Application Note

Characterization of Peptides Associated with Molecules of the Major Histocompatibility Complex

Immunopeptidome Profiling Services

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Key Features

- An optimized workflow for the sensitive and specific analysis of MHC-I peptides, using HCT116 as a case study, is presented.
- Analysis of the enriched peptides by LC-MS/MS resulted in approximately 2,500 unique 8- to 15-mer peptides.
- A greater than 60% enrichment of 9-mer peptides was achieved.
- The peptide sequence data from the observed 9-mer peptides can be probed further to confirm or predict HLA allele information by motif analysis.

Introduction

The major histocompatibility complex (MHC) is a region of highly polymorphic genes encoding for glycoproteins (MHC molecules) that form part of the cell-mediated branch of the acquired immune system. MHCs (HLAs in human) are expressed on the cell surface of all nucleated cells and act as the machinery for antigen presentation to T cells in the acquired immune system. In the cytosol, cellular self and foreign (non-self) proteins are constantly being degraded, and the resultant proteolytic peptides are loaded into the peptide binding cleft of the MHC. The MHC functions to display these peptides (antigens and neoantigens) on the cell surface for inspection by T lymphocytes ($CD8^+$ cytotoxic T lymphocytes or CTLs for MHC Class I and $CD4^+$ helper T lymphocytes for MHC Class II). Recognition of foreign antigens by T cell receptors triggers an immediate T cell activation and expansion, resulting in the destruction of the presenting cell by the CTL.

Characterizing the antigens involved in this process is paramount to understanding the immunogenicity of proteins and generating tools for targeted cell destruction (*e.g.*, immunotherapy for targeting tumors). The molecular level characterization of peptides associated with MHC-I and -II requires a targeted protein complex enrichment by immunoprecipitation (IP), an unbiased peptide elution, and peptide analysis by mass spectrometry (MS). Most frequently, immunoprecipitation is used to isolate the target complex followed by peptide elution performed under conditions minimizing protein contamination. Lastly, peptide analysis is accomplished by mass spectrometry. Using the human colorectal carcinoma cell line HCT116 as a case study, we present an optimized peptide enrichment methodology, state-of-the-art MS, and data processing for the sensitive and specific analysis of MHC-I peptides (**Figure 1**).

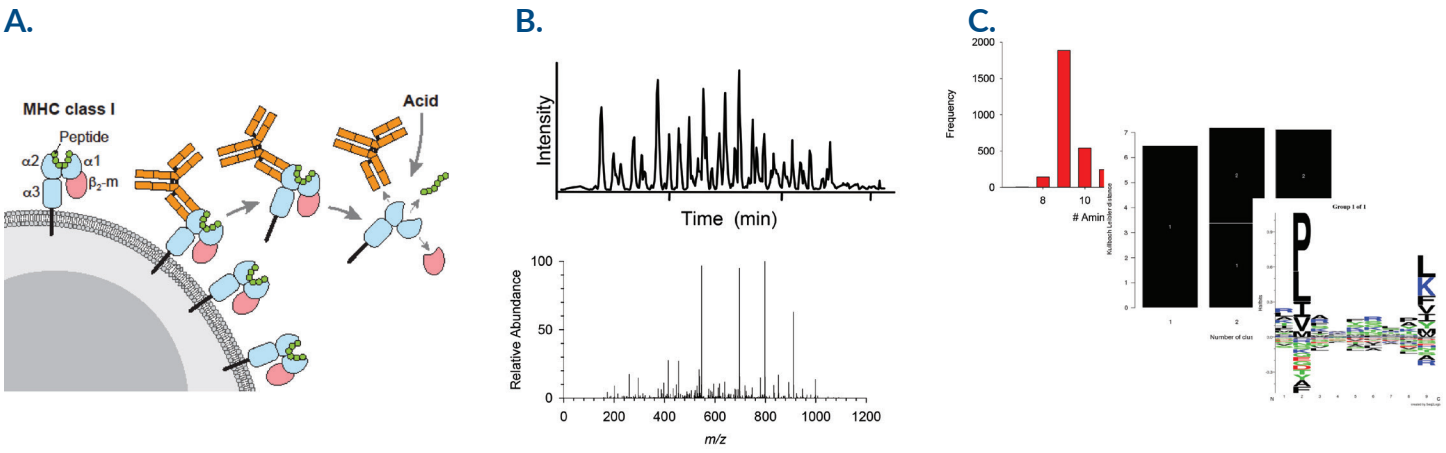

Figure 1. Workflow: A) Cells are lysed and the resulting preparations are subjected to immunoaffinity capture. Following multiple washes, peptides are eluted from the MHC cleft with acid. **B)** Purified peptides are analyzed by LC-MS/MS using nano-scale chromatography combined with Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer using electron-transfer/higher-energy collision dissociation (EThcD) fragmentation.[1] MaxQuant is used for peptide identification and quantitation.[2] **C)** Processed data can be further interrogated using bioinformatics tools. For example, NetMHC or GibbsCluster allow the elucidation of binding strengths and motifs.[3-5]

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Materials and Methods

Cell Culture

HCT116 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1x penicillin/streptomycin. Once the cells reached 80% confluency, they were further passaged 1:5 in HYPERflasks® or harvested by Accutase® treatment. After washing twice in PBS, harvested cell pellets with defined density were stored at -80°C.

HLA-I Complexes Enrichment

HCT116 cells were lysed by homogenization with a Polytron PT2100 benchtop homogenizer at 4°C with 0.25% deoxycholic acid, 1% octylthioglucoside, 1 mM EDTA, 0.2 mM iodoacetamide, and Roche HALT in PBS. The lysate was cleared by centrifugation at 20,000 × g for 20 minutes at 4°C. The HLA-I complex was isolated with MHC Class I Monoclonal Antibody (Clone W6/32) (Cayman Chemical, Item No. 20898) bound to Protein A-Sepharose® 4B beads (Invitrogen). SDS-PAGE was employed to confirm crosslinking. The antibody/bead matrix was washed thoroughly prior to use. The HLA-I complex was isolated from the cleared lysate by overnight incubation at 4°C.

HLA-I Peptide Enrichment

The capture matrix was washed thoroughly with 20 mM Tris-HCl, 150 mM NaCl, and the MHC-I complex was eluted with multiple additions of 5% acetic acid. HLA-I complex and peptides were visualized by SDS-PAGE following elution. The eluent was transferred to a 3 kDa spin filter pre-blocked with angiotensin and centrifuged at 14,000 x g for 20 minutes. HLA-I complex-presented peptides will pass through the spin filter membrane, while the HLA-I complex remains trapped above the membrane.

Liquid Chromatography and Mass Spectrometry (LC-MS/MS)

Isolated peptides were desalted by Stage-Tip and analyzed by nano LC-MS/MS with a Thermo Fisher Scientific nLC-1200 HPLC system interfaced to a Thermo Scientific™ Orbitrap Fusion™ Lumos™ mass spectrometer operating in EThcD mode. Peptides were loaded on a trapping column and eluted over a 75 µm x 50 cm analytical column (Thermo Fisher P/N ES-803) at 300 nl/min using a 2 hour reverse phase gradient; both columns were packed with PepMap RSLC C18, 2 µm resin (Thermo Scientific). The mass spectrometer was operated in data-dependent mode, with the Orbitrap™ operating at 60,000 and 17,500 FWHM for MS and MS/MS, respectively. The instrument was run with a 3 second cycle for MS and MS/MS.

MS Data Analysis

Data were processed with MaxQuant version 1.5.3.17 (Max-Planck Institute for Biochemistry) operating on a dedicated 64 core server with 128GB of RAM. MaxQuant employs the Andromeda search engine. Data were searched against the UniProt Human reference proteome with no-specific enzyme specificity and N-terminal acetylation and methionine oxidation as variable modifications. The second peptide identification option in Andromeda was enabled. A false discovery rate of 1% was required for peptides. A protein false discovery rate was not set.

Results

Due to the low abundance of MHC Class I molecules and its associated peptides, selective enrichment is required prior to the analysis of the MHC Class I peptidome by MS (**Figure 2**). Once the complex is enriched, the MHC-associated peptides can be selectively isolated from the complex for further analysis (**Figure 3**). To address the sensitivity challenge, the selective immuno-enrichment was performed on a preparative scale. To achieve deep coverage of the Class I peptidome, we used 1 x 10^9 HCT116 cells as the input and 5 mg/ml antibody load on the resin. This approach achieved peptide yields on the order of 5 µg total peptide. The sensitivity we obtained was also enabled by the application of EThcD.[2] EThcD is a fragmentation method that generates dual fragment ion series, enabling extensive peptide backbone fragmentation. We found this method of peptide fragmentation generated a 1.5-fold increase in the total peptide yield (**Figure 4**).

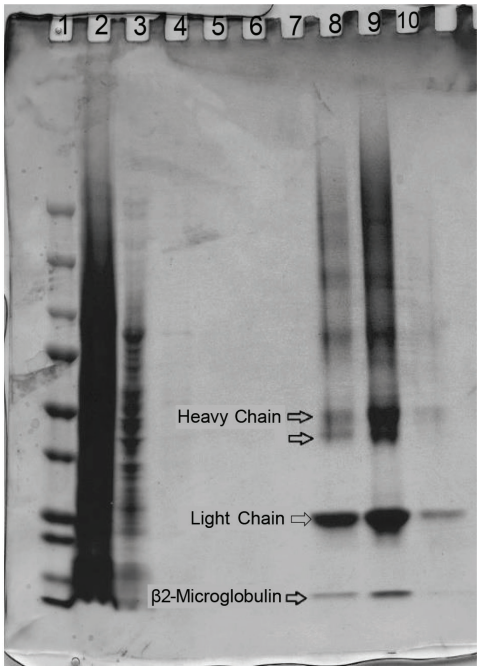

Figure 2. Representative SDS-PAGE of MHC-I Preparations: 1) MW Markers, **2)** Lysate, **3)** Flow Thru, **4-7)** Washes, **8)** Eluate, **9)** 3 kDa MWCO Retentate, **10)** 3 kDa MWCO Filtrate

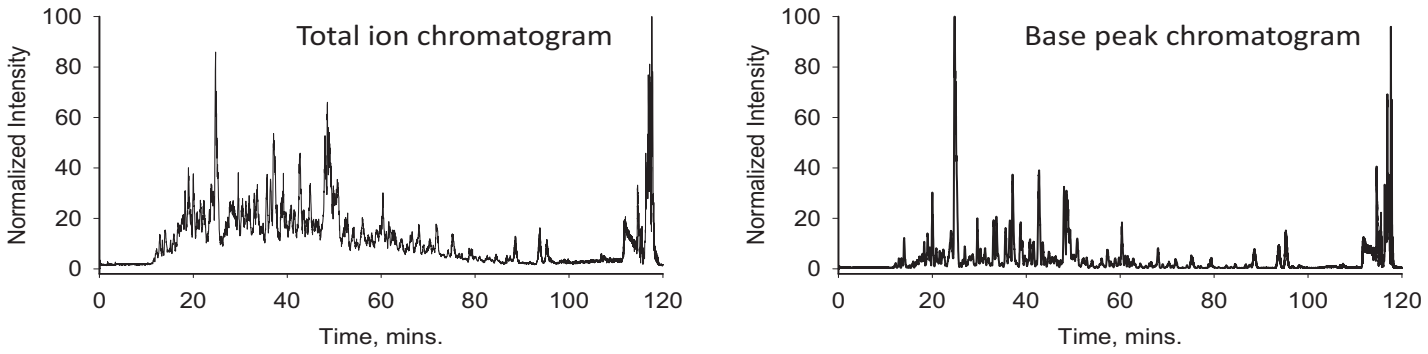

Figure 3. LC-MS/MS: Representative LC-MS/MS chromatograms from 50% load of 500 M cell enrichment.

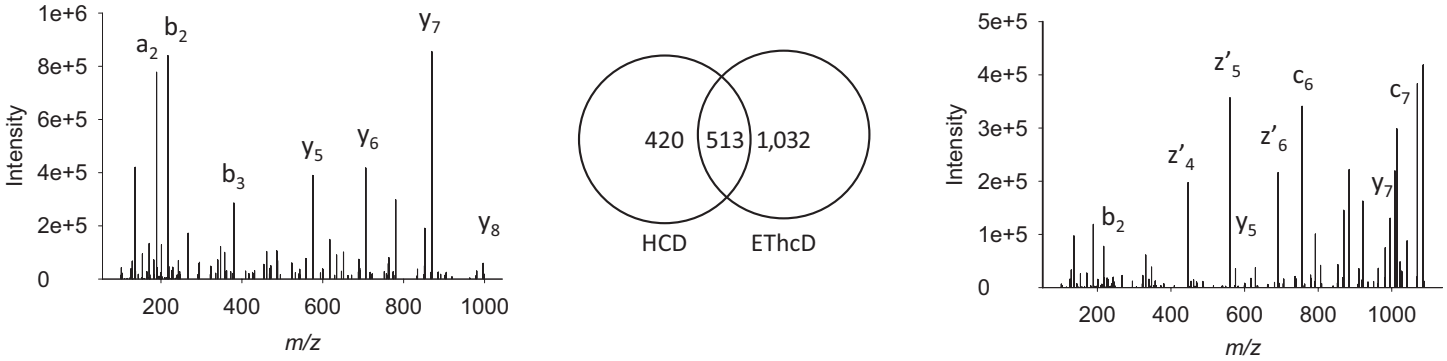

Figure 4. HCD *versus* EThcD: HCD (note b and y ions) and EThcD (note b, y, c, and z ions) spectra of the peptide SEYMNN-KEA. The Venn diagram shows the number of unique peptides acquired from a single sample using both approaches.

To better understand the HLA-I peptide yield and improve practical application of this assay, we varied the input amount while holding the antibody concentration constant (20 mg/ml) and varied the antibody concentration while holding the input constant (500 M cells). We considered the antibody concentration and its impact on the HLA-I peptide yield as potential to reduce costs while improving the practical application of this assay (**Figure 5**). After settling on the appropriate antibody to protein/cell number ratio, we checked the reproducibility of the assay. Figure 6 shows the comparisons of the HCT116 MHC Class I peptidomes obtained from the duplicate analysis of an enriched sample. The Pearson correlation coefficient of 0.98 obtained from the comparison of duplicate injections reflects the high degree of analytical reproducibility obtained with this optimized workflow.

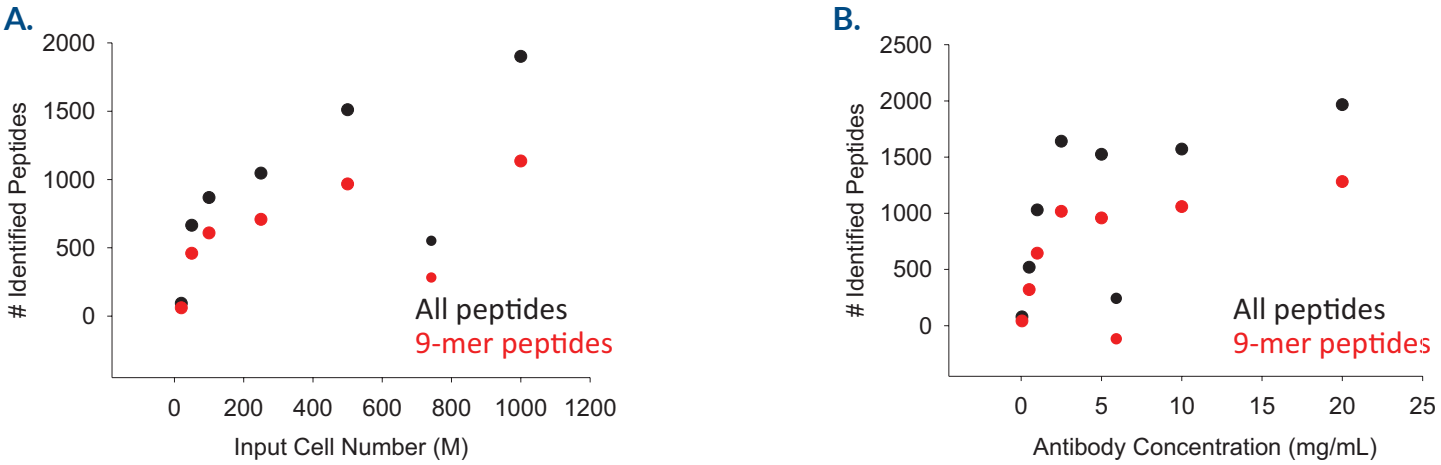

Figure 5. Varied Cell Input and Antibody. A) Varying input cell number with a fixed antibody concentration; **B)** Varying antibody concentration with a fixed input cell number.

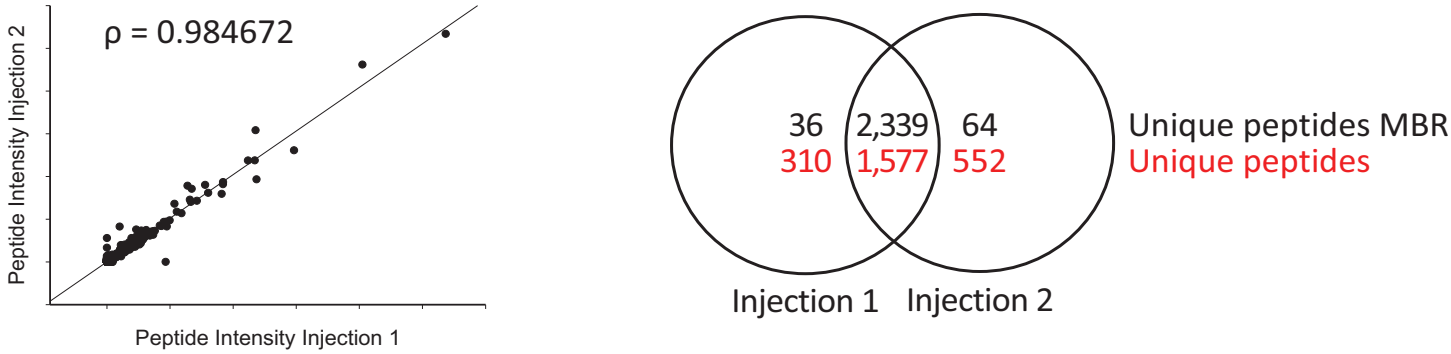

Figure 6. Analytical Replicates. A single sample (500 M cells) was injected twice. The data were processed with and without the MaxQuant Match Between Runs (MBR) feature enabled. The Pearson correlation was calculated with the MBR data.

Utilizing the optimized workflow, the analysis of the enriched MHC-I associated peptides by LC-MS/MS resulted in approximately 2,000 unique 8-15-mer peptides per sample. Using features unique to the MaxQuant data processing platform, this increased to approximately 2,500 peptides per analysis. The scatter plots in Figure 6 shows data for all the observed peptides and exclusively the 9-mers. Figure 7 shows that this workflow resulted in a greater than 60% enrichment of the 9-mer peptide population. The peptide sequence data from this 9-mer population can be used to confirm or predict HLA allele information by motif analysis (Figure 8) and assess binding strength.

The ability to fully characterize the immunopeptidome will help to better understand the immune system and to drive the development of next-generation vaccines and immunotherapies.

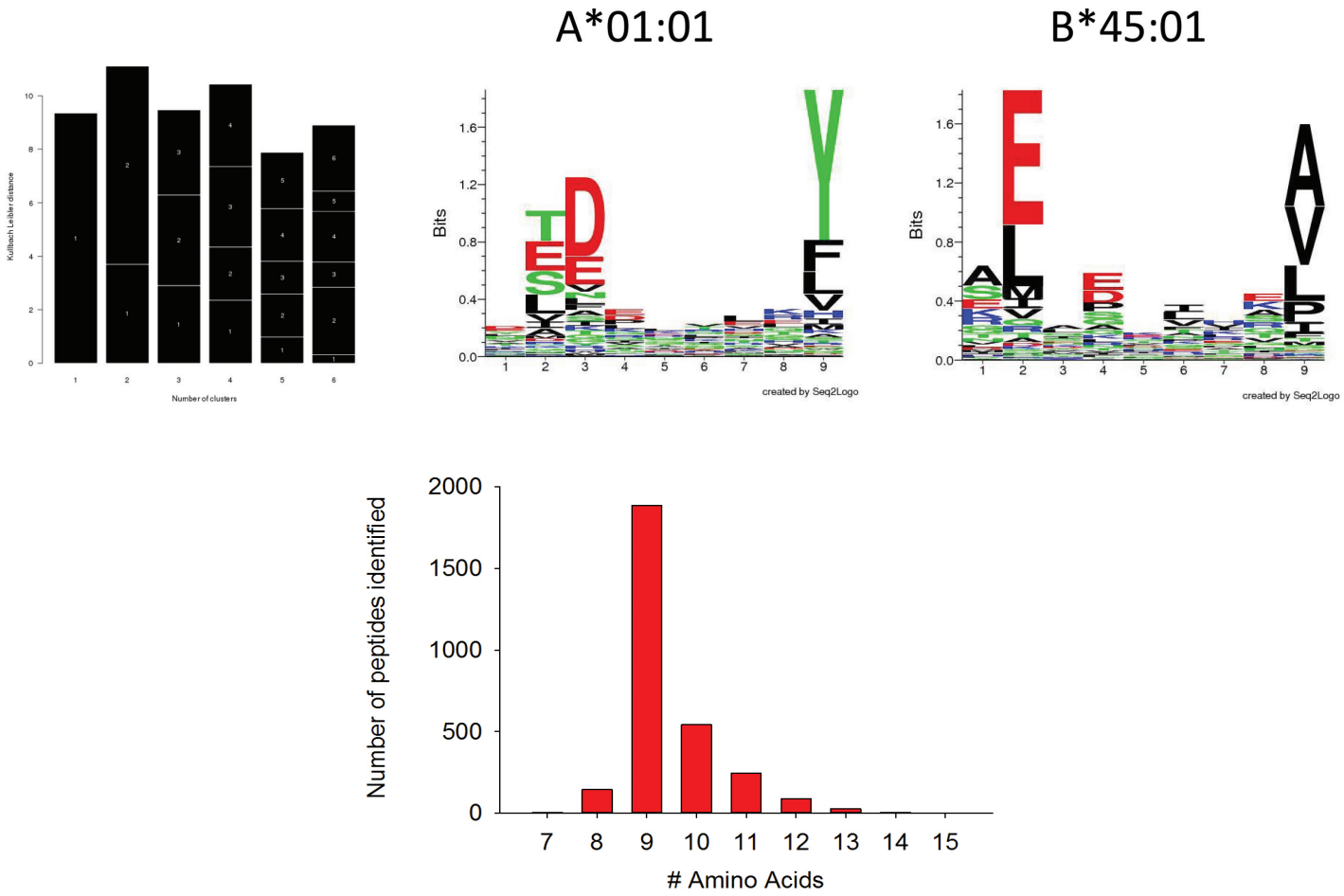

Figure 7. Analytical Replicates. Histogram of the peptide length (# Amino Acids) against the number of peptides from the processed LC-MS/MS data shows enrichment towards 9-mer peptides.

References

1. Cox, J. and Mann, M. *Nat. Biotech.* **26(12)**, 1367-1372 (2008).
2. Swaney, D.L., McAlister, G.C., Wirtala, M., *et al. Anal. Chem.* **79(2)**, 477-485 (2007).
3. Andreatta, M. and Nielsen, M. *Bioinformatics* **32(4)**, 511-517 (2016).
4. Nielsen, M., Lundegaard, C., Worning, P., *et al. Protein Sci.* **12(5)**, 1007-1017 (2003).
5. Andreatta, M., Lund, O., and Nielsen, M. *Bioinformatics* **29(1)**, 8-14 (2013).

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