Poster Reprint

ASMS 2020
ThP 412

Development of an automated MHC-associated peptide enrichment method for immunopeptidomics analysis using AssayMAP large capacity cartridges

Samuel Pollock[1], Shuai Wu[2], Jerry Han[2], Steve Murphy[2]

[1] Genentech, South San Francisco, CA, USA

[2] Agilent Technologies Inc., Santa Clara, CA, USA

Introduction

Immunopeptidomics is generally considered more challenging than conventional proteomics workflows for a number of reasons: First, the MHC-associated peptides are extremely low in abundance compared to other cellular peptides (or proteins), which makes their enrichment and detection very difficult. Second, the mechanism of generating the mature peptide-MHC complex is unclear as it involves multiple proteases and peptidases. The peptides that bind with MHC complex have similarity in terms of length and sequence which are different from proteolytic digested peptides.

In this workflow, we used the AssayMAP Bravo for automated immunoaffinity purification and peptide clean-up that provided users with a high throughput and reproducible method for MHC peptidomics.

Experimental

Antibody Cross-link with PAW Cartridge

1 mg of anti-human MHC-I antibody (w6/32) was loaded on new AssayMAP 25 µL PAW cartridges in parallel (x6) using the Affinity Purification application (Figure 1). Dimethyl pimelimidate (DMP) was used to cross-link the antibody to protein A, before being washed away with TBS. The just-crosslinked cartridges were then washed with 1% acetic acid to remove unbound antibody, equilibrated in TBS, and stored at 4 °C until use.

Immunoaffinity Purification of MHC-I Complex

GRANTA-519 cell pellets were lysed in non-denaturing buffer as previously described. The MHC-I complexes were immunoprecipitated with the antibody cross-linked cartridges. About 3 mg GRANTA lysate was loaded on each of 6 cartridges and the MHC complex was enriched out of the lysate and combined to give sample 1. The same experiment was repeated on different days to give samples 2 and 3. The MHC-associated peptides were separated from MHC protein and desalted on C18 cartridges using Peptide Cleanup Application. The AssayMAP protocols are summarized in Table 1.

Figure 1. AssayMAP Bravo platform with new AssayMAP 25 µL PAW cartridge (Right)

Experimental

LC/MS Analysis

The Agilent 1290 Infinity II LC system was converted to nanoflow LC with the Agilent Infinity UHPLC Nanodapter. This nanoflow LC was connected to the Agilent nanoESI source and coupled to the 6550 iFunnel Q-TOF (Figure 2).

Peptide samples were analyzed with a 90-min gradient using data-dependent acquisition (Table 2). The tandem MS results were analyzed with Byonic software using human UniProt database with no enzyme specificity. Methionine oxidation, deamidation, were used as variable modifications for database search.

Table 2. Nano-LC Parameters

LC Conditions				
Trap-Column	PepMap C18, 75 µm x 2 cm, at 60 °C			
Analytical Column	PepMap C18, 75 µm x 25 cm, at 60 °C			
Solvent A	0.1% Formic Acid in Water			
Solvent B	0.1% Formic Acid in 90 % Acetonitrile			
Flow rate	0.085 mL/min primary flow			
	300 nL/min on-column flow rate			
Gradient	Time (min)	B (%)	Time (min)	B (%)
	0	3	97	70
	90	35	100	3
	95	70	120	3
Injection volume	5 µL			

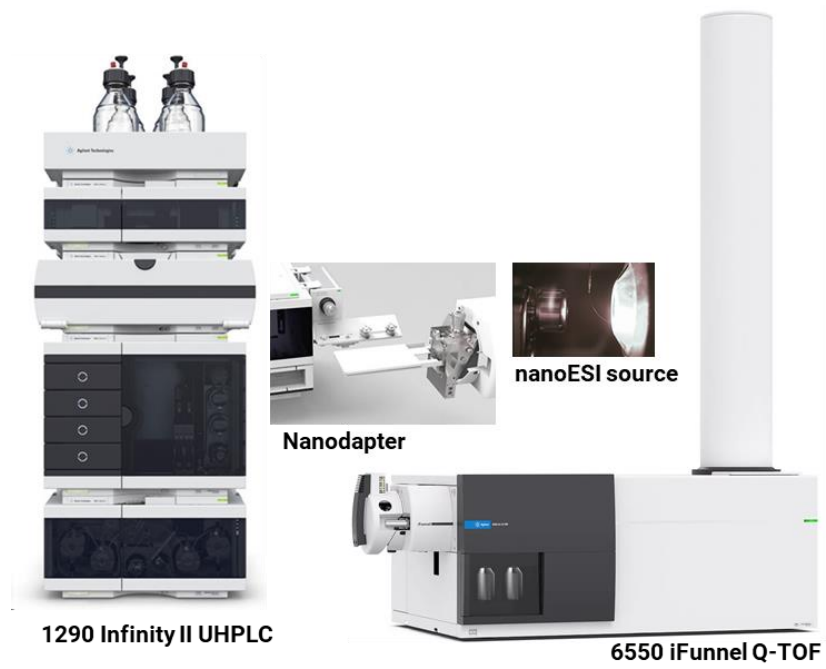

Figure 2. Nanodapter converts standard flow LC to nanoflow LC coupled with Q-TOF

Table 1. AssayMAP Bravo protocols

	Affinity Purification	Cross-linking	Immunoaffinity purification	Peptide Clean-up
Resin volume	25 µL	25 µL	25 µL	5 µL
Affinity Medium	Protein A	Protein A + antibody	Protein A +Xlinked antibody	C18
Prime buffer	PBS, pH=7.4	0.2 M triethanolamine, pH=8.1	TBS, pH=7.4	70% ACN, 0.1% TFA in water
Equilibration buffer	PBS, pH=7.4	0.2 M triethanolamine, pH=8.1	TBS, pH=7.4	2% ACN, 0.1% TFA in water
Loading buffer	Antibody storage buffer	5mM DMP in 0.2M TEA	3 mg/mL GRANTA lysate	1% Acetic Acid
Loading volume	1000 µL	250 µL	1000 µL	100 µL
Loading flow rate	20 µL/min	10 µL/min	20 µL/min	5 µL/min
Washing buffer 1	PBS, pH=7.4	TBS, pH=7.4	TBS, pH=7.4	2% ACN, 0.1% TFA in water
Washing volume 1	250 µL	250 µL	250 µL	50 µL
# washes 1	1	3	3	1
Washing buffer 2	NA	1% Acetic Acid	25mM Tris, pH=8.0	NA
Washing volume 2	NA	100 µL	250 µL	NA
# washes 2	NA	3	3	1
Elution buffer	NA	TBS, pH=7.4	1% Acetic Acid	30% ACN, 0.1% TFA in water
Elution volume	NA	250 µL	100 µL	100 µL

MHC Peptide Identification and Quantitation

Figure 3 is the total ion chromatogram (TIC) of the three samples using a 90-minute gradient. The TIC shows reproducible retention time and peak abundance between the samples. The tandem MS data were analyzed by Byos workflow with Byonic for peptide identification and Byologic for peptide quantitation (Figure 4). In Byonic, both singly and multiply charged ions were considered as precursor ions. A manual score cut, 150, was used for filtering identified peptides.

The identified peptides were imported into Byologic with their sequences. Byologic extracted each identified peptide with its peak area and further filtered peptides with certain number of decoys defined by user. The final peptide IDs are summarized in Figure 5. The unique MHC class I peptides identified in each sample ranged from 2282 to 2424 with a CV% at about 3.0%. The number of unique peptides identified across all three samples is 3604.

The total peptide abundances in each sample are summarized in Figure 6 with a CV% at about 11.1%. Considering multi-steps were used including GRANTA lysate loading on PAW cartridges at different days with a following C18 cleanup, the CV% calculated from the three samples are within a good range, which showed a good reproducibility from the automated sample preparation.

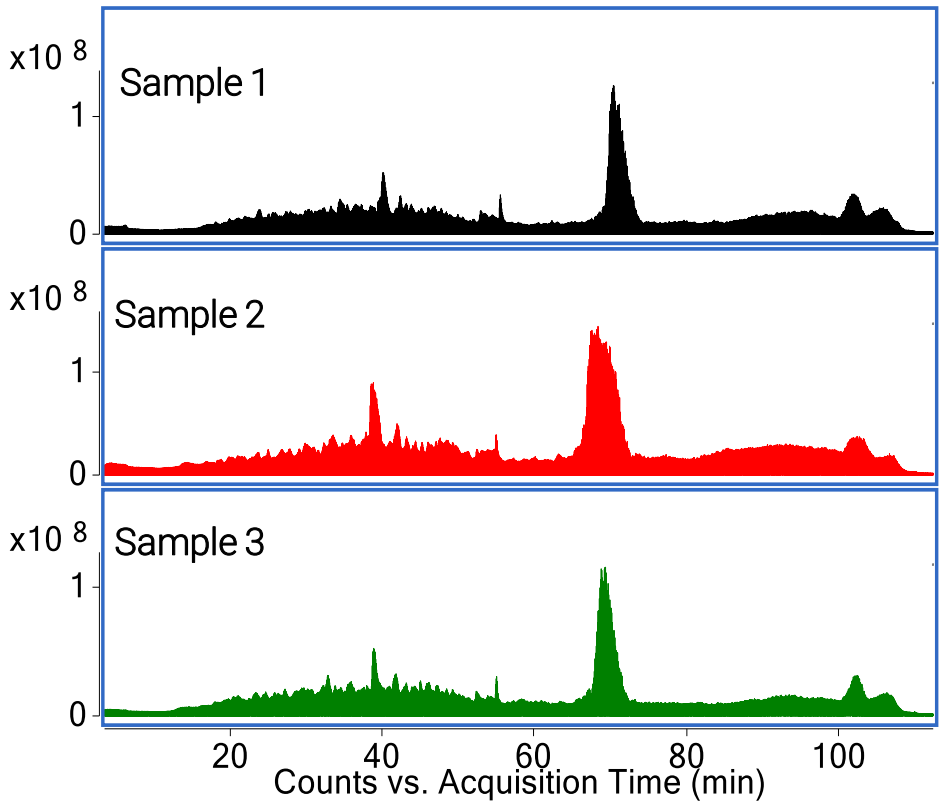

Figure 3. TIC of MHC peptides using 90 minute gradient

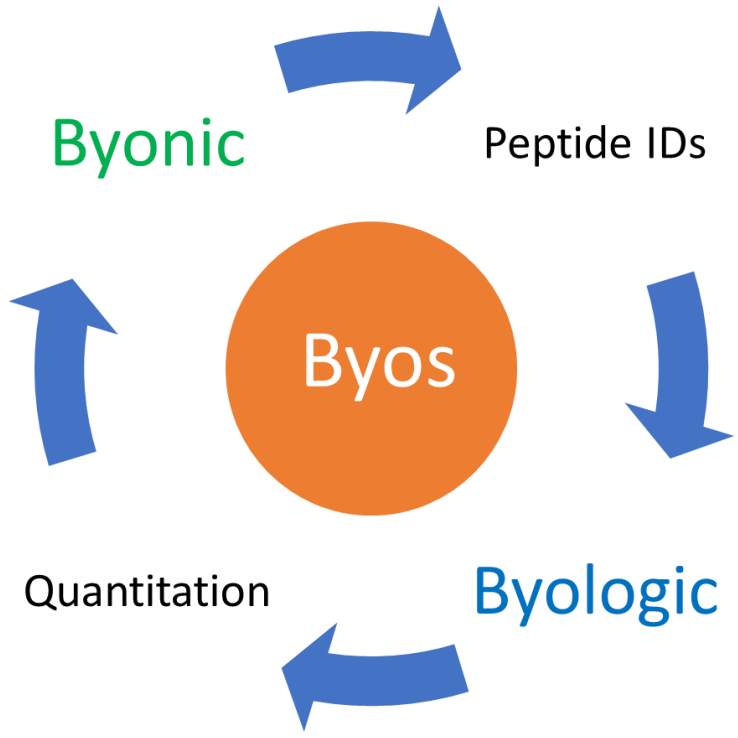

Figure 4. Byos workflow for peptide analysis

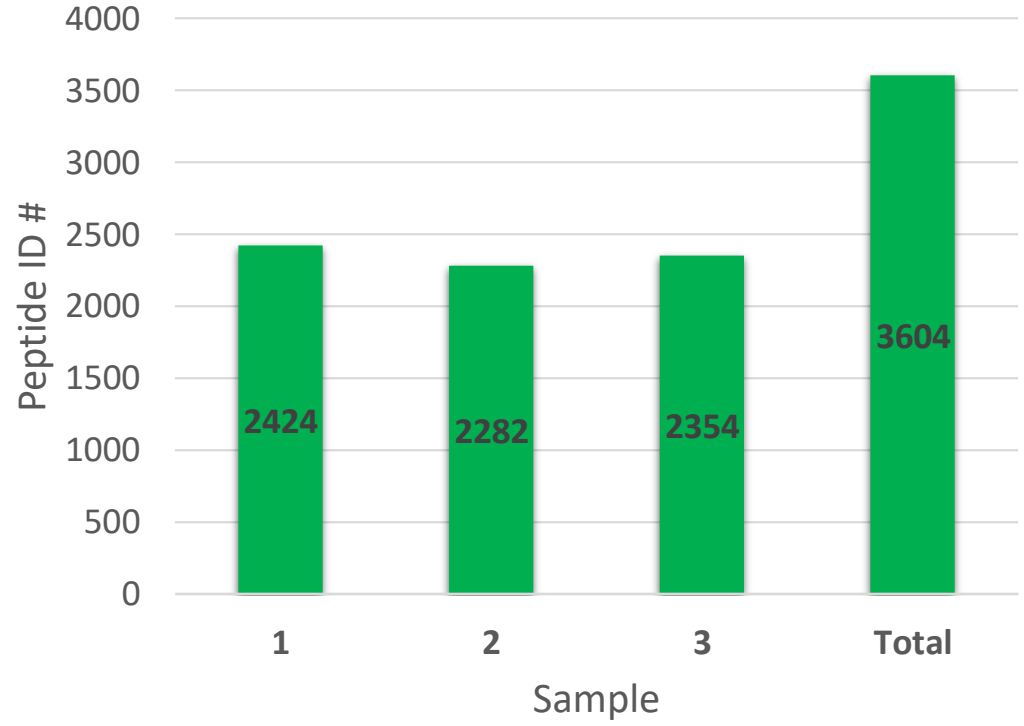

Figure 5. The unique peptide number identified using 6550 iFunnel Q-TOF LC/MS

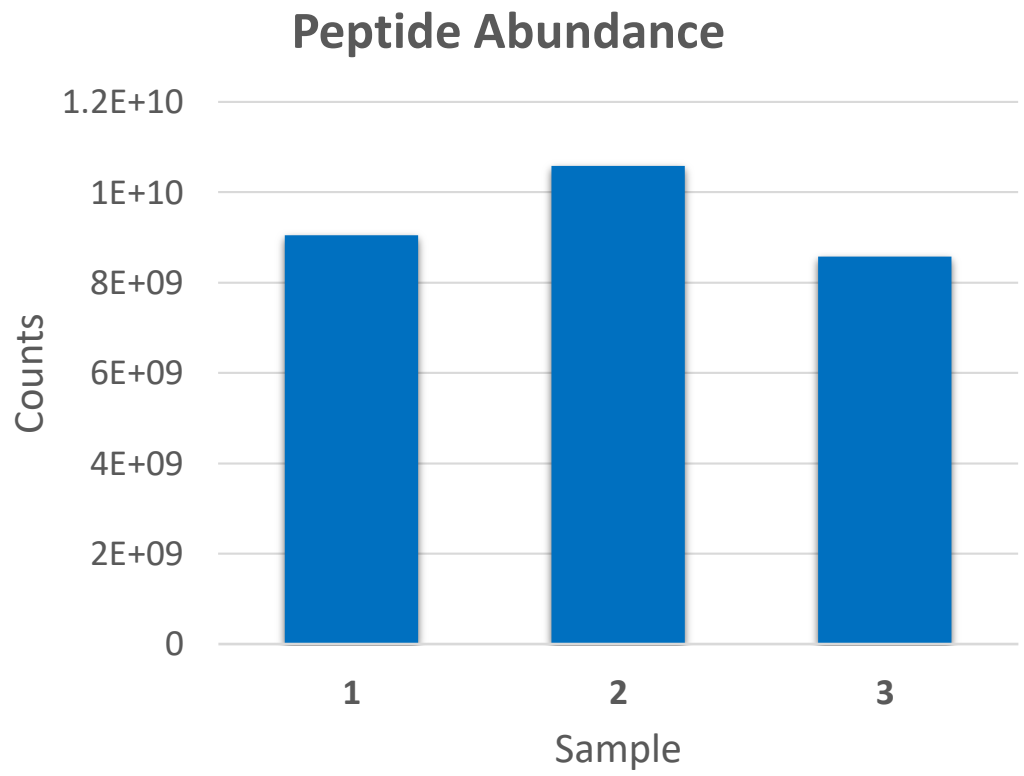

Figure 6. MHC Class I peptide abundance for each sample with CV% at about 11.1%

Results and Discussion

Peptide Length Distribution

The frequency distribution of peptide length is plotted in Figure 7 to further confirm the identification of MHC Class I peptides. Data shown in figure 7 are compiled from the overall peptide identified after Byos workflow analysis. The 3604 unique peptides (Figure 5) spanned peptide lengths from 3 to 17 residues. However, the vast majority of peptides (94%) were 8 to 11 residues long, with most (75%) at 9 residues. This is well in line with what has been reported in literature.

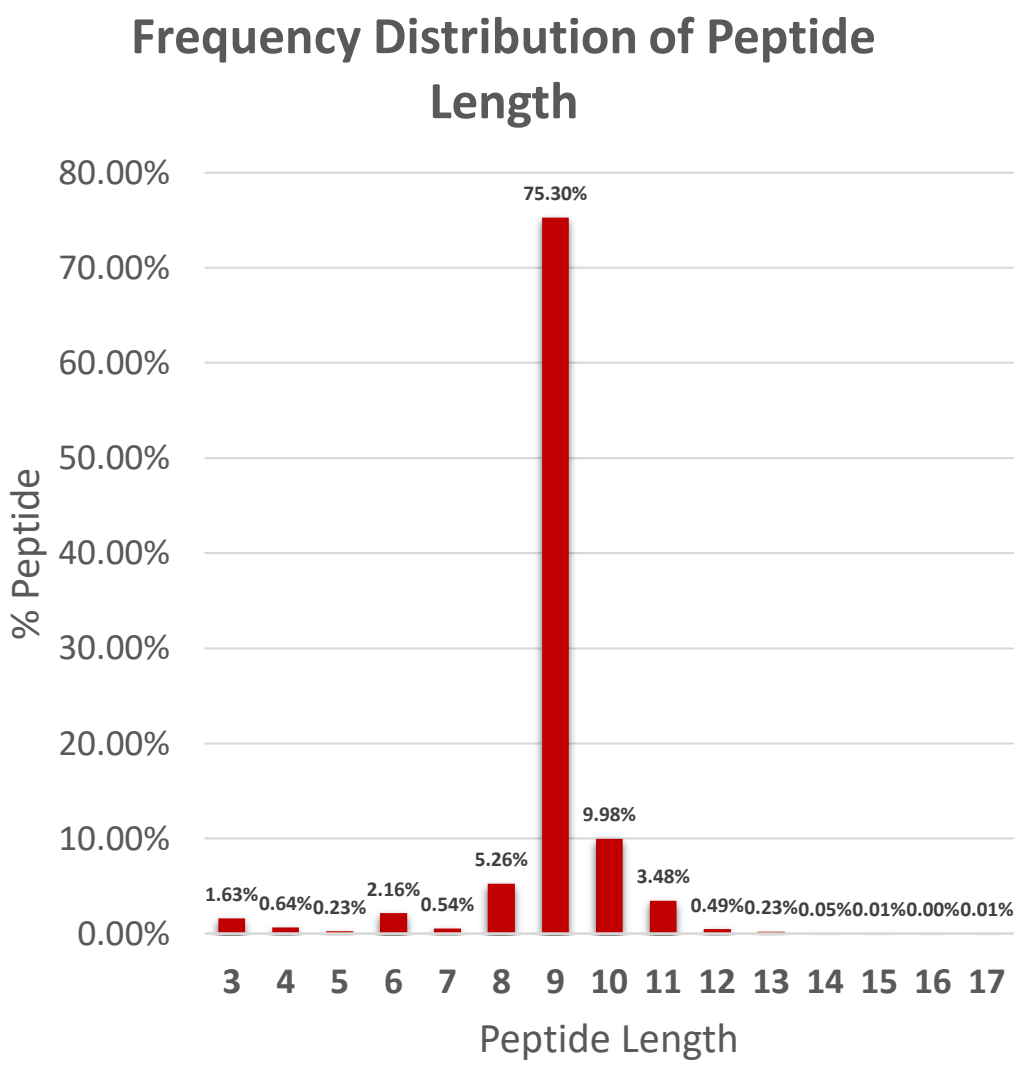

Figure 7. The frequency distribution of the peptide length of MHC class I peptides from the average of 3 samples

Peptide-binding Motif Analysis

It is critical to carefully evaluate the HLA-bound peptide data to ensure the quality of the results. One popular method is to visualize positions of residue preference within the immunopeptidomic datasets. This can be achieved using online tools such as Seq2Logo. http://www.cbs.dtu.dk/biotools/Seq2Logo/

Since the majority of the peptides identified were 8-, 9-, 10- and 11-mers with 9-mers being the most abundant peptides (> 75%), all 9-mer sequences were uploaded to the Seq2Logo website and generated the HLA peptide-binding motif in Figure 8. The analysis of the HLA motif showed a strong preference for L or V at position 9 (C terminus) and at position 2.

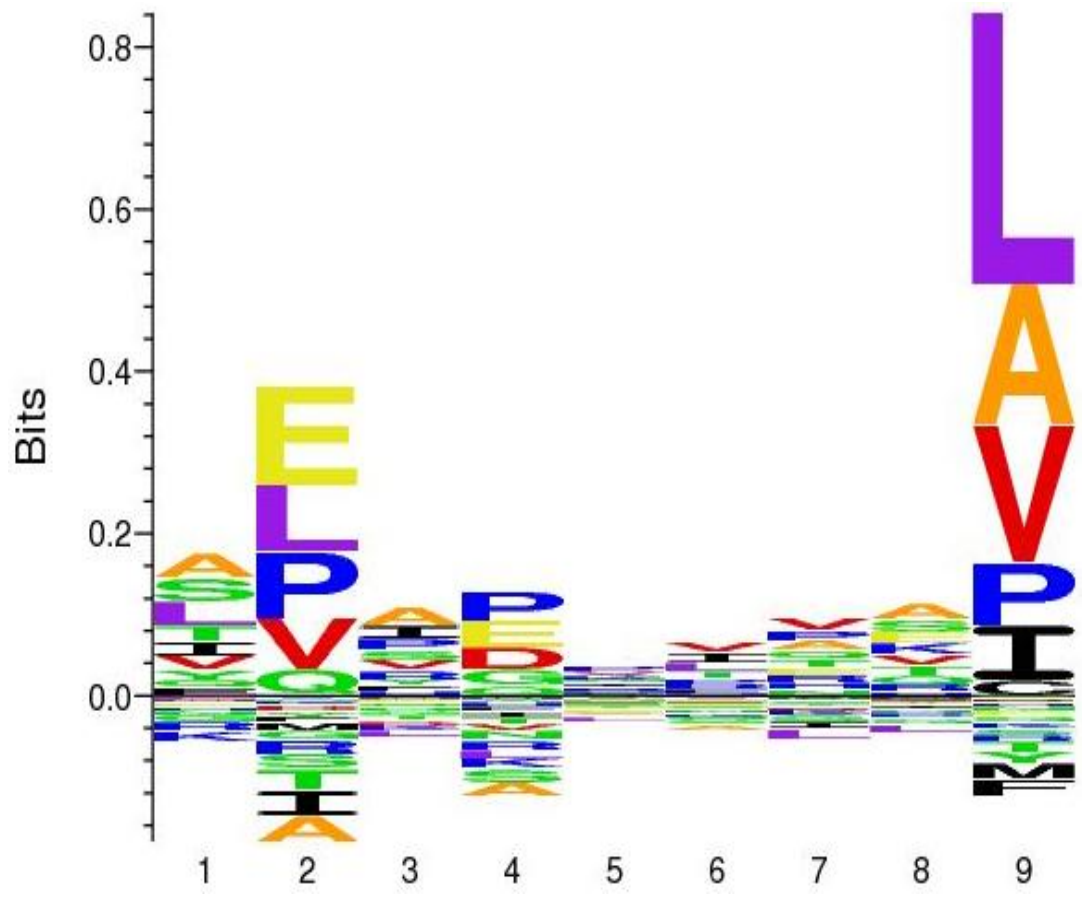

Figure 8. HLA peptide-binding motif was constructed on the basis of nonamer peptides (created by Seq2Logo)

Conclusions

An automated MHC-associated peptide enrichment for immunopeptidomics analysis has been developed. This workflow provides a high throughput, reproducible and easy-to-use enrichment for MHC peptide analysis.

- AssayMAP 25 µL PAW cartridges are well suited for low concentration MHC-complex enrichment. 5 µL C18 cartridge provides an efficient peptide separation and cleanup from protein complexes.
- The number of unique peptides identified from the samples are highly consistent.
- The peptide abundance between the samples showed good reproducibility with predominantly 9-mer peptides.

For Research Use Only. Not for use in diagnostic procedures.

Published in USA, June 1, 2020

Agilent
Trusted Answers