An Automated MHC-associated Peptide Enrichment for Immunopeptidomics Analysis

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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 peptides, 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. Third, the peptides that bind with MHC complex have similar length and often contain sequence mutations different from proteolytic digested peptides, which makes their analysis more challenging.

In this workflow, we used 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

2 mg of anti-human MHC-I antibody (x 3) or anti-gp120 isotype control (x 3) were loaded on new AssayMAP 25 µL PAW cartridges in parallel using the Affinity Purification application. Dimethyl pimelimidate (DMP) was used to cross-link the antibody to protein A.

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

Immunoaffinity purification of MHC-I complex

The GRANTA-519 cell pellets were lysed and membrane fraction were isolated. The MHC-I complexes were immunoprecipitated with the antibody cross-linked cartridges. For each cross-linked cartridge, about 0.5 mg membrane protein was used. The MHC-associated peptides were separated from MHC protein and desalted on C18 cartridges using Peptide Cleanup application. The protocols of AssayMAP were summarized in Table 1.

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	25mM DMP in 0.2M TEA	0.5 mg/mL GRANTA membrane fraction	1% Acetic Acid
Loading volume	1000 µL	250 µL	1000 µL	100 µL
Loading flow rate	10 µL/min	5 µL/min	5 µL/min	5 µL/min
Washing buffer 1	PBS, pH=7.4	0.1 M ethanolamine, pH=8.8	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	TBS, pH=7.4	25mM Tris, pH=8.0	NA
Washing volume 2	NA	250 µL	250 µL	NA
# washes 2	NA	3	3	1
Elution buffer	NA	NA	1% Acetic Acid	30% ACN, 0.1% TFA in water
Elution volume	NA	NA	100 µL	100 µL

Experimental

MHC peptide 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 6545XT AdvanceBio 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, cysteinylation, methylation and phosphorylation 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	0.080 mL/min primary flow			
	300 nL/min on-column flow rate			
	Time (min)	B (%)	Time (min)	B (%)
Gradient	0	3	97	70
	90	35	100	3
	95	70	120	3
Injection volume	5 µL			

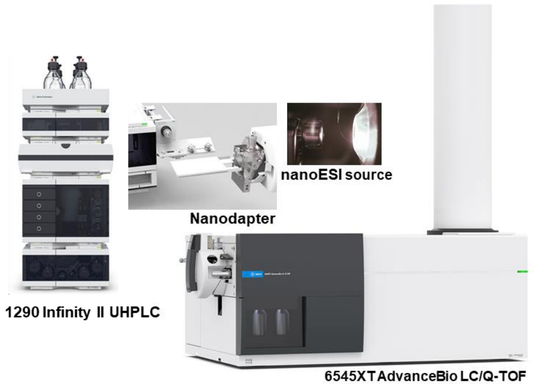

Figure 2. Nanodapter converts standard flow LC to nanoflow LC coupled with Q-TOF

Results and Discussion

Peptide Identification and relative quantitation

Peptide samples were analyzed on both Genentech and Agilent site. Figure 3 showed distinct peptide number identified from Orbitrap Fusion Lumos (Data provided by Genentech).

Peptide samples were also analyzed on 6545XT Q-TOF with a 90-minute gradient. Data were processed by Byonic and the peptide extracted ion chromatogram (XIC) peak area were summed in Figure 4 with the CV% annotated on each sample. The relative quantitation showed a good reproducibility between the replicates with the CV% < 6%.

The frequency distribution of the peptide lengths of class I peptides from the three samples were plotted in Figure 5. Peptides in the samples are predominantly 9-, 10- and 11- mers which is in line with literatures.

HLA peptide-binding motif was constructed based on nonamer peptides which showed a strong preference for V or L at position 2 and for L or V at the C terminus. (Figure 6)

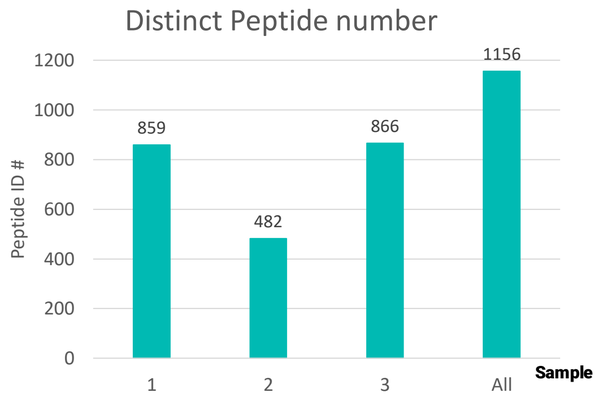

Figure 3. The distinct peptide number identified using Orbitrap Fusion Lumos (Data provided by Genentech).

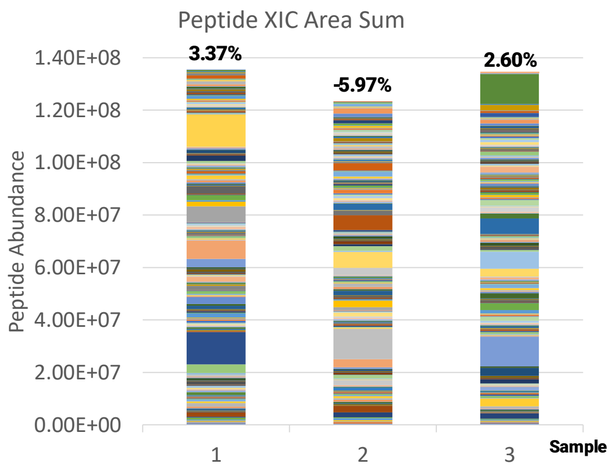

Figure 4. Sum of Class I peptide XIC area for each sample with CV% annotated (Data collected on 6545XT Q-TOF)

Results and Discussion

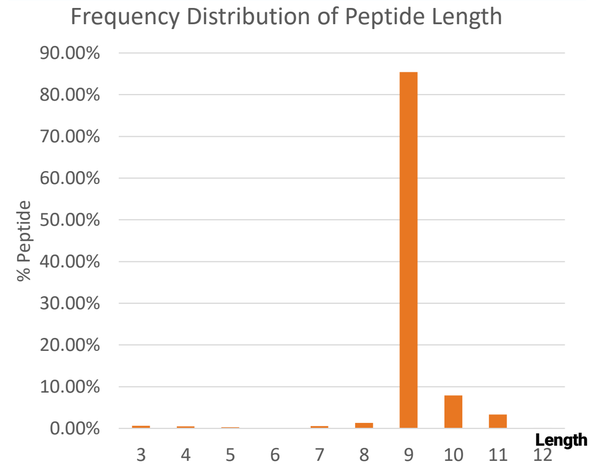

Figure 5. The frequency distribution of the peptide length of class I peptides from the average of 3 samples using 6545XT Q-TOF

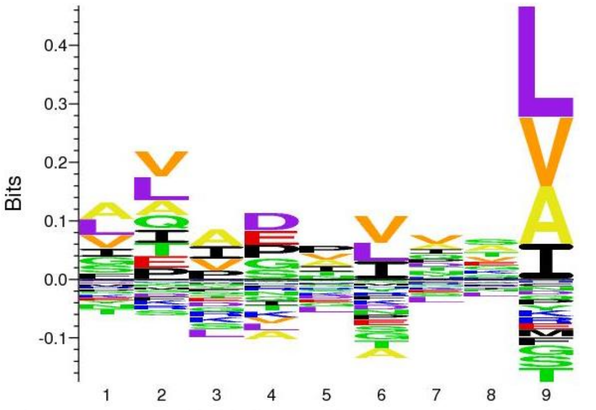

Figure 6. HLA peptide-binding motif was constructed on the basis of nonamer peptides (created by Seq2Logo)

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 cartridge is well suited for low concentrated MHC-peptide complex enrichment with large sample loading volume. 5 µL C18 cartridge provides an efficient peptide separation and cleanup from protein complexes.
- AssayMAP Bravo platform provides an automated and highly reproducible sample preparation for MHC peptides.
- The quantitation result from 6545XT Q-TOF showed a good reproducibility of peptide abundance between the samples with predominantly 9- mers in peptides.