Application Note

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Automated MHC-Associated Peptide Enrichment for Immunopeptidomics Analysis Using Agilent AssayMAP Bravo Large Capacity Cartridges

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Abstract

This application note describes a method for automated MHC-I associated peptide enrichment using the Agilent AssayMAP Bravo platform with large-capacity (25 µL) cartridges for immunopeptidomics analysis. For accompanying LC/MS analysis, an Agilent 6550 iFunnel Q-TOF LC/MS was enabled for nanoflow operation using an Agilent Infinity UHPLC Nanodapter. This workflow provides a reproducible, straightforward means of enrichment for MHC-I peptide analysis.

Introduction

When there is a viral infection, cytotoxic T lymphocytes (CTLs) are activated after recognizing host cells presenting viral antigens via the MHC-I display system. These antigens are generated after viral proteins are degraded into peptides in the cytoplasm and transported to the endoplasmic reticulum (ER), where they are loaded into class I major histocompatibility complexes (MHC-I), which then migrate to the cell surface. These MHC-I complexes are of interest not only in viral defense, but also in cancer immunotherapy, as CTLs can also recognize peptides derived from endogenous, mutated proteins.[1,2] Therefore, characterization of MHC-I presented peptides, or immunopeptidome, is of great interest in the design of vaccines and immunotherapies against cancer and infectious diseases.[3]

Immunopeptidomics is generally considered more challenging in part due to the fact that most MHC-associated peptides are extremely low in abundance compared to other cellular peptides. One way to address this challenge is to immunoprecipitate the MHC-I complexes from large amounts of cell lysate using an anti-MHC-I antibody coupled to a solid phase. The MHC-associated peptides are then isolated from the complex and identified by mass spectrometry.[1–3]

High-quality sample preparation is critical for obtaining good peptide signal and highly reproducible results. This workflow used the AssayMAP Bravo platform for automated immunoaffinity purification and peptide cleanup. The AssayMAP Bravo platform is composed of three main components (Figure 1): a high-precision liquid handler fitted with probe syringes that provides exquisite flow control; cartridges with a packed resin bed that connect to the probe syringes to allow positive flow over the resin bed; and user-friendly software with applications optimized to give great results straight out of the box with minimal optimization.

In addition to high-quality sample preparation, a very sensitive and reproducible LC/MS system is required. For this study, an Infinity UHPLC Nanodapter converted standard LC flow to nanoflow. The nanoflow LC system was then coupled with a 6550 iFunnel Q-TOF LC/MS using data-dependent acquisition (DDA) for peptide analysis. The combination of high-quality sample preparation and a sensitive LC/MS system provide a reproducible and scalable solution to meet the challenges of studying MHC-I peptidomics.

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

Experimental

Material

- Anti-human MHC-I antibody (w6/32) was expressed and purified at Genentech.
- The GRANTA-519 cell line was obtained from Genentech's cell bank.
- AssayMAP 25 µL Protein A cartridge (PAW) and 5 µL C18 cartridges are from Agilent Technologies, Inc. (Santa Clara, CA, USA) (Figure 1).
- All other chemicals were ordered from Sigma-Aldrich (St. Louis, MO, USA).

Antibody immobilization and crosslinking to 25 µL protein A cartridge

Anti-human MHC-I antibodies were immobilized on 25 µL protein A (PAW) cartridges using the Affinity Purification software application on the AssayMAP Bravo (see Table 1, Immobilization column, for details). Briefly, phosphate buffered saline (PBS) pH = 7.4 was used to prime and equilibrate AssayMAP 25 µL PAW cartridges. Six of these cartridges were then loaded in parallel with 1 mg (1 mg/mL, 1,000 µL) of anti-human MHC-I antibody (w6/32) per cartridge using a loading flow rate of 20 µL/min. Lastly, the cartridges were washed once with 250 µL PBS (pH = 7.4).

The PAW cartridges with a resin bed containing anti-MHC-I antibodies crosslinked to protein A resin were generated by treating the cartridges as described above, with a crosslinking reagent using the Affinity Purification application on the AssayMAP Bravo (see Table 1, crosslinking column, for details). Briefly, the 25 µL anti-MHC-I cartridges were primed and equilibrated with 0.2 M triethanolamine (pH = 8.1). Dimethyl pimelimidate (DMP) was used to crosslink the antibody to protein A by flowing 200 µL of 5 mM DMP in 0.2 M TEA over the cartridge beds with a loading flow rate of 5 µL/min. The crosslinking reaction occurred at room temperature during the 40-minutes loading. Unreacted DMP was washed away with TBS and Tris buffer, and non-crosslinked antibody was washed away using 1% acetic acid. The newly crosslinked cartridges were then equilibrated in TBS buffer (pH = 7.4) and stored at 4 °C until use. The chimneys on the cartridge plate where the crosslinked cartridges were stored were filled in with TBS buffer (pH = 7.4) + 0.025% sodium azide as storage buffer. About 190 µL buffer was added in each chimney.

Table 1. Affinity purification and peptide cleanup protocol using the Agilent AssayMAP Bravo platform.

	Immobilization	Crosslinking	Immunoaffinity Purification	Peptide Cleanup
Resin Bed Volume	25 µL	25 µL	25 µL	5 µL
Affinity Medium	Protein A	Protein A + antibody	Protein A + X-linked 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	1,000 µL	200 µL	1,000 µL	200 µL
Loading Flow Rate	20 µL/min	5 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
No. Washes 1	1	1	1	1
Washing Buffer 2	NA	25 mM Tris, pH = 8.0	25 mM Tris, pH = 8.0	NA
Washing Volume 2	NA	250 µL	250 µL	NA
No. Washes 2	NA	1	1	1
Elution Buffer	NA	1% acetic acid	1% acetic acid	30% ACN/0.1% TFA in water
Elution Volume	NA	50 µL	50 µL	50 µL

Immunoaffinity purification of MHC-I complex

GRANTA-519 cell pellets were lysed in nondenaturing buffer as previously described.[5] The Affinity Purification software application on the AssayMAP Bravo was used to purify MHC-I complexes from the cell lysate (see Table 1, Immunoaffinity purification column, for details). Briefly, TBS buffer (pH = 7.4) was used to prime and equilibrate the antibody crosslinked PAW cartridges. The MHC-I complexes were immuno-captured with the antibody crosslinked cartridges. About 3 mg (3 mg/mL, 1 mL) GRANTA lysate was loaded onto each of six cartridges with a loading flow rate of 20 µL/min. This was followed by two sequential wash steps. The first wash was a 250 µL wash with TBS buffer (pH = 7.4) followed by a 250 µL wash with 25 mM Tris buffer (pH = 8). Lastly, the enriched MHC complexes were eluted with 50 µL of 1% acetic acid. The eluate from six cartridges was combined to generate sample 1 (Table 1, Figure 2). The same experiment was repeated on different days to generate samples 2 and 3.

MHC-I peptide cleanup

The MHC-I peptides were separated from MHC protein and desalted on C18 cartridges using the Peptide Cleanup application software on the AssayMAP Bravo system (see Table 1, Peptide Cleanup column, for details). Briefly, 70% ACN/0.1% TFA in water was used to prime the cartridge. The cartridge was then equilibrated with 2% ACN/0.1% TFA in water. The MHC complex in 200 µL of 1% acetic acid was loaded on the C18 cartridge with a loading flow rate of 5 µL/min. Cartridges were washed once with 50 µL of 2% ACN/0.1% TFA in water. The MHC-I peptides were eluted with 50 µL of 30% ACN/0.1% TFA in water (Table 1, Figure 2). Samples were combined as appropriate and dried down using Speed-Vac at room temperature. The dried samples were stored at –80 °C until analysis.

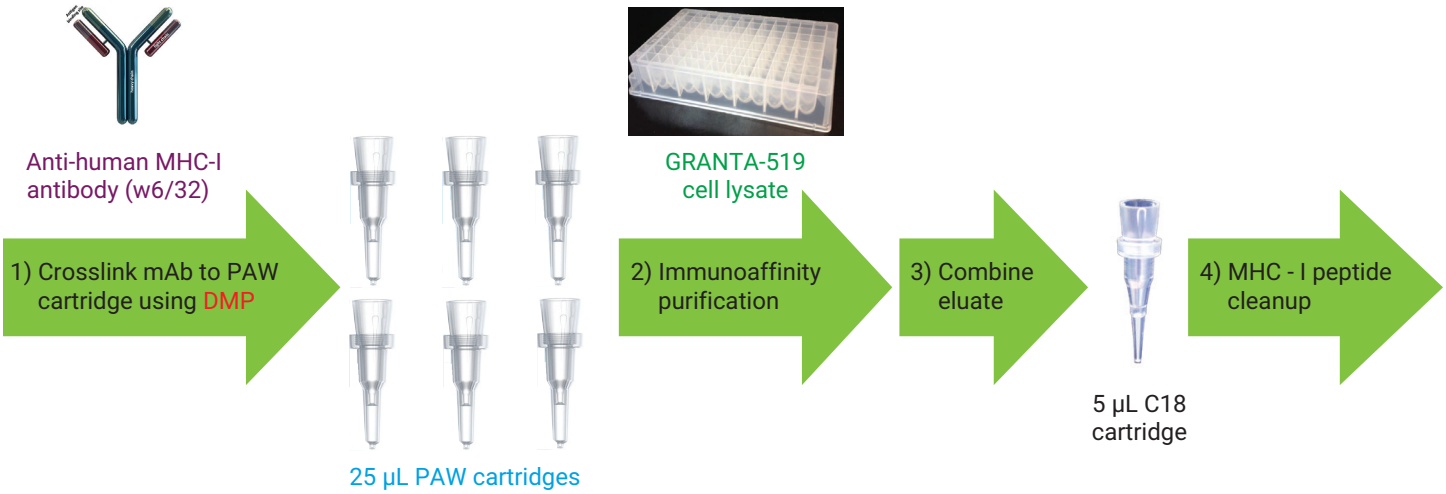

Figure 2. MHC-I peptide enrichment and cleanup using the Agilent AssayMAP Bravo platform.

Peptide identification using DDA

The dried samples were resuspended in 5 µL of 10% ACN/0.1% FA. The sample vials were vortexed and sonicated for two minutes. Samples were further diluted in 5 µL of 0.1% FA such that the final samples were suspended in 10 µL of 5% ACN/0.1% FA.

An Agilent 1290 Infinity II LC was converted to a nanoflow LC by coupling with the Agilent Infinity UHPLC Nanodapter. This nanoflow LC was connected to the Agilent nanospray source and coupled with the Agilent 6550 iFunnel Q-TOF LC/MS for peptide identification (Figure 3). The LC parameters were listed in Table 2. The Nanodapter was configured in a direct injection mode. A 75 µm × 25 cm C18 column was kept at 60 °C and used for peptide separation with a 90-minute gradient in a total 120-minute LC run time.[2,6]

For peptide identification, 5 µL reconstituted MHC-I peptide samples were injected once for each sample. DDA was used with a selection of top 20 precursor ions. The detailed settings for the 6550 iFunnel Q-TOF LC/MS are listed in Table 3. For MHC-I peptide analysis, singly charged ions were selected as precursor ions even though they were given lower priorities than doubly and triply charged ions. Based on literature, many MHC-I peptides form singly charged ions, which should be considered in DDA experiments. However, higher collision energy should be applied on singly charged ions than multiply charged ions.[2]

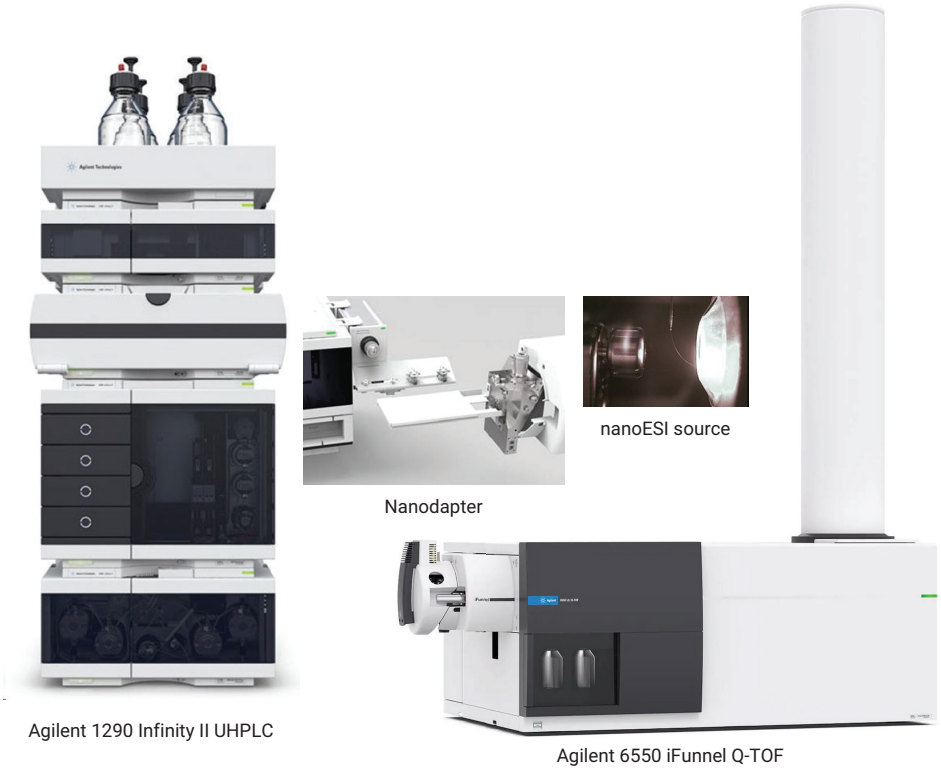

Figure 3. Agilent Infinity UHPLC Nanodapter converts standard-flow LC to nanoflow LC coupled with an Agilent 6550 iFunnel Q-TOF LC/MS.

Table 2. Nano-LC parameters.

LC Conditions				
Trap Column	PepMap C18, 75 µm × 2 cm, at 60 °C			
Analytical Column	PepMap C18, 75 µm × 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) 0 90 95	%B 3 35 70	Time (min) 97 100 120	%B 70 3 3
Injection Volume	5 µL			

Qualitative and quantitative analysis using Byos workflow

Data analysis was processed using Byos workflow from Protein Metrics Inc. The Byos workflow includes peptide identification using Byonic search engine and peptide quantitation using Byologic software (Figure 4). Byos workflow processes peptide identification and quantitation consecutively with the parameters preset by users. Table 4 lists some of the key parameters used in Byonic and Byologic software for MHC-I peptide analysis. Byonic was used to search against the Uniprot human protein database with nonenzyme specific and no limitation on missed cleavage. Oxidation (M, W) and deamidation (N, Q) were set as variable modifications. A manual score cut, 150, was used for filtering identified peptides (Table 4A). The MS profiles for the identified peptides were then extracted in Byologic with certain retention times and *m/z* windows. A decoy number of 50 was used as a second filter for protein and peptide identification (Table 4B). The unique peptides were summarized and quantified in Byologic.

Table 3. Agilent 6550 iFunnel Q-TOF LC/MS parameters.

Parameter	Value	
Spray Needle	New objective noncoated needle 25 µm id, 10 µm tip id, 5 cm length orthogonally positioned	
Gas Temperature	200 °C	
Drying Gas	11 L/min	
Acquisition Mode	Extended Dynamic Range (2 GHz) *m/z* 100 to 1,700 High analytical sensitivity	
	MS	**MS/MS**
Mass Range	*m/z* 300 to 1,700	*m/z* 50 to 1,700
Acquisition Rate	3 spectra/sec	>3 spectra/sec
Isolation Width	Medium (~*m/z* 4)	
Collision Energy	(Slope)*(*m/z*)/100+Offset Charge Slope Offset 2 3.1 1 3 3.6 -4.8 1 4 6 >3 3.6 -4.8	
Max Precursor/Cycle	20	
Precursor Threshold	1,000 counts and 0.01%	
Active Exclusion	Excluded after 1 spectra Released after 0.2 min	
Isotope Mode	Peptides	
Sort Precursors	By charge state then abundance; +2, +3, 1, >+3	
Scan Speed Varied Based On Precursor Abundance	Yes	
Target	25,000 counts/spectrum	
Use MS/MS Accumulation Time Limit	Yes	
Purity Stringency	100%	
Purity Cutoff	30%	

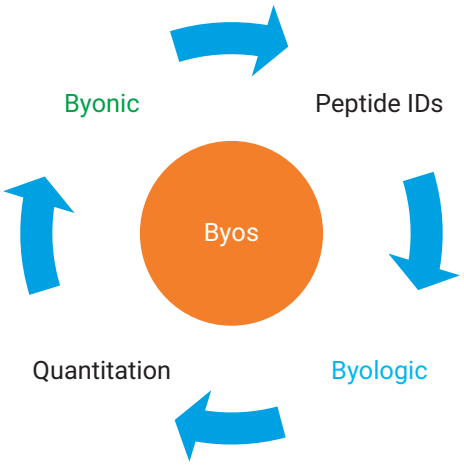

Figure 4. Byos workflow for peptide analysis.

Table 4A. Byonic search parameters.

Byonic Settings	
Database	Uniprot human proteins
Digestion Specificity	Nonspecific
Cleavage Side	C-terminal
Maximum Missed Cleavage	No limit
Modifications	Oxidation (M, W) Deamidated (N, Q)
Fragmentation Type	QTOF/HCD
Mass Tolerance	MS1 MS2 20 ppm 40 ppm
Manual Score Cut	150

Table 4B. Byologic quantitation parameters.

Byologic Settings	
Enable In-Silico	No
Relevant Peptides	All types
m/z Window	20 ppm
Apex Search Window	2 minutes
XIC Area Window	2 minutes
Enable Lock-Mass Calibration	No
Centroid Smoothing Width	0.02
Decoys	50

Results and discussion

MHC peptide identification and quantitation

Figure 5 is the total ion chromatogram (TIC) of the three samples using a 90-minute gradient with a total 2-hour nano-LC run time. The TIC shows reproducible retention time and peak abundance between the samples. The tandem MS data were analyzed by Byos workflow and peptide IDs were summarized in Figure 6. The unique MHC class I peptides identified in each sample ranged from 2,284 to 2,426 with a CV% at 3.0%. The number of unique peptides identified across all three samples is 3,604. A Venn diagram shows a more detailed relation of peptide IDs between the samples. The number of unique peptides identified in all three samples is about 1,351, constituting about 55.7% to 59.2% in each sample. The same peptides identified between two samples ranged from 65.4% to 71.3% for each sample (Figure 7).

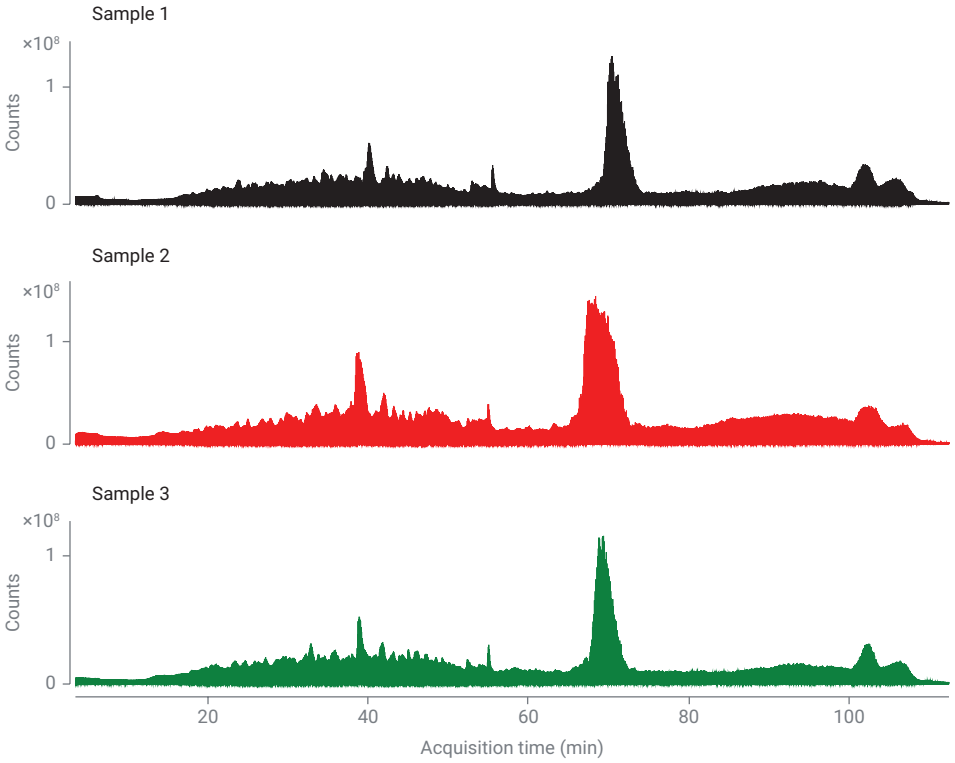

Figure 5. TIC of MHC-I peptides using a 90-minute gradient.

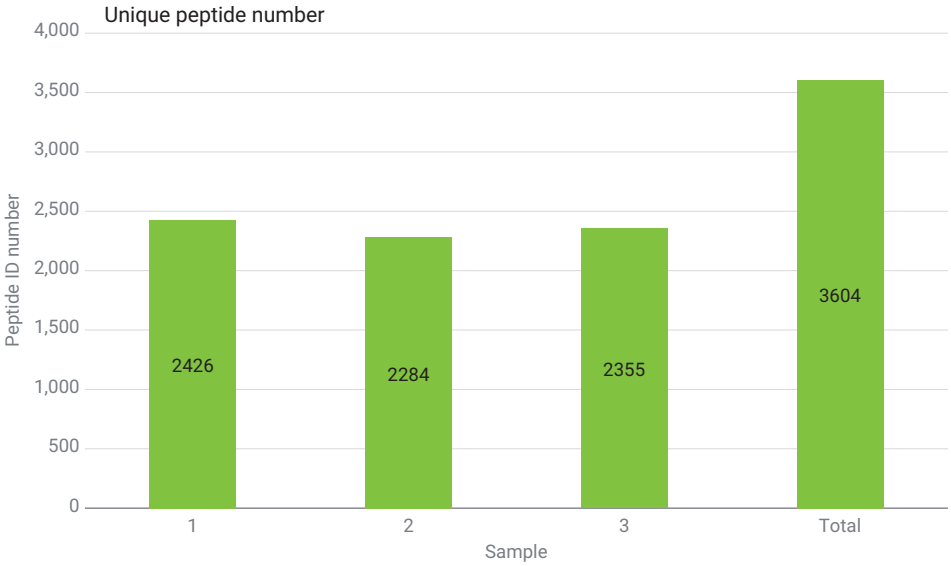

Figure 6. The unique peptide number identified using an Agilent 6550 iFunnel Q-TOF LC/MS.

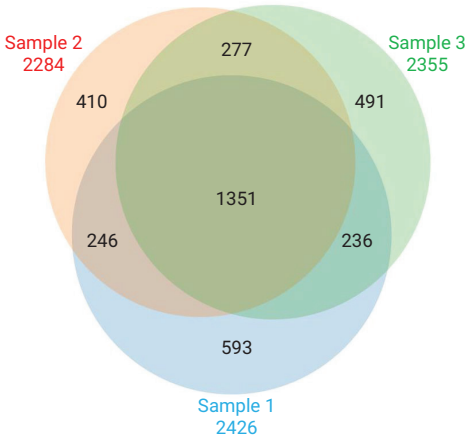

Figure 7. Venn diagram of unique peptides identified from the three samples.

Byologic extracts peptide abundance based on the MS1 scan for each identified peptide. By adding the peptide abundance, the total peptide abundances in each sample are summarized in Figure 8 with a CV% at 11.1%. The three samples were prepared using the same six crosslinked PAW cartridges on three different days. Multiple steps were used to prepare the samples, including crosslinking of mAb with PAW, GRANTA lysate loading on each cartridge, combining the eluate, and a subsequent C18 cleanup. Therefore, the CV% of the total peptide abundance between the samples showed a good day-to-day reproducibility using the automated sample preparation. It also demonstrated that the crosslinked cartridges can be re-used and maintain a good performance.

Peptide length distribution

The frequency distribution of peptide length is plotted in Figure 9 to further confirm the identification of MHC class I peptides. Data shown in Figure 9 are compiled from the overall peptide identified in Byos workflow analysis. The 3,604 unique peptides (Figures 6 and 7) 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.[2]

MHC peptide-binding motif analysis

It is critical to carefully evaluate the MHC peptide-binding motif 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 10. The analysis of the HLA motif showed a strong preference for L or V at position 9 (C terminus) and at position 2.[2]

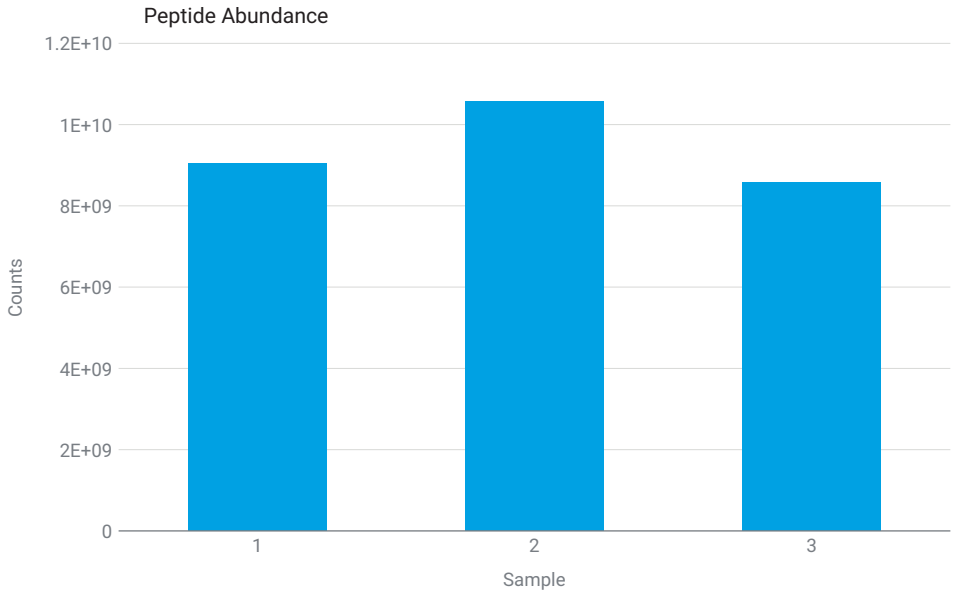

Figure 8. MHC-I peptide abundance for each sample with CV% at approximately 11.1%.

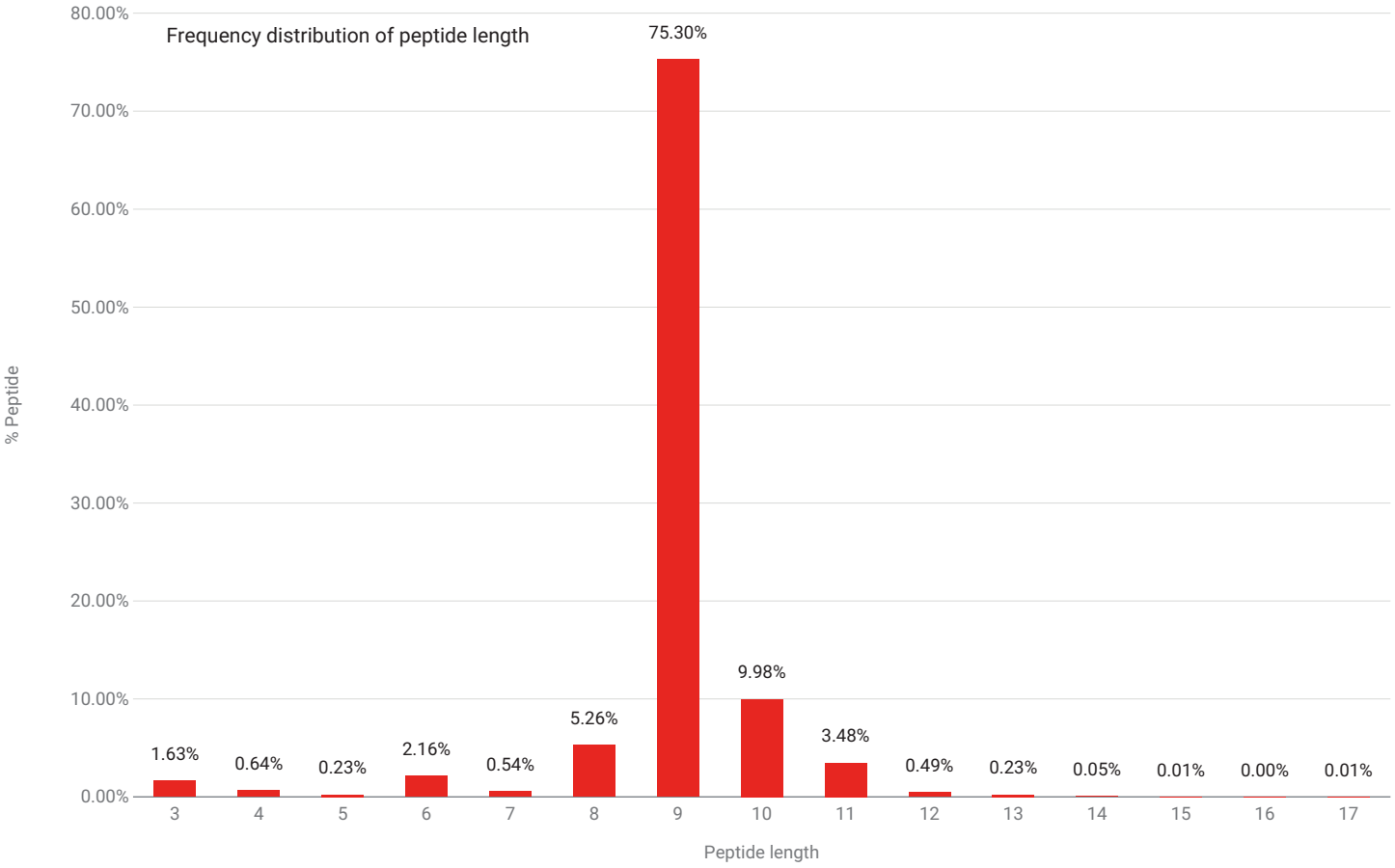

Figure 9. The frequency distribution of the peptide length of MHC class I peptides from the average of three samples.

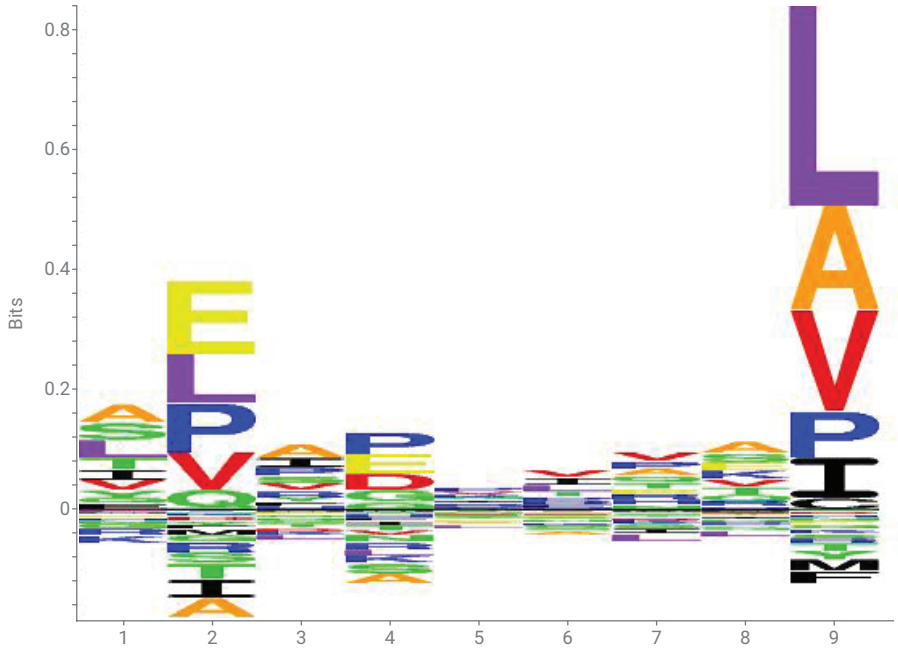

Figure 10. MHC peptide-binding motif was constructed on the basis of nonamer peptides (created by Seq2Logo).

Conclusion

An automated MHC-I associated peptide enrichment using large-capacity (25 µL) PAW cartridges for immunopeptidomics analysis has been developed. This workflow provides a reproducible and easy-to-use enrichment for MHC peptide analysis.

Antibody crosslinking with PAW cartridge, immunoaffinity purification, and peptide cleanup can all be automated on the AssayMAP Bravo in a stepwise manner. AssayMAP 25 µL PAW cartridges are well suited for processing low-concentration MHC complex with a high loading speed and high enrichment efficiency. 5 µL C18 cartridges provide an efficient peptide separation and cleanup from protein complexes. The unique MHC-I peptides identified from the three samples prepared on different days ranged from 2,284 to 2,426 with a CV% less than 3%, which showed a highly consistent peptide ID result. The peptide quantitation result showed a good reproducibility of peptide abundance from the three samples, with a CV% at about 11.1%. The day-to-day sample comparison demonstrated a good reproducibility with an automated workflow, even with multiple sample preparation steps. The peptide length distribution from all the unique peptides showed over 94% peptides identified have lengths from 8 to 11 residues, which is well in line with the literature. The HLA peptide-binding motif generated from the 9-mer sequence logo further confirmed the preference of L and V at position 9 and position 2.

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