

METHODS IN MOLECULAR BIOLOGY™

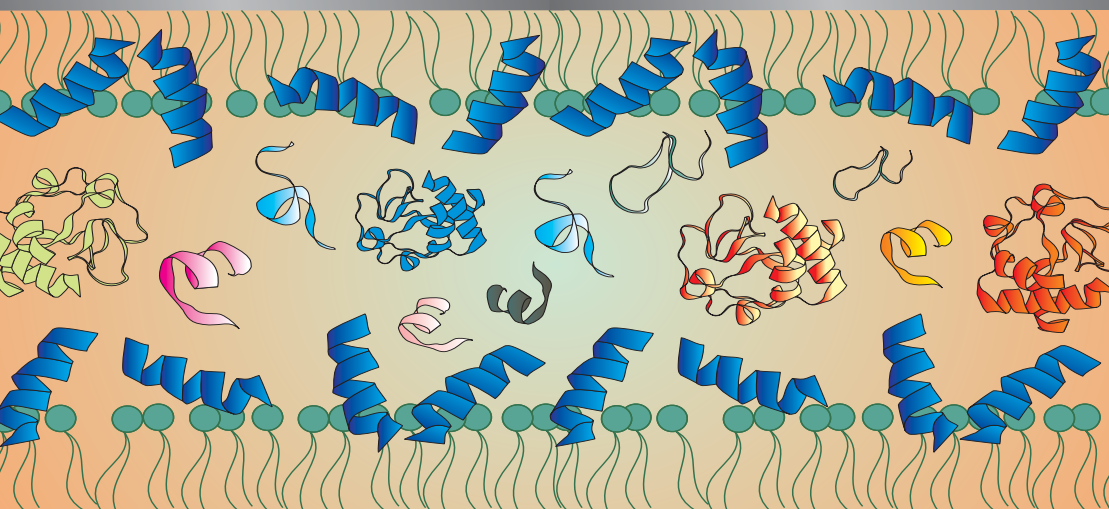
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## **Isolation and Characterization of Naturally Processed MHC-Bound Peptides From the Surface of Antigen-Presenting Cells**

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### **1. Introduction**

The human major histocompatibility complex (MHC) is located on the short arm of chromosome 6 and encompasses approx 4 Mb, or 0.1%, of the genome. This region is, by far, the most polymorphic of the human genome. More than 220 genes have been identified in this region and at least 10% of these genes have a direct function in immune responses. The human MHC can be divided into three regions that encode the class I, class II, and class III human leukocyte antigen (HLA) gene products. These HLA molecules demonstrate tremendous polymorphism, which reflects the natural evolution of these genes in response to various microbial pathogens in different ethnic populations. HLA class I molecules are expressed on all nucleated cells and associate with short peptides (8–11 amino acids in length) derived from both self and foreign antigens. These peptide ligands are primarily generated in or transported into the cytoplasm and subsequently translocated into the endoplasmic reticulum (ER) where they assemble with nascent MHC class I molecules. These mature, peptide-loaded, complexes are then transported to the cell surface where they are scrutinized by CD8<sup>+</sup> cytotoxic T lymphocytes (CTL). Should the peptide ligand be derived from a pathogen and be recognized as foreign in an immunocompetent host, the cell is killed via the cytotoxic armory of the CTL. The expression of HLA class II molecules is confined to a small subset of highly specialized cells called antigen-presenting cells (APCs). The class II molecules associate with longer peptides (9–25 amino acids in length) than class I mole-

cules and this association occurs in late endosomal compartments, a distinct and separate cellular compartment to the ER-Golgi route inhabited by assembling MHC class I molecules. Class II molecules are recognized by CD4<sup>+</sup> T helper cells and functional recognition of these complexes is intimately involved in both the humoral and cellular immune response. MHC class I and class II molecules form membrane-distal structures that comprise a cleft in which the antigenic peptide ligands reside (*1–3*). The T-cell receptor (TCR) on CD4<sup>+</sup> or CD8<sup>+</sup> T cells recognizes MHC molecules in the context of both the class I or class II molecule and the peptide antigen presented in the antigen binding groove of these cell surface molecules (*4*). Technologies that allow the direct isolation and identification of peptide antigens associated with class I or II molecules have highlighted the ligand specificity of different MHC molecules and allowed direct identification of naturally processed and presented antigens derived from infectious micro-organisms as well as self-peptides associated with autoimmune disorders and cancers.

Several different approaches have been used to isolate MHC-bound peptides from cells, these include analysis of acidified cell lysates (*5–7*), elution of peptides from the cell surface (*8,9*), and immunoaffinity purification of the MHC-peptide complexes from detergent solubilized cell lysates (*10,11*). Each approach has advantages, with the latter providing the best chance of epitope identification owing to the additional specificity of the immunoaffinity chromatography step and subsequent simplification of the range of cellular peptides isolated. However, they all share common features: (i) that upon cell lysis, peptides bound to MHC molecules (and other chaperones/receptors) are protected from intracellular and extracellular proteolysis; and (ii) acid treatment dissociates bound peptides from their MHC complexes. In the first approach, peptides are extracted from whole cell lysates following treatment with trifluoroacetic acid (TFA). The presence of TFA also aids in the precipitation of larger proteins leaving a complex mixture of intracellular and extracellular polypeptides, a proportion of which were bound to and protected from proteolysis by MHC molecules. Typically, these preparations are fractionated by reversed-phase high-pressure liquid chromatography (RP-HPLC) and screened with an immunological readout, such a T-cell assay to confirm the presence of a particular T-cell epitope or to quantitate known T-cell epitopes in different cell types (*5–7*). An alternative to this method utilizes a nonlytic approach for recovering cell surface-associated peptides. The cells are washed in an isotonic buffer containing citrate at pH 3.3, the acidic nature of this buffer facilitates dissociation of MHC-bound peptides from the cell surface without affecting cell viability (*8*). The great advantage of this technique is that the same cells may be harvested daily in an iterative approach for obtaining MHC-bound material. Again, although the specificity of this process is a little better for

MHC-bound material than whole cell lysates, some form of biological readout is generally necessary to locate peptides of interest prior to attempts at biochemical characterization.

The use of immunoaffinity chromatography dramatically improves the specificity of the peptide extraction process. The use of appropriate monoclonal antibodies can select a single MHC allele and some antibodies can even select a subpopulation of MHC molecules with defined molecular or functional properties (12,13). The use of immunoaffinity chromatography to isolate specific MHC molecules provides the most appropriate material for identifying individual peptide ligands restricted by a known MHC allele. Furthermore, in all the approaches discussed, the complexity of the eluates/lysates can be reduced by using cell lines that express reduced numbers of HLA alleles (e.g., homozygous lymphoblastoid or mutant cell lines, such as C1R which express very low levels of endogenous class I molecules, but support high-level expression of transfected class I molecules [14]). This property makes these cells very attractive for examining endogenous peptides presented by individual class I alleles under normal physiological conditions (15–18) or during infection (19,20).

This chapter explores methods for the direct isolation of MHC class I or II molecules and focuses on the use of immunoaffinity chromatography and the subsequent separation and identification of the bound peptide antigens.

## 2. Materials

### 2.1. Generation of Cell Lysate by Whole Cell Lysate Method

1. A pellet of  $1\text{--}5 \times 10^8$  cells (may be stored frozen for up to 6 mo at  $-70^\circ\text{C}$ ). The cells should express high levels of the class I or II molecule of interest (see Note 1).
2. Lysis buffer: 0.1% TFA (HPLC grade) v/v in Milli-Q  $\text{H}_2\text{O}$ .

### 2.2. Generation of Cell Lysate by Citrate Shock Method

1. Cells live in culture (either adherent or suspension)  $1\text{--}5 \times 10^8$  cells are sufficient.
2. Citrate shock buffer: 0.131 M citric acid, 0.066 M  $\text{Na}_2\text{HPO}_4$ , 150 mM NaCl, pH 3.3.

### 2.3. Detergent Lysate for Immunoaffinity Chromatography

1. A cell pellet of between  $5 \times 10^9$  and  $5 \times 10^{10}$  cells (may be stored frozen for up to 6 mo at  $-70^\circ\text{C}$ ). The nature of the cell type predicates the nature of the immunoaffinity matrix employed in subsequent purification steps and the ease with which individual peptides may be sequenced (see Notes 1 and 2).
2. Lysis buffer: 0.5% NP-40 (IGEPAL 630 from Sigma (St. Louis, MO) is the equivalent), 50 mM Tris-HCl, pH 8.0 (from a 1 M stock solution composed of Tris-HCl base (121.1 g/L) with 42 mL conc. HCl), 150 mM NaCl, protease inhibitor cocktail (Total Protease inhibitor from Roche or equivalent, should be made up fresh each time), Milli-Q  $\text{H}_2\text{O}$  (make sure this is freshly drawn and is filtered).

## 2.4. Preparation of Immunoaffinity Column

1. Purified monoclonal antibody at 1–10 mg/mL in Protein A loading buffer. Ideally, the mAb should only recognize the class I or II allele of interest, although affinity and specificity issues frequently require a compromise (*see Note 2*).
2. Suitable column (e.g., disposable plastic Econo-Column from Bio-Rad).
3. Protein A loading buffer: 0.05 M borate buffer pH 8.0 (composed of 500 mL of a 0.1 M boric acid/0.1 M KCl stock solution plus 39.7 mL 0.1 N NaOH and 460.3 mL Milli-Q H<sub>2</sub>O).
4. Protein A wash buffer: 0.2 M triethanolamine, pH 8.2 at room temperature (RT) (prepare this solution fresh and pH just prior to use).
5. DMP cross-linker: 40 mM DMP-2HCl (Pierce), 0.2 M Triethanolamine, pH 8.2 (38 mL), pH to 8.3 with NaOH. Bring to 40 mL with 0.2 M triethanolamine pH 8.2 (do not filter).
6. Termination buffer: ice-cold 0.2 M Tris-HCl, pH 8.0.

## 2.5. Immunoaffinity Purification of MHC Class I/Class II Molecules

1. Wash buffer 1: 0.005% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM ethylene diamine tetraacetic acid (EDTA), 100  $\mu$ M phenyl methyl sulfanyl fluoride (PMSF), Sigma (0.1 M stock in absolute ethanol), 1  $\mu$ g/mL Pepstatin A, Sigma (1 mg/mL stock in iso-propanol), in Milli-Q H<sub>2</sub>O.
2. Wash buffer 2: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl in Milli-Q H<sub>2</sub>O.
3. Wash buffer 3: 50 mM Tris-HCl, pH 8.0, 450 mM NaCl in Milli-Q H<sub>2</sub>O.
4. Wash buffer 4: 50 mM Tris-HCl, pH 8.0 in Milli-Q H<sub>2</sub>O.
5. Elution buffer: 10% acetic acid in Milli-Q water (use best grade glacial acetic acid, e.g., Sigma ACS grade).

## 2.6. RP-HPLC Fractionation

1. Buffer A: 0.1% TFA in Milli-Q (filtered with a 0.22- $\mu$ m filter).
2. Buffer B: 0.09% TFA in neat acetonitrile (HPLC grade, filtered with a 0.22- $\mu$ m filter).

## 3. Methods

### 3.1. Generation of Cell Lysate (Whole Cell Lysis)

1. Cells ( $5 \times 10^8$  to  $1 \times 10^9$ ) can be grown in spinner flasks, small bioreactors, or roller bottles to appropriate numbers and harvested by centrifugation (2000g, 10 min at 4°C). If necessary, the cells may be harvested by centrifugations and stored at –80°C for up to 6 mo.
2. Wash cells twice in phosphate-buffered saline (PBS).
3. Lyse cells in 0.1% TFA at a cell density of  $5 \times 10^7$  per mL.
4. Clarify cell lysate by centrifugation and ultrafiltration (e.g., Ultrafree 15 centrifugal filter with a 5-kDa cutoff membrane, Millipore).
5. Concentrate flowthrough (i.e., <5 kDa fraction) by vacuum centrifugation, taking care not to allow the sample to dry completely.

6. Reconstitute in 0.1% TFA (or equivalent and compatible RP-HPLC equilibration buffer) and fractionate by RP-HPLC (*see Subheading 3.6.*).

### 3.2. Generation of Cell Lysate (Citrate Shock)

1. Adherent cells can be treated while still in a tissue-culture flask or attached to microbead/ceramic disk carriers in spinner flask or bioreactor. Suspension cells ( $5 \times 10^8$  to  $1 \times 10^9$ ) can be grown in spinner flasks, small bioreactors, or roller bottles to appropriate numbers and harvested by centrifugation (2000g, 10 min at 4°C) and resuspended directly in citrate shock buffer.
2. Wash cells twice in PBS.
3. Resuspend cells in citrate shock buffer (at approx  $1 \times 10^8$  cells/mL density for cells cultured in suspension or enough to just cover adherent cells (e.g., 2–5 mL for T175 flask, 15 mL for rollerbottle with continued rolling).
4. Incubate cells at RT for up to 15 min (less time if you want to retain maximum viability of the cells, typically around 5 min is sufficient for most cell types).
5. Wash cells twice in PBS and resuspend in fresh culture media. They will be ready for reharvesting in 12–24 h, depending on cell type and duration of the previous citrate shock.
6. Spin harvested citrate wash to remove cellular debris, filter with a 0.22- $\mu$ m filter and then ultrafilter (e.g., Ultrafree 15 centrifugal filter with a 5-kDa cutoff membrane, Millipore).
7. The citrate wash is then desalted by passage over a solid phase extraction column (e.g., C18 Sep-Pak cartridge [Waters]), washed with 0.1% TFA, and bound peptides eluted with 60% acetonitrile/0.09% TFA.
8. The eluate is then dried by vacuum centrifugation, taking care not to totally dry the sample, and reconstituted in 0.1% TFA or equivalent.
9. The reconstituted SPE eluate is then subjected to RP-HPLC fractionation (*see Subheading 3.6.*).

### 3.3. Generation of Cell Lysate (Immunoaffinity Isolation)

1. Cells ( $5 \times 10^9$  to  $1 \times 10^{10}$ ) can be grown in spinner flasks, bioreactors or roller bottles to appropriate numbers and harvested by centrifugation (2000g, 10 min at 4°C). Cells may be harvested iteratively and the washed cell pellet stored frozen at –80°C for up to 6 mo.
2. Prepare a 2X concentrated Lysis buffer.
3. Determine the amount of lysis buffer required. Cells are lysed at  $1.25 \times 10^8$  cells per mL of (1X) lysis buffer.
4. Add correct volume of 2X lysis buffer to the frozen cell pellets and thaw the pellets quickly in a bath of tepid (i.e., RT) water. The temperature of the material should remain cold to touch, so do not let the material equilibrate, thaw until small ice clumps are left, and add ice-cold Milli-Q to a final volume so as the lysis buffer is now at 1X strength.
  - **Tip:** Check volume of cell pellet, if the volume of the cell pellet is close or over 50% of the final volume required, you may need to lyse at a lower cell density.

5. Give mixture a brief homogenize (e.g., using a Polytron Disperser) to disperse any left over ice pellets.
6. Mix the lysate by end-over-end mixing at 4°C for 1 h.
7. Centrifuge lysate for 10 min at 2000g (4°C). This step removes the nuclei.
8. Centrifuge supernatant for 30 min at 38,000g at 4°C—Sorvall high-speed centrifuge, SS34 rotor, 18,000 rpm.
9. Take supernatant from previous step and spin for 1 h 15 m in a Sw28 rotor, Beckman ultracentrifuge 25,000 rpm (100,000g) at 4°C. Multiple spins at **steps 7 and 8** may be necessary to fully clarify the lysate.
10. Collect the supernatant. It should be clear. If there is an unclear layer at top of the tubes carefully remove this layer and filter through a 0.8- $\mu$ m and a 0.45- $\mu$ m filter.

### **3.4. Preparation of Cross-Linked Immunoaffinity Column**

#### **3.4.1. Swelling Resin and Packing Column**

1. Add 3.0 g of protein A-sepharose beads (Pharmacia CL-4B) per 10 mL column bed volume to a 50-mL tube and swell resin in Milli-Q water.
2. Wash twice in Milli-Q and create a 50% slurry.
3. Pour the slurry into column and allow to settle by gravity—check for air bubbles at this stage and agitate the slurry if necessary to remove air bubbles.
4. Equilibrate in Protein A loading buffer.

#### **3.4.2. Adding Antibody to Column**

1. Wash column in 10 column volumes (c.v.) of borate buffer.
2. Load antibody onto column at a flow rate of approx 0.5 mL/min.

#### **3.4.3. Washing the Column of Unbound Material**

1. When all of the antibody has gone through twice, wash the column with at least 20 c.v. of buffer or until the UV<sub>280nm</sub> returns to baseline level.
2. Wash column with 15 c.v. of 0.2 M triethanolamine, pH 8.2 at RT (prepare this solution fresh and pH just prior to use).

#### **3.4.4. Cross-Linking the Sepharose Protein A to the Fc Region of the Monoclonal Antibody**

1. Prepare dimethyl pimelimidate (DMP) cross-linker solution.
2. Save a small aliquot of beads before cross-linking to run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel to check for efficient antibody capture by the protein A Sepharose resin.
3. Pump 5 c.v. of DMP crosslinker through the column at RT leaving a meniscus just over the Sepharose column bed.
4. Seal the bottom of the column and allow to sit at RT for 1 h.



### 3.4.5. Termination of the Cross-Linking Reaction

1. Add 10 c.v. of ice-cold 0.2 M Tris-HCl, pH 8.0.
2. Pump through 10 c.v. of ice cold 0.1 M borate buffer, pH 8.0. (It may be convenient to stop here—Wash and store the column in Borate buffer, pH 8.0 supplemented with 0.02% NaN<sub>3</sub>.)

### 3.5. Immunoaffinity Purification of MHC Class I/Class II

1. Load cell lysate onto a 0.5 mL protein A Sepharose precolumn that has been preequilibrated in wash buffer 1.
2. Multiple columns may be required and should be replaced upon clogging. These steps are done either by gravity or using a peristaltic pump in the cold room.
3. Collect precleared lysate and load onto the mAb column slowly—use peristaltic pump because gravity feed is too quick, at least initially.
4. For maximal yield, the lysate should be run through the columns twice (this may or may not be practicable!).
5. Wash the columns in the following order:
  - Wash buffer 1: 20 column volumes.
  - Wash buffer 2: 20 column volumes (to remove detergent).
  - Wash buffer 3: 20 column volumes (to remove nonspecifically bound material).
  - Wash buffer 4: 20 column volumes (removes salt to prevent crystal formation).
6. Take a 25- $\mu$ L aliquot of column for SDS-PAGE analysis.
7. Elute MHC molecules in 5 c.v. of elution buffer.
8. Dispose of column.
9. As soon as possible, run the eluates over an an centrifugal ultrafiltration device (e.g., Ultrafree 15 centrifugal filter with a 5-kDa cutoff membrane, Millipore) **(the filter should be prewashed with 5 mL of 10% AcOH and the acid flowthrough removed)**.
10. Retain the retentate for SDS-PAGE analysis ( $-70^{\circ}\text{C}$ ).
11. Freeze flow through which contains the eluted peptides at  $-70^{\circ}\text{C}$  (or progress straight on to RP-HPLC fractionation).

### 3.6. RP-HPLC Fractionation

1. Concentrate flowthrough either by: (i) freeze-drying overnight; or by (ii) centrifugal vacuum centrifugation (this is preferable, but take care not to dry the sample too long using this method). Ideally, the sample volume should be reduced up to 95%, but not to complete dryness, because this results in unacceptable sample loss caused by adsorption to the plasticware.
2. Take concentrated filtrate and fractionate on a HPLC system. A minimum of two dimensions of RP-HPLC will be necessary to achieve sufficient separation for biochemical studies, however, a single RP-HPLC step may be sufficient if using an



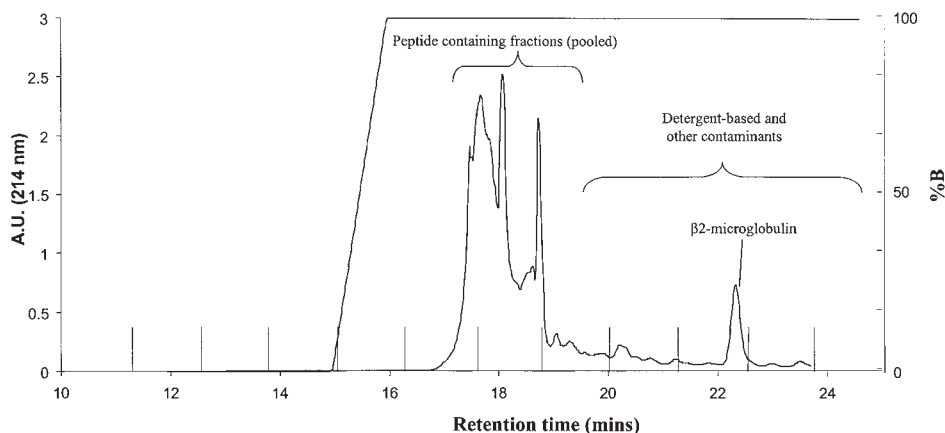


Fig. 1. Rapid isolation of MHC-bound peptides concentrates the eluate and removes contaminants that interfere with MS and cellular-based assays. Purification of HLA molecules from cell lines was performed as described in methods. In this case, human B-LCLs were grown in miniPERM bioreactors (HEREAUS, Hanau, Germany) in EXCEL 610 serum free media (CSL, Melbourne, Australia) supplemented with 1% fetal bovine serum (CSL).  $5 \times 10^9$  cells were lysed at  $4^\circ\text{C}$  in 0.5% Nonidet P-40, 20 mM Tris-HCl, and 150 mM NaCl (pH 7.4) supplemented with complete protease inhibitor cocktail (Roche). Cell lysates were clarified by two rounds of centrifugation, and the supernatant was filtered and passed over a Tris-blocked Sepharose 4B precolumn. The precleared lysates were then applied to columns containing 2 mL of W6/32 affinity matrix, and the columns were washed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 0.005% Nonidet P-40 (pH 8.0). The columns were subsequently washed extensively with 50 mM Tris-HCl and 150 mM NaCl (pH 8.0), a high salt buffer, to remove nonspecifically bound material (50 mM Tris-HCl and 500 mM NaCl [pH 8.0]), and finally, into 50 mM Tris-HCl (pH 8.0). Bound HLA-peptide complexes were eluted with 10% acetic acid, which also facilitates dissociation of the peptide ligands. The eluate was then passed through a Centricon 3 membrane (Millipore), and the flowthrough was concentrated by vacuum centrifugation to a final volume of 300  $\mu\text{L}$ . This ultrafiltered peptide flowthrough ( $<3$  kDa) was fractionated by RP-HPLC using a SMART system HPLC (Pharmacia Biotech, Uppsala, Sweden). Peptides were separated using a  $\mu\text{RPC C2/C18}$  (2.1 mm [id]  $\times$  10-cm column; Pharmacia Biotech, Uppsala, Sweden) and resolved from contaminating detergent polymers by employing a rapid gradient from 0 to 60% acetonitrile in 0.1% aqueous TFA (12%/min, 200 mL/min). This material is suitable for pool Edman sequencing (46) and matrix-assisted laser desorption ionization–time-of-flight/mass spectrometry (MALDI-TOF/MS; as shown in Fig. 3).

immunological readout to assay fractions. For mass spectrometric-based analysis of fractions, use a rapid gradient of buffer A to B, which results in 2–3 peptide-containing fractions (see Fig. 1). Using this approach, a single fraction contains greater than 95% of the peptides, whereas the latter fractions contain

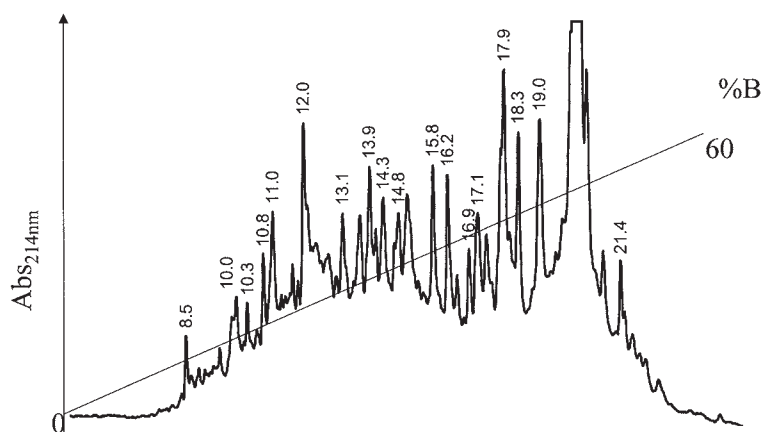


Fig. 2. Optimized RP-HPLC separation of peptide-containing fractions reveals the complexity of HLA-bound peptides and is a portal to their ultimate biochemical and immunological characterization. Peptide-containing fractions from initial RP-HPLC based isolation (*see Fig. 1*) were further purified using an optimized gradient based on the RP-HPLC retention behavior of a number of synthetic analogs of known naturally presented peptide ligands. Peptides were separated using a  $\mu$ RPC C2/C18 (2.1 mm (id)  $\times$  10-cm column and SMART system HPLC (Pharmacia Biotech) and resolved using linear gradient from buffer A (0.1% TFA) to 40% B (acetonitrile/0.09% TFA; 1.3%/min), then 40% B to 60% B (4%/min) at a flow rate of 150  $\mu$ L/min. Fractions (75  $\mu$ L) were retained and analyzed by MALDI-TOF/MS. This material can subsequently be used for MS-based sequence analysis of individual species, screening for T-cell recognition or can be further fractionated using different RP-HPLC modalities and narrow-bore or capillary columns.

NP-40 polymers which hamper MS analysis severely and  $\beta$ 2-microglobulin (class I purification only).

3. Further fractionation can be afforded using optimized RP-HPLC protocols. These can vary from allele to allele and are best modeled using synthetic mixtures of known ligands of the class I or II molecule. An example following a general approach is shown in **Fig. 2**. At this stage, further LC-MS or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) should be attempted.
4. Where a specific ligand is targeted, fractions can be screened by  $^{51}\text{Cr}$ -release, cytokine assays (e.g., ELISPOT) or proliferative responses using TCR systems. The design and execution of such screening assays requires a great deal of immunological expertise and care must be taken to minimize sample toxicity to T cells. Typically, assays involve T-cell line, clone or T-T hybridoma recognition of fraction-pulsed APC. Several excellent recent reports that use these assays are referred to for more detail (21–38).

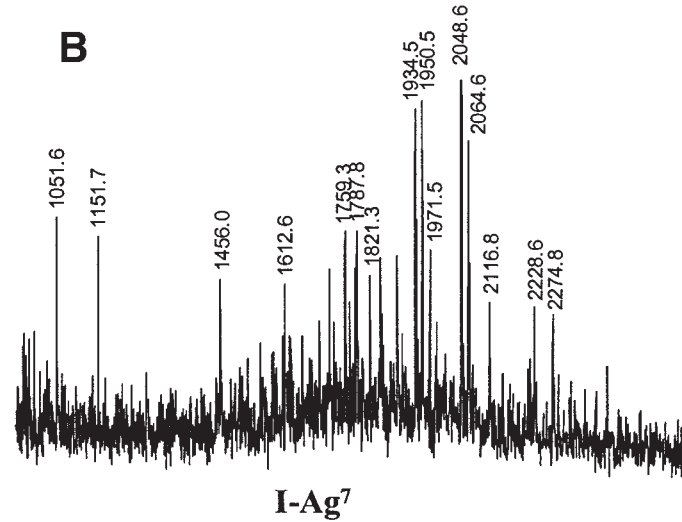
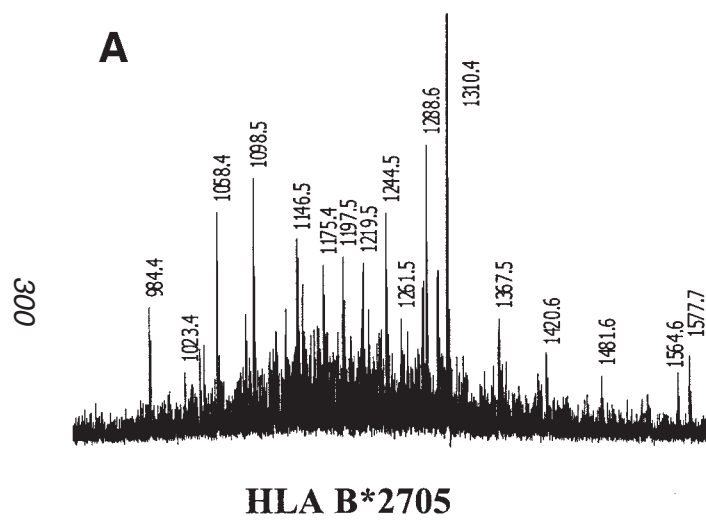


Fig. 3. Examples of biochemical analysis of peptides eluted from MHC class I and II molecules. Peptides eluted from (A) human class I (HLA B\*2705) and (B) murine class II (I-Ag<sup>7</sup>) MHC molecules following one round of RP-HPLC separation and visualized by MALDI-TOF/MS.

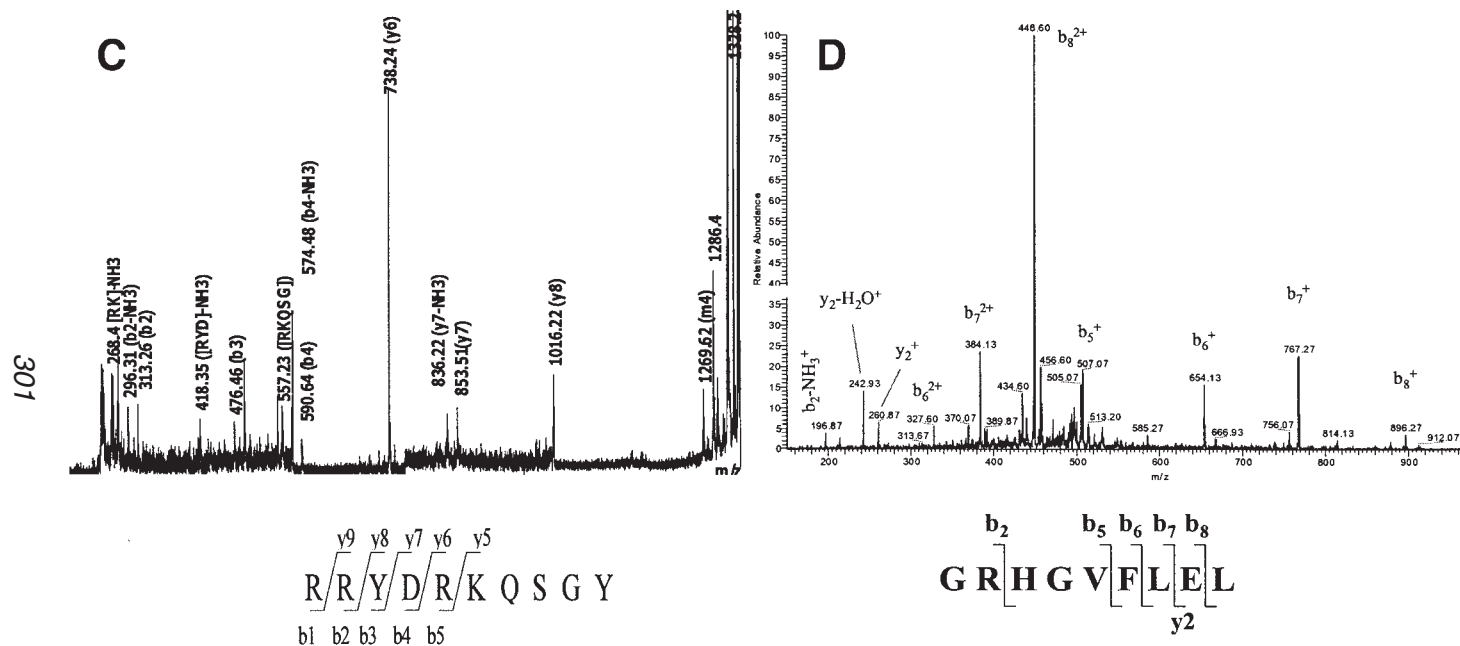


Fig. 3. (C) PSD-MALDI and (D) ESI-ion trap MS<sup>2</sup> based fragmentation and sequence assignment for a B\*2705-restricted self peptides isolated from the surface of human B-lymphoblastoid cells following two dimensions of optimized RP-HPLC separation (40,41).

5. Additional fractionation can be afforded using either narrow bore or capillary HPLC systems preferably with on-line liquid chromatography–tandem mass spectroscopy (LC-MS/MS) capabilities (*see* **Note 3**).

### 3.7. Analysis of Fractions Using MS

Although outside the scope of this chapter, most forms of modern MS can be applied directly to fractionated material produced using these protocols. MALDI-TOF/MS can be performed directly by combining 0.5  $\mu$ L of fraction with 0.5  $\mu$ L of matrix (e.g.,  $\alpha$ -cyano-4-hydroxycinnamic acid, 10 mg/mL in ethanol) to give highly informative insights into fraction complexity and ligand repertoire (**39,40**). Moreover, for instruments fitted with a reflectron and post-source decay capacity, individual ligands may be sequenced as recently reviewed (**41**). The use of electrospray ionization or, more aptly, nanoelectrospray ionization-based instruments can also be applied directly to the analysis of fractionated MHC ligands (**40**). Several of these types of instrumentation are also excellent means to obtain sequence information on individual species, including ion-trap, triple quadrupole, and Qq-TOF-based instruments (**41,42**). Some examples are shown in **Fig. 3** (pp. 300, 301).

## 4. Notes

1. Choice of cell type: In order to maximize the yield of MHC class I or II molecules, the cell line used must be given serious consideration. Epstein Barr virus transformed B cell lines that express high levels of HLA A, B, or C class I molecules or HLA DR, DQ, or DP molecules are easily sourced from depositories such as ATCC, and can be used to great effect in biochemical studies of bound ligands. These B lymphoblastoid cell lines (B-LCLs) grow to high density in cell culture and express high levels of HLA gene products. Homozygous cell lines for most common class I or II alleles are well documented and have been used for years by the tissue typing community. The use of B-LCLs dictates the use of a discriminating antibody should a single allele be required to be purified. In the absence of such an antibody, the ideal cell type for these experiments would express a single MHC molecule and have intact antigen processing and presentation pathways. Several mutant cell lines have been generated that approximate such a cell type. The B-lymphoblastoid cell line Hmy2.C1R was generated by gamma irradiation of LICR.LON.Hmy2 (**43**) and selected with antibodies against HLA A and HLA B alleles and complement. This resulted in a cell line with no detectable HLA A or B gene products, yet with intact antigen processing and presentation pathways (**44**). Thus, these cells are able to support high level expression of individually transfected HLA A, B, or C gene products (**44**). Similar cell lines exist for class II elution studies. For example, the murine cell line M12.C3 lacks endogenous Ia and functional I-A<sup>k</sup> expression can be restored by introduction of I-A<sup>k</sup>  $\alpha$  and  $\beta$  chains via transfection (**45**). It should be noted, however, that not all cells express class II molecules endogenously, thus restricting the array of antigen pre-

senting cells amenable to mutagenesis for the creation of appropriate cell lines. It is for this reason that B-LCLs homozygous at the DR, DQ, and DP locus are used in studies of human class II ligands whereas splenocytes and lymphomas are typically used in murine studies.

2. Choice of monoclonal antibody: Closely allied to the choice of cell line, and its impact upon the complexity of the total pool of bound peptide ligands, is the specificity and efficacy of the monoclonal antibody/antibodies used in the immunoaffinity isolation of MHC molecules. Monoclonal antibodies with specificity toward classes of MHC molecule, families of MHC molecule, individual alleles of MHC molecules, and even subsets of molecules of an individual allotype have been generated over the years and are hybridomas are readily accessible commercially through bodies such as the ATCC ([www.atcc.org](http://www.atcc.org)).
3. Column and mobile phase choice for multidimensional RP-HPLC are dictated by sample composition, but considerations should include altered ion pair agent, altered mobile phase pH, altered stationary phase ligand or mode of chromatography. A detailed discussion of these considerations can be found in Chapters 1, 2, and 12.

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