration of biotinylated pMHCII.

- To calculate the concentration of biotinylated pMHCII in your sample, first multiply the molar amount of SA-PE in the saturation point by a factor of 4 to determine the molar amount of biotinylated pMHCII that was loaded in that lane. Divide this amount of biotinylated pMHCII by the

5 μL volume in which it was delivered to determine the concentration of biotinylated pMHCII in the “200 $\mu\text{g}/\text{mL}$ ” diluted stock of pMHCII monomer prepared in Subheading 3.5, step 1. Then multiply this concentration by the dilution factor to calculate the concentration of biotinylated pMHCII in the original sample.

3.6 pMHCII Tetramerization

It is important to mix at least 4 moles of biotinylated pMHCII for each mole of SA-PE (or SA-APC) to ensure that the resulting tetramer is fully multimerized. For making calculations, use the concentration of biotinylated pMHCII empirically determined in the previous section (Subheading 3.5) and the concentration of SA-PE specified in the data sheet for each production lot from the manufacturer.

1. Calculate how many moles of tetramer to generate (*see* Notes 11 and 12).
2. Determine the volume of SA-PE that is needed by dividing the desired molar amount of tetramer by the concentration of the SA in the SA-PE.
3. Determine the molar amount of biotinylated pMHCII that is needed by multiplying the desired molar amount of tetramer by 4 and then again by a safety factor of 2 to ensure that there is an excess of pMHCII to SA-PE. To determine the volume of biotinylated pMHCII needed, divide this molar amount by the empirically determined concentration of biotinylated pMHCII from the previous titration step (Subheading 3.5) (*see* Note 13).
4. Mix the calculated volumes of SA-PE and biotinylated pMHCII together, and incubate at room temperature for 30 min.
5. Spin the tetramerized product through a 0.2 μm microfuge spin filter to remove aggregates.
6. Measure the OD566 (try a 1:20 dilution first), and calculate the PE concentration.

concentration = $\text{OD566} \times \text{dilution factor} / \text{extinction coefficient}$ (final units in μM).

The extinction coefficient for PE is $1.96 \text{ cm}^{-1} \mu\text{M}^{-1}$ (i.e., an OD566 of 1.96 corresponds to a concentration of $1.0 \mu\text{M}$). The extinction coefficient for APC at 650 nm is $0.70 \text{ cm}^{-1} \mu\text{M}^{-1}$ (i.e., an OD650 of 0.70 corresponds to a concentration of $1.0 \mu\text{M}$).

7. Ideally, there should be exactly one molecule of SA conjugated to each molecule of PE in the SA-PE stock. However, this stoichiometry is usually slightly off. The data sheet for each lot of SA-PE should specify the individual concentrations of SA and PE. Convert the measured PE concentration of your sample to that of SA by multiplying by the $[\text{SA}]:[\text{PE}]$ ratio obtained

from the data sheet. Because SA constitutes the core of the tetramer, the concentration of SA should be the actual concentration of the tetramer.

8. Dilute the concentration of tetramer to 1.0 μM , and store at 4 °C in the dark. Do not freeze.
9. The final staining concentration for cells is usually 10 nM, but every production lot of tetramer should be titrated in a pull-down experiment on mice immunized with the corresponding antigen (usually peptide + CFA immunized by subcutaneous injection and assessed 7 days after immunization).
10. After determining how much pMHCII is needed to make an ideally sized batch of tetramer, freeze down appropriately sized aliquots of your remaining pMHCII at $-80\text{ }^{\circ}\text{C}$ for future batches.

4 Notes

1. In general, S2 cells are grown at 28 °C without CO_2 . Maintain humidity in the incubator with a tray of water.
2. Optimal medium volumes for tissue culture vessels for S2 cells are:

T25	5 mL
T75	15 mL
T225	50 mL
2 L shaker	1 L
3. A T25 flask of parental cells in 5 mL complete medium should be continuously maintained, split no more than 1:10 every 3–4 days until all planned transfections are completed.
4. Transfected S2 cell cultures can be maintained indefinitely by splitting them 1:10 in complete medium + drug about once per week. S2 cells require a soluble autocrine growth factor for optimal growth, so any split beyond 1:10 will result in a substantial slowdown in growth and possible loss of the culture. Because the cells do not do well at low densities, subcloning is very difficult.
5. Avoid the use of shaker flasks with baffled sides, as these will increase the generation of bubbles and foam which can damage cells.
6. Pluronic F-68 is a surfactant that is added to the shaker or spinner culture to prevent cell shearing. It may also inhibit aggregation of secreted pMHCII in the media.
7. This protocol describes pMHCII purification via nickel column chromatography because this process is easy to set up with commercially available kits and does not pose a high risk

for pMHCII precipitation. However, the purity of the resulting pMHCII is low with large amounts of contaminants from the S2 cell culture. While these contaminants do not significantly impact the function of the final tetramer reagents, they complicate calculations of pMHCII protein concentration and are aesthetically unpleasing due to their visible brown hue. If desired, size exclusion chromatography (S-300 Sepharose or similar) may be used to remove most of these contaminants, which are much larger than pMHCII complexes. Alternatively, immunoaffinity chromatography may be used to achieve much greater purities of pMHCII. We have found that the I-A^b-specific antibody clone Y3P, but not M5/114, works very well for this purpose. However, an associated risk with immunoaffinity purification is that the pMHCII may sometimes precipitate from solution during elution with the low or high pH buffers that are necessary to disrupt antibody-antigen binding. The tendency for pMHCII to precipitate varies for different constructs and can only be reliably predicted by trial and error.

8. The HisBind resin has a binding capacity of about 8 mg protein per mL. 4 ml of resin should therefore be capable of binding 32 mg of protein, which is above the 3–30 mg yields that are typical of 1 L cultures. If a yield of more than 25 mg is achieved, it may be worth running the supernatant through a second time to harvest any protein that was missed due to saturation of the column.
9. The quality of tetramers is highly dependent on the quality of the streptavidin reagents used. We use SA-PE and SA-APC from ProZyme, which have worked very well compared to other vendor sources and come with detailed specifications including relative concentrations of SA and fluorochrome in each production lot.
10. Note that tetramer concentrations will only be as accurate as the listed concentration of SA-PE from the manufacturer, so it is best to titrate your sample to the exact tube of streptavidin that you will be using to tetramerize your pMHCII.
11. The ideal concentration of the final tetramer is 1 μ M, which is used at 1:100 for staining at a final concentration of 10 nM. The testing of most cell samples will therefore only require 1–2 μ L of reagent, so 500 μ L of tetramer would be sufficient for a few hundred tests and would represent a reasonable amount of tetramer to make in a single batch such that most of it is used before getting too old. 500 μ L of tetramer at 1 μ M corresponds to 500 pmol, but because some tetramer is lost during tetramerization, you may wish to start off making somewhere between 500 pmol and 1.0 nmol tetramer.

12. The shelf life of tetramers varies considerably for different MHC and antigens, but a good arbitrary expiration date is 1 year.
13. When tetramerizing pMHCII, always err on the side of excess pMHCII. The affinity of monomeric pMHCII for TCR is several orders of magnitude lower than that of tetramer, so excess free pMHCII in the final reagent is not a concern.

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