Application Note

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Human Breast Cancer Cell Line Phosphoproteome Revealed by an Automated and Highly Selective Enrichment Workflow

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Introduction

Phosphopeptide enrichment has been a challenging task in that the reproducibility of the experiment has large variation between sample-to-sample and person-to-person caused by manual sample preparation. The Agilent AssayMAP Bravo platform provides a fully automated, highly selective, and reproducible enrichment workflow using high-capacity Fe(III)-NTA cartridges[1]. To evaluate how the ratio of total peptide sample amount to affinity resin (sample/resin) affects the performance and reproducibility of phosphopeptide enrichment, and monitor the yield and reproducibility of the enrichment using phosphopeptide standards, this Application Note joined the PME11 part II initiative proposed by the European Proteomics Association (EuPA).

The samples analyzed in the study (PME11-2A) consisted of a tryptic digest of the human MCF7 breast cancer cell line (C-18 purified), prespiked with a mixture of 20 human phosphopeptide standards containing light isotopes (Phosphomixes 1 and 2 from Sigma-Aldrich[2]). The Agilent AssayMAP Phosphopeptide Enrichment application was used for automated phosphopeptide enrichment with Fe(III)-NTA cartridges. The cell line digest samples were loaded onto the cartridges with four different sample/resin ratios. The other 20 phosphopeptide standards containing heavy isotopes (PME11-2B, from Sigma-Aldrich) were spiked into the enriched samples at the same amount as the light isotopic standards. An Agilent Infinity UHPLC nanodapter converted standard flow to nanoflow. For discovery proteomics, the enriched samples were analyzed with nanoflow LC coupled with an Agilent 6550 iFunnel Q-TOF using data-dependant acquisition (DDA). To provide phosphopeptide identification, MS/MS spectra were analyzed with Spectrum Mill. The same nanoflow LC system was then coupled with an Agilent 6495B triple quadrupole (TQ) LC/MS to perform peptide quantitation. Multiple reaction monitoring (MRM) was performed to calculate the enrichment yield based on the 20 phosphopeptide standards (Figure 1). This Application Note demonstrates that this automated, highly selective, and reproducible workflow provides excellent performance for phosphopeptide identification and quantitation that can both easily be implemented in the research lab.

Experimental

Material

- Human MCF7 breast cancer cell line tryptic digest (PME11-2A) was provided by ProteoRed (Barcelona, Spain) as part of the PME11 phosphoproteomics study.
- PME11-2A: 125 µg of cell digest, plus 200 fmol of each of the 20 light Posphomix phosphopeptide standards (1.1L -1.10L and 2.1L-2.10L) was included in each tube. Twelve sample tubes were received in dried form, lyophilized from a water-acetonitrile mixture.
- One tube of PME11-2B containing 3 pmol of each of the corresponding isotopically labeled heavy Phosphomix standard peptides (1.1H-1.10H and 2.1H-2.10LH), was received in dried form.
- AssayMAP Fe(III)-NTA cartridges were from Agilent Technologies, Inc. (Santa Clara, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Sample plate preparation

Twelve original sample tubes were taken from the freezer and placed on the bench until they were restored to room temperature. Then, 160 µL of 80 % ACN with 0.1 % TFA were added to each tube. To ensure complete dissolution, all tubes were gently sonicated for two minutes. The unenriched sample was loaded into the LoBind 96 Eppendorf plate with four different sample/resin ratios according to Figure 2. For Sample R1, the 160-µL sample was loaded onto A1, A2, and A3. For sample R2, 80 µL of original sample was diluted with 80 µL of 80 % ACN with 0.1% TFA. The 160-µL R2 sample was added to B1, B2, and B3. For sample R3, 80 µL of original sample were diluted with 240 µL of 80 % ACN with 0.1 % TFA. The 160-µL R3 sample was aliquoted to rows C and D (two rows, three replicates, six samples). For sample R4, 80 µL of original sample were diluted with 560 µL of 80 % ACN with 0.1 % TFA. A 160-µL R4 sample was aliquoted to rows E and H (four rows, three replicates, 12 samples).

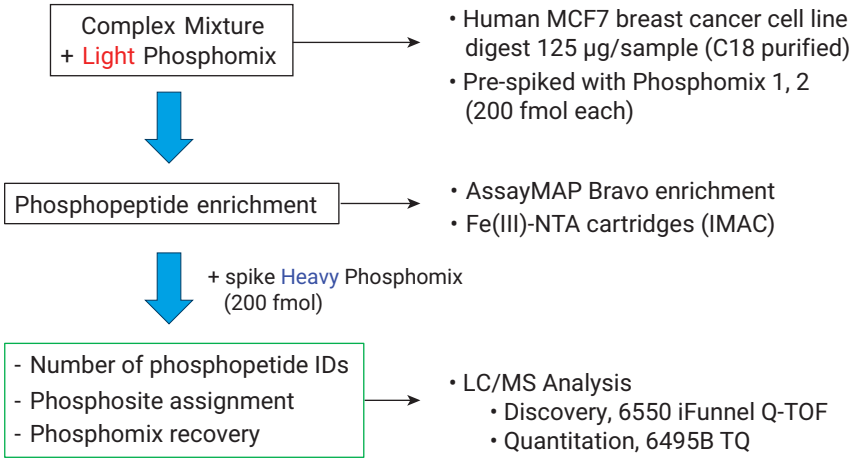

Figure 1. PME11.2 experimental design.

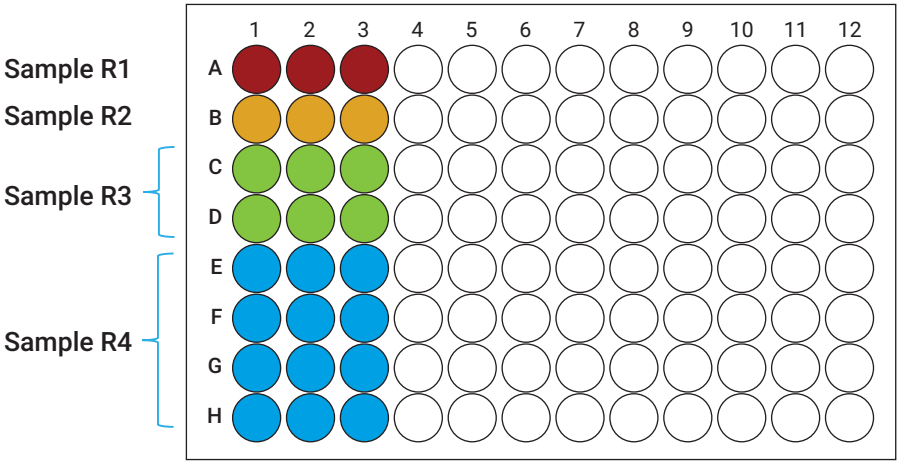

Figure 2. Phosphopeptide sample location on the 96-well plate.

The rest of the original sample was lyophilized in a speedVac, then reconstituted with 40 µL of 10 % ACN, 0.1 % FA first. To dilute the sample to 5 % ACN, 40 µL of water with 0.1 % FA were added. A 1.5-µL amount (approximately 1 µg) of unenriched sample was injected for LC/Q-TOF analysis.

Phosphopeptide enrichment using AssayMAP Fe(III)-NTA cartridges

The AssayMAP Phosphopeptide enrichment v2.0 App was used for automated phosphopeptide enrichment with Fe(III)-NTA cartridges. Figure 3 shows the user interface and application settings[1]. Three full columns of the Fe(III)-NTA cartridges were pretransferred to deck location 2. Labware was chosen according to the labware table, except that LoBind 96 Eppendorf plates were used on deck locations 4 and 9. The cartridges were primed with 50 % ACN containing 0.1 % TFA using a high flow rate of 300 µL/min. Then, the cartridges were equilibrated using 80 % ACN containing 0.1 % TFA, the same solvent as the binding buffer (Table 1). Sample loading onto the cartridge was a critical step. According to our previous study, the flow rate was set at 3.5 µL/min to give enough time to efficiently bind the phosphopeptides. An internal cartridge wash step was used after loading, also using 80 % ACN containing 0.1 % TFA. The enriched phosphopeptides were eluted with 20 µL of 1 % ammonium hydroxide (pH ~11) directly into a LoBind PCR plate containing 5 µL of 50 % formic acid (Table 1 and Figure 3).

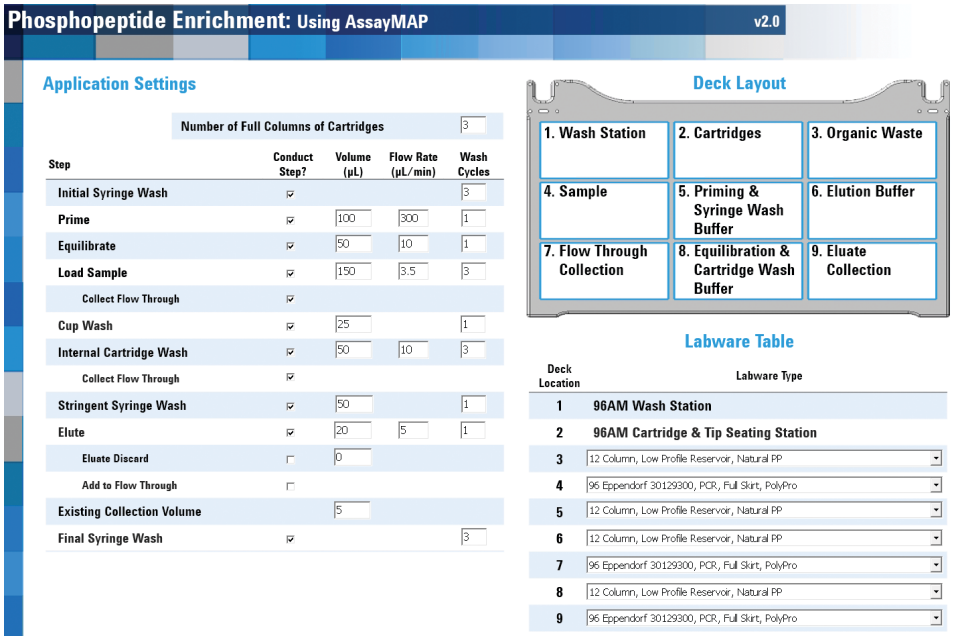

Figure 3. AssayMAP phosphopeptide enrichment v2.0 App.

Table 1. Phosphopeptide enrichment protocol using Fe(III)-NTA cartridge with four sample/resin ratios.

Sample number	R1	R2	R3	R4
Affinity medium (TiO_2/IMAC)	IMAC	IMAC	IMAC	IMAC
Affinity medium amount	5 µL	5 µL	5 µL	5 µL
Support	Fe(III)-NTA, 100 nmol Fe(III)	Fe(III)-NTA, 100 nmol Fe(III)	Fe(III)-NTA, 100 nmol Fe(III)	Fe(III)-NTA, 100 nmol Fe(III)
Sample amount	125 µg	62.5 µg	31.25 µg × 2	15.625 µg × 4
Affinity medium/sample ratio	5 µL:125 µg	5 µL:62.5 µg	5 µL:31.25 µg	5 µL:15.625 µg
Binding buffer	80 % ACN, 0.1 % TFA in water	80 % ACN, 0.1 % TFA in water	80 % ACN, 0.1 % TFA in water	80 % ACN, 0.1 % TFA in water
Binding volume	150 µL	150 µL	150 µL	150 µL
Washing buffer 1	80 % ACN, 0.1 % TFA in water	80 % ACN, 0.1 % TFA in water	80 % ACN, 0.1 % TFA in water	80 % ACN, 0.1 % TFA in water
Washing volume 1	50 µL	50 µL	50 µL	50 µL
No. washes 1	1	1	1	1
Elution buffer 1	1 % Ammonium hydroxide	1 % Ammonium hydroxide	1 % Ammonium hydroxide	1 % Ammonium hydroxide
Elution volume 1	20 µL	20 µL	20 µL	20 µL
No. replicates	3	3	3	3

The final sample plate was centrifuged for two minutes. Samples from rows C and D were combined into row C. Samples from rows E through H were combined into row E. One hundred fifty microliters of 80 % ACN containing 0.1 % TFA were added into the PME11-2B vial (contains 3 pmol heavy labelled phosphomix standards), and sonicated for two minutes to ensure complete dissolution. A 10-µL (200 fmol) amount of heavy isotopic labeled B standard was spiked into sample R1 (three samples). For samples R2, R3, and R4, only 5 µL (100 fmol) of standard B were spiked in since only half of the unenriched sample was used for enrichment (Figure 2).

All 12 enriched samples were dried and reconstituted with 10 % ACN containing 0.1 % FA, and sonicated for two minutes. Samples were diluted further with the same volume of water containing 0.1 % FA. The final volume of sample R1 was 40 µL, and samples R2, R3, and R4 were 20 µL since they started with half of the unenriched sample compared to sample R1.

Nano-LC Q-TOF MS analysis

An Agilent 1290 Infinity II LC system was converted to nanoflow LC by coupling it with an Agilent Infinity UHPLC Nanodapter. This nanoflow LC was connected to an Agilent nanoESI source, and coupled with a 6550 iFunnel Q-TOF LC/MS for peptide identification (Figure 4)[3]. Table 2 lists the LC parameters. The Nanodapter was configured in direct injection mode[3]. A 75 µm × 25 cm C18 column was kept at 60 °C, and used for peptide separation with a 90-minute gradient in a 120-minute LC run time. For discovery proteomics, 1/5 of the enriched sample from 125 µg of original sample (8 µL) was injected for analysis. Data-dependent acquisition was used with a selection of the top 20 precursor ions. Table 3 lists the detailed setup for the 6550 iFunnel Q-TOF.

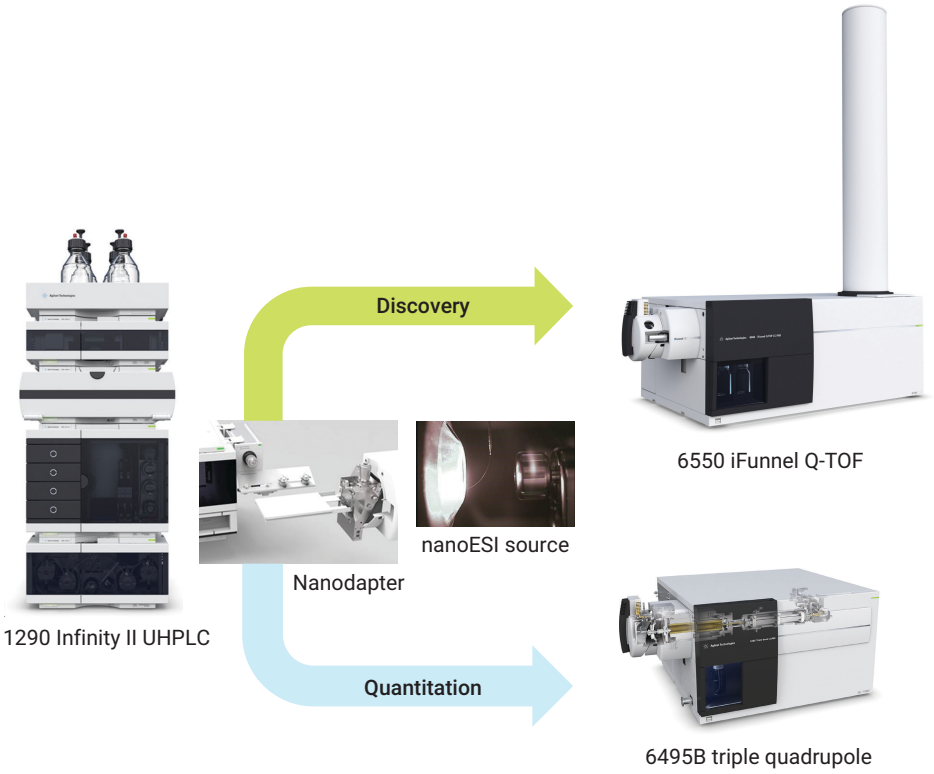

Figure 4. Nanodapter converts standard flow LC to nanoflow LC coupled with either Q-TOF or TQ LC/MS.

Table 2. Liquid chromatography parameters.

Parameter	Value
Nanodapter configuration	Direct injection mode
Trap-column	Thermo Acclaim PepMap C18, 75 µm × 2 cm
Analytical column	Thermo Acclaim PepMap C18, 75 µm × 25 cm
Column temperature	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	**Q-TOF** (Time (min), %B): 0, 3; 90, 37; 95, 70; 97, 70; 100, 3; Stop time, 115; Post time, 5 **TQ** (Time (min), %B): 0, 3; 30, 35; 33, 70; 38, 70; 41, 3; 56; 4
Injection volume	8 µL (Q-TOF); 4 µL (TQ)

Spectrum Mill was used to search against the customized database containing SwissProt human proteins with a 1.5 % false discovery rate. Trypsin was the digestion enzyme, and up to two missed cleavages were allowed. Carbamidomethylation was set as the fixed modification, while N-term Acetyl deamidation and phosphorylation of serine, threonine, and tyrosine were set as the variable modifications. Table 4 lists the detailed search parameters.

Nano-LC TQ MS analysis

The same nanoflow LC system was coupled to a 6495B triple quadruple LC/MS for quantifying the recovery of phosphopeptide enrichment using an MRM method (Figure 4)[4]. A 30-minute gradient in a 60-minute LC run was used to separate the enriched peptides. For quantitation analysis, 1/10 of the enriched sample from 125 µg of original sample (4 µL) was injected (Table 2). A suggested transition list for all 40 phosphopeptide standards (20 light and 20 heavy) was used in Skyline. Agilent has an automation tool implemented with Skyline that automates MRM analysis and method development. Starting from a list of target peptides in Skyline, the automation tool allows users to select all the parameters stepwise during optimization. First, it runs selected peptides in MRM mode to determine the retention time; then, it optimizes collision energy based on a retention time scheduled MRM method. At the end, it creates the dynamic MRM (DMRM) method by testing the sample with the optimized retention time window and collision energy[5]. This automation tool saves user time compared to optimizing the MRM method manually. The final DMRM result was imported into Skyline, and the yield of enrichment was calculated automatically by Skyline using the ratio between the largest transition of light over heavy peptide.

Table 3. 6550 iFunnel Q-TOF 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* 100–1,700 High Sensitivity
	MS **MS/MS**
Mass range	*m/z* 300–1,700 *m/z* 50–1,700
Acquisition rate	3 spectra/sec >3 spectra/sec
Isolation width	Narrow (~1.3 *m/z*)
Collision energy	(Slope)*(*m/z*)/100+Offset **Charge Slope Offset** 2 3.1 1 3 3.6 −4.8 >3 3.6 −4.8
Max precursor/cycle	20
Precursor threshold	1,000 counts and 0.01 %
Active exclusion	Excluded after 1 spectrum Released after 0.1 minutes
Isotope mode	Peptides
Sort precursors	By abundance only; +2, +3, > +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 %

Table 4. Spectrum Mill search parameters.

Parameter	Value
Database	Swissprot human proteins
Enzyme	Trypsin
Maximum missed cleavage	2
Modifications	**Fixed** **Variable** Carbamidomethylation Acetyl (N-term) Deamidated (N) Phosphorylated (S) Phosphorylated (T) Phosphorylated (Y)
Mass tolerance	**MS1** **MS2** 15 ppm 30 ppm
Maximum ambiguous precursor charge	5
Calculate reversed database scores	Yes
Dynamic peak thresholding	Yes
Peptide FDR	1.50 %
Precursor charge range	2 to 7

Results and discussion

Phosphopeptide discovery with data-dependent acquisition

Figure 5 compares the total ion chromatogram (TIC) of the unenriched sample to the enriched sample R2. The triplicate analysis of R2 shows good reproducibility of the enrichment using a 90-minute gradient. With the injection of 1 µg of unenriched sample, approximately 4,700 distinct peptides were identified. Prior to enrichment, approximately 1.5 % of the peptides were identified as phosphopeptides. After enrichment, the selectivity (phosphopeptides/total peptides %) of the phosphopeptide enrichment was routinely above 90 % across all 12 samples (Table 5 and Figure 6). This demonstrates a very reproducible and robust phosphopeptide enrichment from AssayMAP. Approximately 55 to 60 % of the phosphorylation sites were assigned across all enriched samples. Table 5 lists the detailed Spectrum Mill search results.

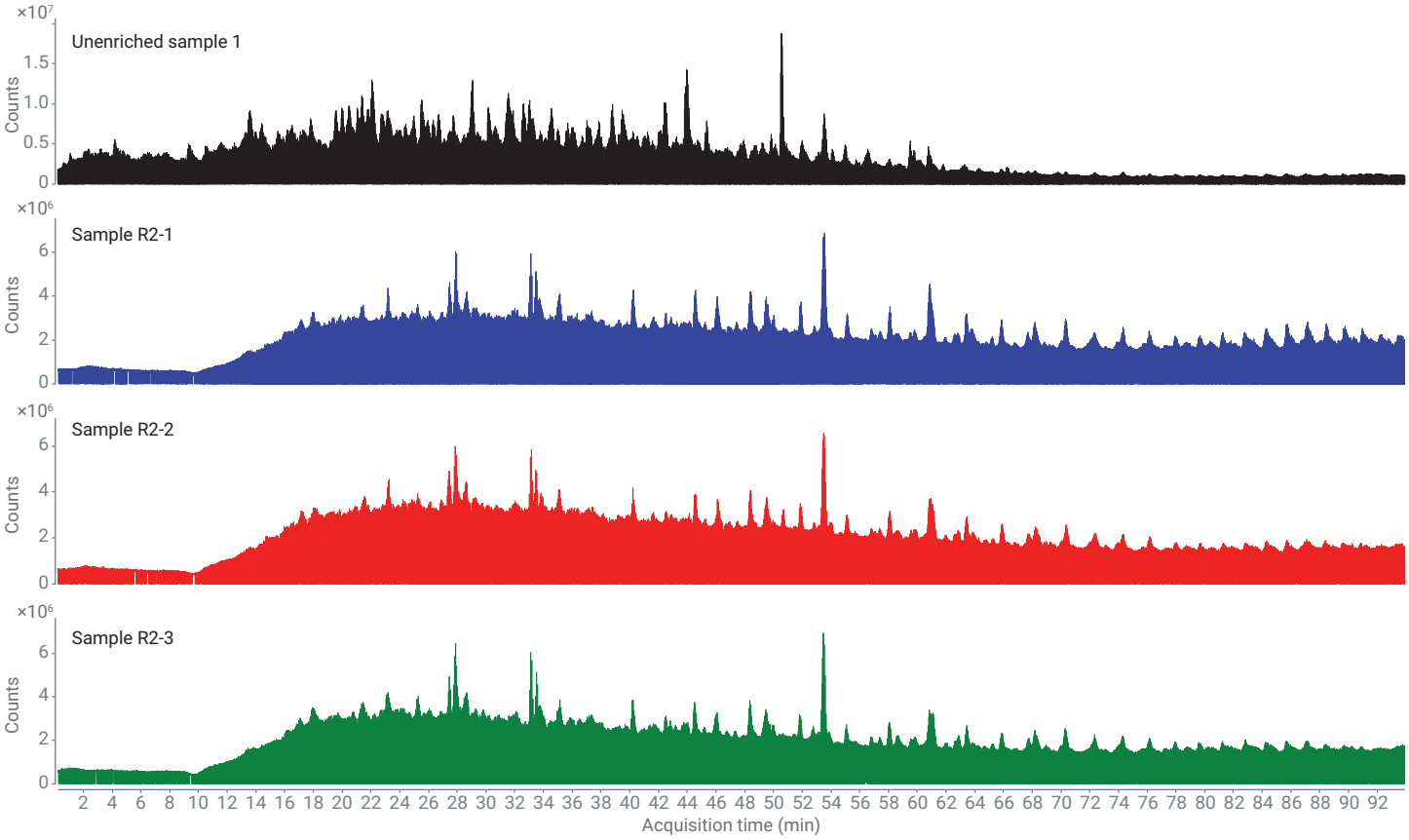

Figure 5. TIC of unenriched sample compared to enriched sample R2 using a 90-minute LC gradient.

Table 5. Spectrum Mill search results.

	Pre-enrichment			Sample R1			Sample R2			Sample R3			Sample R4		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
Total proteins	1,212	1,273	1,338	507	481	485	474	415	447	379	410	507	410	420	430
Total peptides	4,533	4,693	4,794	1,049	965	976	956	830	864	699	809	1050	782	795	813
PSMs	7,854	8,652	8,875	2,255	1,998	2,006	1,993	1,686	1,756	1,435	1,662	2,358	1,701	1,747	1,865
Total phosphopeptides	73	64	71	996	930	945	906	766	836	664	752	961	728	745	774
Mono-	57	56	60	908	859	870	844	727	789	624	701	911	674	689	721
Di-	12	6	9	80	68	71	56	36	43	37	47	49	52	52	49
Tri-	4	1	2	3	3	4	6	3	3	2	3	1	2	3	3
Tetra-	0	1	0	5	0	0	0	0	1	1	1	0	0	1	1
% Phosphopeptides/ total peptides	1.6	1.4	1.5	94.9	96.4	96.8	94.8	92.3	96.8	95.0	93.0	91.5	93.1	93.7	95.2
Total phosphosites	93	75	84	1,097	1,004	1,024	974	808	888	708	808	1,012	784	806	832
Sites assigned %	59.5	57.8	55.4	56.4	55.9	57.7	56	58.3	58.9	53.6	56	57.9	59.2	56.5	60.4
Sites not assigned	38	30	34	444	407	415	394	327	360	287	327	410	318	326	337

Figure 6 compares the number of distinct phosphopeptides before and after enrichment in a histogram. For the 12 enriched samples, the average number of distinct phosphopeptides ranged from 957 for R1 samples to 749 for R4 samples. Higher phosphopeptide ID numbers were achieved with more sample loading, but not proportional to the sample/resin ratio. The combined number of distinct phosphopeptides from three replicates was from 1,513 for R1 samples to 1,188 for R4 samples.

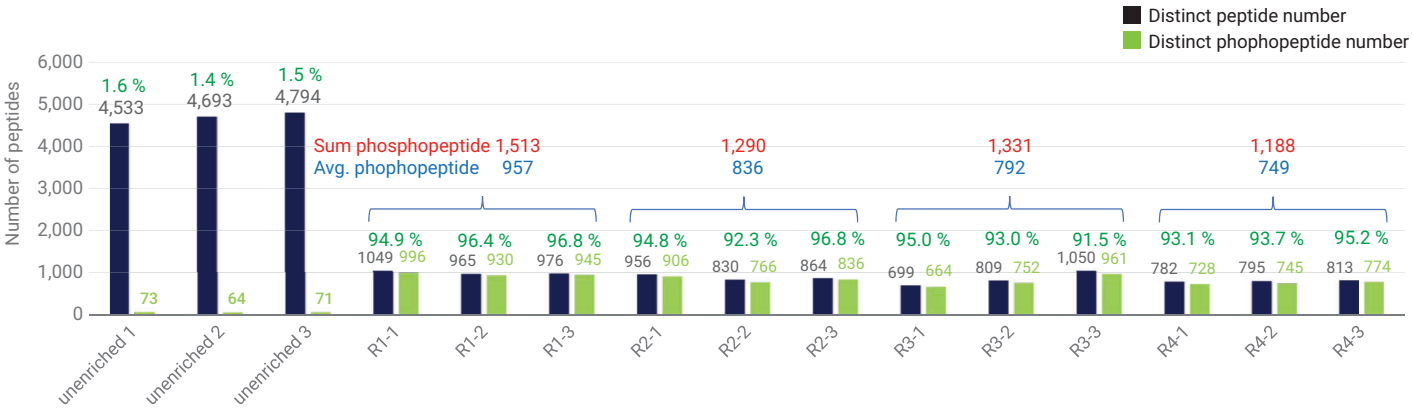

Figure 6. Number of distinct phosphopeptides identified before and after enrichment.

Quantitation of the standard phosphopeptide enrichment yield using MRM

Since the same amount of light and heavy isotope standard phosphopeptides were spiked into the samples before and after enrichment separately, MRM of both light and heavy standards in the final sample can be used to calculate the recovery of the enrichment. Figure 7 shows the DMRM results of 20 standard phosphopeptides (10 light and 10 heavy isotope) from the 6495B TQ MS. The light and heavy isotopic peptides have the same retention time. Since the molecular weight difference between the light and the heavy peptides are either 8 or 10 Da, TQ can easily quantify them based on the peak ratio between light and heavy transitions[2].

The MRM results were imported into Skyline, and the yield of enrichment was calculated based on the ratio between the most abundant transitions of light and heavy peptides. Figure 8 shows the quantitation result generated from Skyline for sample R1-1. The yield of each phosphopeptide standard was calculated using the ratio between light and heavy peak areas. The transitions of the light and heavy peptide 1.4 (tKLItQLRDAK) are shown as an example.

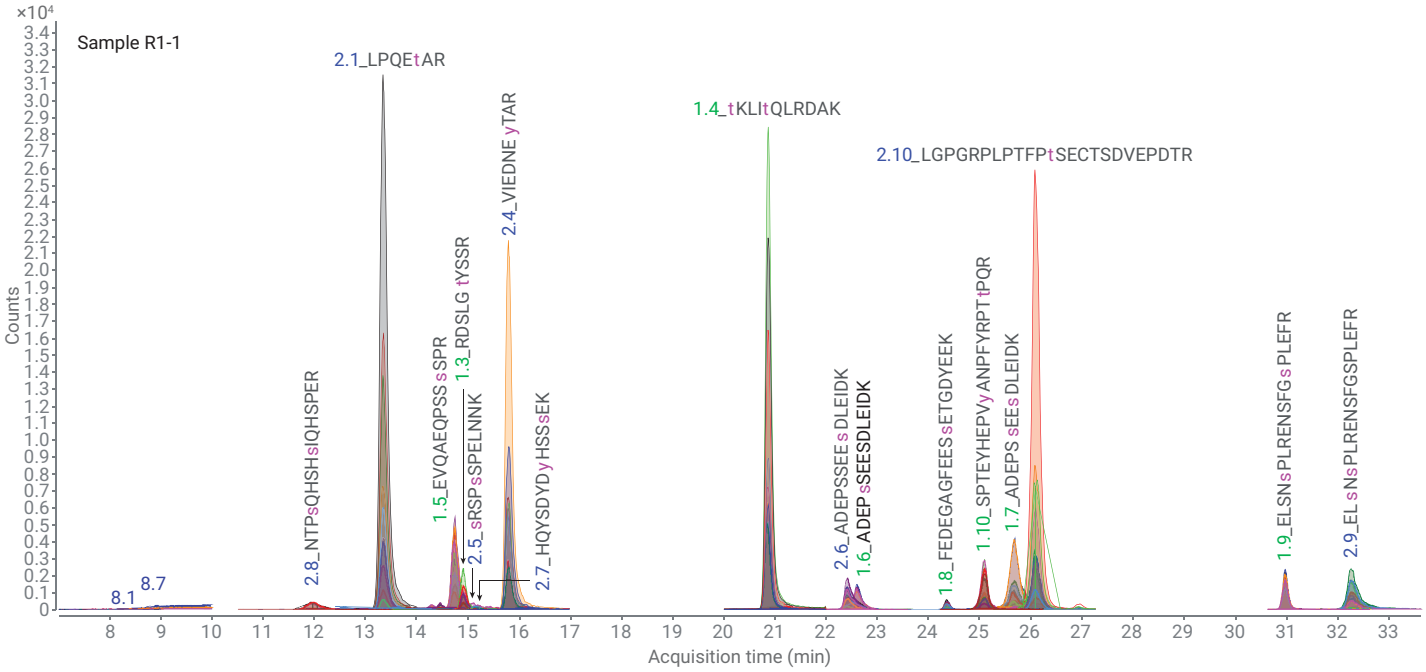

Figure 7. The dynamic MRM profile of phosphoMix standards.

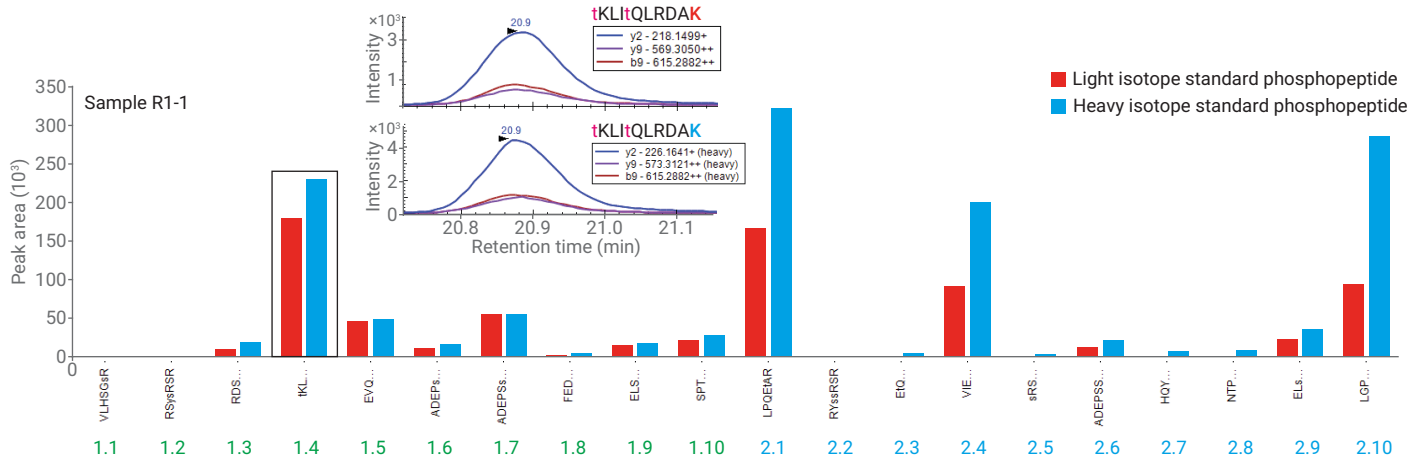

Figure 8. Yield of the standard phosphopeptide enrichment in sample R1-1.

Table 6. Average yield of enrichment for the phosphopeptide standards.

Sequence	PhosphoMix	Phosphosite	Average yield of enrichment (%)	Average yield of enrichment (%)	Average yield of enrichment (%)	Average yield of enrichment (%)	RSD (%)
			Sample R1	Sample R2	Sample R3	Sample R4	
VLHSG sR	1_1	S6	not found	not found	not found	not found	NA
RS ys RSR	1_2	Y3, S4	not found	not found	not found	not found	NA
RDSLG tYSSR	1_3	T6	54.3	51.9	58.0	58.7	3.2
tKLItQLRDAK	1_4	T1, T5	82.0	78.9	83.7	87.7	3.6
EVQAEQPSS sSPR	1_5	S10	94.7	95.7	94.0	96.0	0.9
ADEP sSEESDLEIDK	1_6	S5	73.6	67.1	73.7	76.7	4.0
ADEPS sEE sDLEIDK	1_7	S6, S9	95.7	92.3	99.0	101.3	3.9
FEDEGAGFEES sETGDYEEK	1_8	S12	79.7	77.3	81.0	92.0	6.5
ELSN sPLRENSFG sPLEFR	1_9	S5, S14	87.3	83.3	88.0	88.0	2.3
SPTEYHEPV yANPFYRPT tPQR	1_10	Y10, T19	84.7	79.5	84.7	74.0	5.1
LPQE tAR	2_1	T5	53.0	50.5	56.7	52.0	2.6
RY ss RSR	2_2	S3, S4	not found	not found	not found	not found	NA
EtQSPEQVK	2_3	T2	not found	not found	not found	not found	NA
VIEDNE yTAR	2_4	Y7	48.3	46.5	51.3	50.7	2.2
sRSP sSPELNNK	2_5	S1, S5	65.0	71.3	58.3	65.0	5.3
ADEPSSEE sDLEIDK	2_6	S9	61.0	56.6	68.3	66.3	5.3
HQYSDYD yHSS sEK	2_7	Y8, S12	6.0	5.3	5.7	4.0	0.9
NTP sQHSH sIQHSPER	2_8	S4, S9	12.0	18.0	14.3	not found	3.0
EL sN sPLRENSFGSPLEFR	2_9	S3, S5	70.3	67.0	70.0	77.3	4.4
LGPGRPLPTFP tSECTSDVEPDTR	2_10	T12	35.7	33.3	36.3	35.0	1.3
			62.7	60.9	63.9	64.0	1.4

Table 6 summarizes the average yield for the 20 peptides in the four sample/resin ratios. We identified 16 out of 20 phosphopeptides. The four missing phosphopeptides showed either no peak or a very low-abundance peak on the vendor's datasheet[2]. The enrichment yield was consistent for the same phosphopeptide across the four ratios, with approximately 63 % overall recovery. Note that the recovery of each phosphopeptide within the same sample could be very different. The yield for some phosphopeptides were very high, with nearly 100 % recovery, while a few others were less than 10 %. This difference was deduced to be structure-related. The recovery variation (%RSD) for each of the 16 phosphopeptide standards across samples with different sample/resin ratios was less than, or approximately, 5 %. This shows very good reproducibility of the phosphopeptide enrichment and the robustness of the LC/TQ MS analysis.

Conclusions

A fully automated phosphopeptide enrichment from a complex cell line digest was implemented using the Agilent AssayMAP Bravo. AssayMAP enabled high-throughput and reproducible phosphopeptide enrichment through a cartridge-based platform. The enrichment result showed excellent selectivity (>90 %) across all samples with different sample/resin ratios.

The nanodapter effectively converted a standard-flow UHPLC to a nanoflow UHPLC, allowing users to have both flow options in one system. The nanoflow and nanoESI source enabled both sensitive and quantitative analysis.

The 6550 iFunnel Q-TOF LC/MS offered the ultimate analytical sensitivity, identifying 1,200 to 1,500 distinct phosphopeptides by injecting 1/5 of the enriched sample with four different sample/resin ratios. Phosphopeptide selectivity increased from 1.5 % before enrichment to over 90 % after enrichment for all enriched samples. AssayMAP Bravo enabled reproducible enrichment resulting in consistent high selectivity for all samples. Different degrees of phosphorylation were identified with approximately 55 to 60 % phospho-sites assigned. Across the four sample/resin ratios, we found that the distinct number of phosphopeptides increased with more sample loading, but not proportional to the ratio.

The 6495B TQ LC/MS worked with Skyline seamlessly to provide an automated MRM analysis for calculating the enrichment yield. The yield for each phosphopeptide within the same sample varied, ranging from less than 10 % to nearly 100 %. However, the same phosphopeptide standards showed consistent yield across different sample/resin ratios, with approximately 63 % overall recovery for the 20 phosphopeptide standards. The yield for each phosphopeptide standard showed excellent reproducibility, with less than 5 % RSD of the enrichment and robustness of the peptide quantitation.

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