Automated High-throughput Phosphopeptide Enrichment Using TiO_2 Dispersive Pipette Extraction

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INTRODUCTION

Phosphorylation events are key signal transduction mechanisms within the eukaryotic cells and further understanding such signaling pathways can elucidate crucial molecular signatures of various human diseases (1-3). The global deep profiling of phosphopeptide has been successfully optimized in the procedures of enrichment, mass spectrometry, and data analysis (4,5). However, phosphopeptide enrichment is highly labor-intensive and a low-throughput process that leads to poor reproducibility (6-8). Here, we introduce a phosphopeptide enrichment leveraging dispersive pipette extraction technology on IMCStips. In comparison to conventional solid phase extraction technologies, the patented dispersive pipette extraction process uses loose resin that mixes during aspiration and dispense steps. This increases the interaction between the resin and sample via turbulent mixing within the pipette tips. The tips containing titanium oxide (TiO_2) resin were used to explore the initial feasibility of DPX technology for phosphopeptide enrichment. The reported method uses automated liquid handling systems, such as Integra ViaFlo96, to enrich phosphopeptides from cell lysates for high-throughput and reproducible sample preparation to study phosphorylation events.

MATERIALS AND METHODS

HEK293T cells were treated with 5 mM H_2O_2 for 15 minutes and washed three times with cold PBS. The cells were lysed with 8 M Urea buffer containing a protease and phosphatase inhibitor cocktail. 10 mM dithiothreitol was added to reduce at 56 °C for 30 minutes, then 25 mM iodoacetamide was added for alkylation for 30 minutes in the dark followed by overnight tryptic digestion at 37 °C. For automatic sample processing, we developed a method for the VIAFLO96 from Integra and other high-throughput robotic system (*Figure 1, 2*).

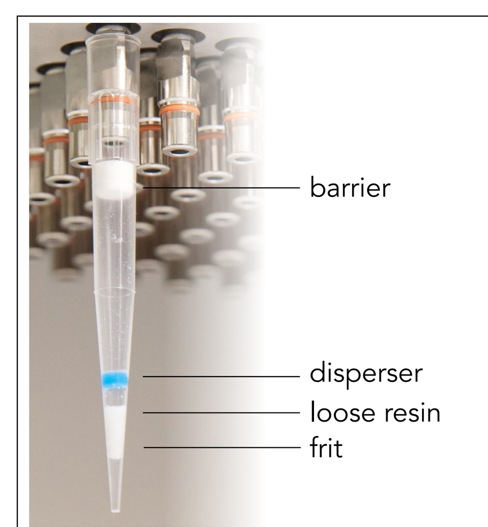

Figure 1. Components in dispersive pipette extraction, IMCStips

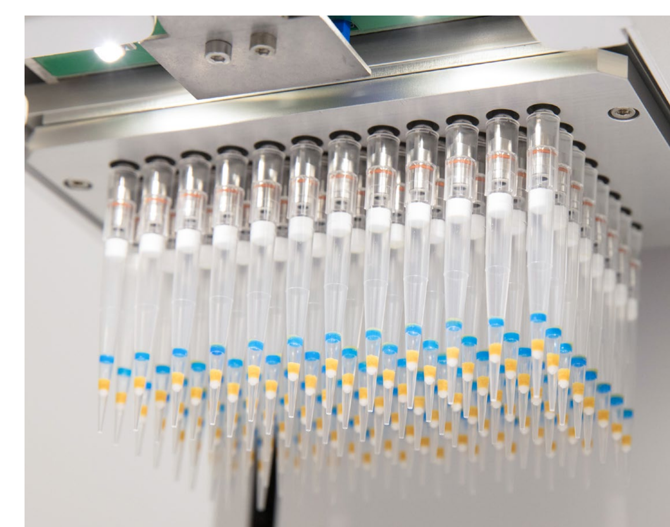

Figure 2. Desalting IMCStips with RP resin on a VIAFLO96 from Integra

To measure the enriched phosphopeptide quantity, we used a UPLC TSQ Endura triple quadrupole mass spectrometry with optimized conditions. For global phosphopeptide identification, we used a Q-Exactive HF mass spectrometer coupled with Ultimate 3000 nano-UHPLC system.

MS spectra were acquired by data dependent scans consisting of MS/MS scans of the twenty most intense ions from the full MS scan with dynamic exclusion option which was 10 seconds. Spectra were searched using Sequest HT algorithm within Proteome Discoverer v2.1 (Thermo Scientific) in combination with the human UniProt protein FASTA database (2017 March, 20,119 entries). Search parameters were as follow; FT-trap instrument, parent mass error tolerance of 10 ppm, fragment mass error tolerance of 0.02 Da (monoisotopic), variable modifications of 15.995 Da (oxidation) on methionine and 79.966 Da (phosphorylation) on serine, threonine and tyrosine, fixed modification of 57.021 Da (carbamidomethylation) on cysteine.

	Quantification	Identification
Mass spectrometer	TSQ Endure, Thermo Fisher	Q-Exactive Thermo Fisher
Liquid chromatography	Vanquish UPLC	Ultimate 3000 nano-UHPLC
Mobile phase A	0.1% formic acid (F.A.) in water	0.1% F.A. in water
Mobile phase B	0.1% F.A. in acetonitrile	0.1% F.A. in acetonitrile
LC gradient	5% - 35% B for 15 minutes	2% - 30% B for 170 minutes
Trap column	N/A	Acclaim PepMap 100 (C_{18}, 5 µm, 100 Å, 300 µm X 5mm)
Analytical column	Syncronis C_{18}, 100 X 2.1 mm, 1.7 µm	Acclaim PepMap RSLC (C_{18}, 2 µm, 100 Å, 75 µm X 15 cm)
Column oven temperature	40 °C	Room temperature

RESULTS

We established a selected reaction monitoring (SRM) method for TSQ triple quadrupole mass spectrometer to test recovery and non-specific binding of TiO_2-IMCStips enrichment using phosphopeptide and non-phosphopeptide standards (**Table 1**).

Table 1. Selected Reaction Monitoring Transitions for the Standard Peptides

Peptide Name	Sequence	Parent Mass m/z	Product Ion 1, m/z	Product Ion 2, m/z	Collision Energy, V
Bradykinin	RPPGFSPFR	354.2	506.3	419.2	15.7
Angiotensin 1	DRVYIHPFHL	432.9	647.4	619.4	18.7
Angiotensin 2	DRVYIHPF	349.5	513.3	371.2	15.6
Leptin	NVIQISNDLENLR	509.9	644.4	531.3	21.7
ACTH18-39	RPVKVYPNGAEDESAEAFPLEF	822.4	505.3	981.0	33.5
Neurotensin	ELYENKPRRPYIL	558.3	483.6	197.1	23.5
b-Casein	FQpSEEQQQTEDELQDK	687.9	977.4	747.4	28.4
PKA Regulatory Subunit 2	DLDVPIPGRFDRRVpSVAAE	731.4	875.4	826.9	30.1
UOM9, pPKC Substrate - 3	KRPpSQRHGSKY	475.2	436.8	572.8	20.3
Insulin Receptor kinase domain	TRDIYETDpYYRK	568.3	925.4	824.3	23.9
pAngiotensin 2	DRVpYIHPF	376.2	371.2	756.3	15.0
pCholecystokinin	IKNLQpSLDPSH	444.6	340.2	455.2	25.0
pCalcitonin	DFNKFHpTFPQTAIGV	601.3	757.8	814.4	15.0

**Phosphorylated amino acid.*

Automatic sample preparation using aVIAFLO96 and a high-throughput automatic liquid handling system was optimized for 1 mL tips packed with 5 or 10 mg TiO_2 resin. The whole enrichment process took less than 30 minutes with minimal hands-on time (**Table 2**).

Table 2. TiO_2 Phosphopeptide Enrichment Protocol using 1 mL IMCStips.

Steps	Process	Solvent	Aspiration µL	Volume µL	Repeat #	Duration minutes
1	Activation	100 % ACN	600	800	2	0.6
2	Condition	TiO_2 Buffer A	400	800	3	1.0
3	Equilibrate	TiO_2 Buffer B	400	800	3	1.0
4	Bind	TiO_2 Buffer B	400	500	20	6.8
5	Wash 1	TiO_2 Buffer B	400	800	3	1.0
6	Wash 2	TiO_2 Buffer A	400	800	3	1.0
7	Elution 1	1.5% NH_4OH	400	400	5	1.6
8	Elution 2	1.5% NH_4OH, 10% ACN	400	400	5	1.6
					Total	14.6

- TiO_2 Buffer A: 80% ACN, 0.4% TFA
- TiO_2 Buffer B: 25% Lactic acid, 60% ACN, 0.3% TFA

We optimized pre- and post-enrichment desalting with IMCStips to increase enrichment efficiency. We compared five types of reverse phase resins in the spin column format to five reverse phase resins in IMCStips for the pre-enrichment desalting. Spin-C_{18}T and Spin-C_{18}N are competitor resins to IMCStips resins (**Table 3**).

Table 3. Comparison of the desalting methods and materials using six standard peptides.

Resin Type	Bradykinin	Angiotensin I	Angiotensin II	Leptin	ACTH18-39	Neurotensin
Spin-C_{18}T	80 ± 12.6%*	79 ± 4.3%	74 ± 0.4%	79 ± 9.1%	93 ± 5.4%	73 ± 0.6%
Spin-C_{18}N	107 ± 3.1%	106 ± 0.6%	94 ± 0.2%	110 ± 3.6%	122 ± 2.0%	94 ± 0.9%
Spin-C_{18}A1	104 ± 1.3%	107 ± 2.1%	95 ± 2.3%	95 ± 7.9%	119 ± 2.7%	93 ± 4.0%
Spin-C_{18}A3	102 ± 2.0%	102 ± 2.3%	91 ± 2.0%	90 ± 3.5%	114 ± 3.9%	93 ± 5.5%
Spin-RP	85 ± 1.1%	77 ± 0.7%	75 ± 2.3%	65 ± 9.2%	88 ± 1.4%	73 ± 0.7%
Tip-C_{18}A1	63 ± 9.3%	64 ± 10.8%	62 ± 5.9%	56 ± 4.8%	70 ± 8.8%	58 ± 7.7%
Tip-C_{18}A1/RP	72 ± 4.8%	69 ± 6.6%	68 ± 4.0%	62 ± 4.5%	84 ± 5.5%	67 ± 2.5%
Tip-C_{18}A3	96 ± 9.7%	98 ± 7.4%	88 ± 6.8%	93 ± 7.3%	117 ± 6.9%	89 ± 8.1%
Tip-C_{18}A3/RP	95 ± 1.6%	94 ± 4.7%	85 ± 0.5%	85 ± 3.6%	115 ± 3.1%	88 ± 3.4%
Tip-RP	85 ± 0.4%	86 ± 0.2%	78 ± 1.2%	82 ± 8.8%	105 ± 4.7%	79 ± 2.0%

** Average recovery (%) ± Relative standard deviation (RSD), 8mg of resin was used for spin columns and IMCStips.*

With the optimized automatic liquid handling protocol, the average recovery of the three exogenous phosphopeptide standards was greater than 81% with minimal non-phosphopeptide binding to the TiO_2 IMCStips (*Figure 3.*)

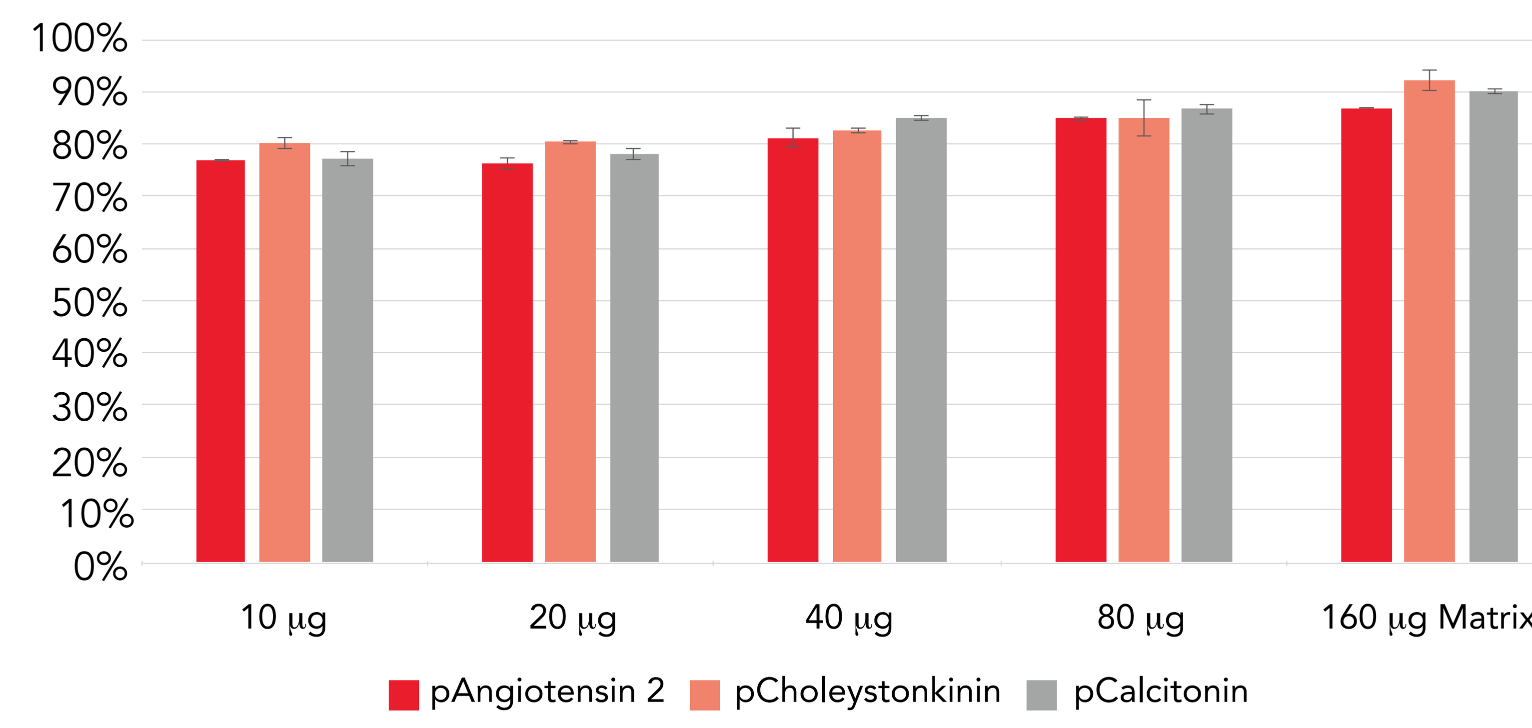

Figure 3. Average phosphopeptide recovery from 10, 20, 49, 80, 160 µg α-, β-casein tryptic digests using 2 mg TiO_2 300 µL IMCStips on a high-throughput automatic liquid handling system.

To test global phosphoproteomics application, we used 500 µg HEK293T cell digest and enriched phosphopeptides using automatic TiO_2 dispersive pipette extraction technology in IMCStips. With the optimized pre- and post-enrichment desalting procedures, we identified over 1300 phosphopeptides with 88.6% specificity (*Figure 4.*)

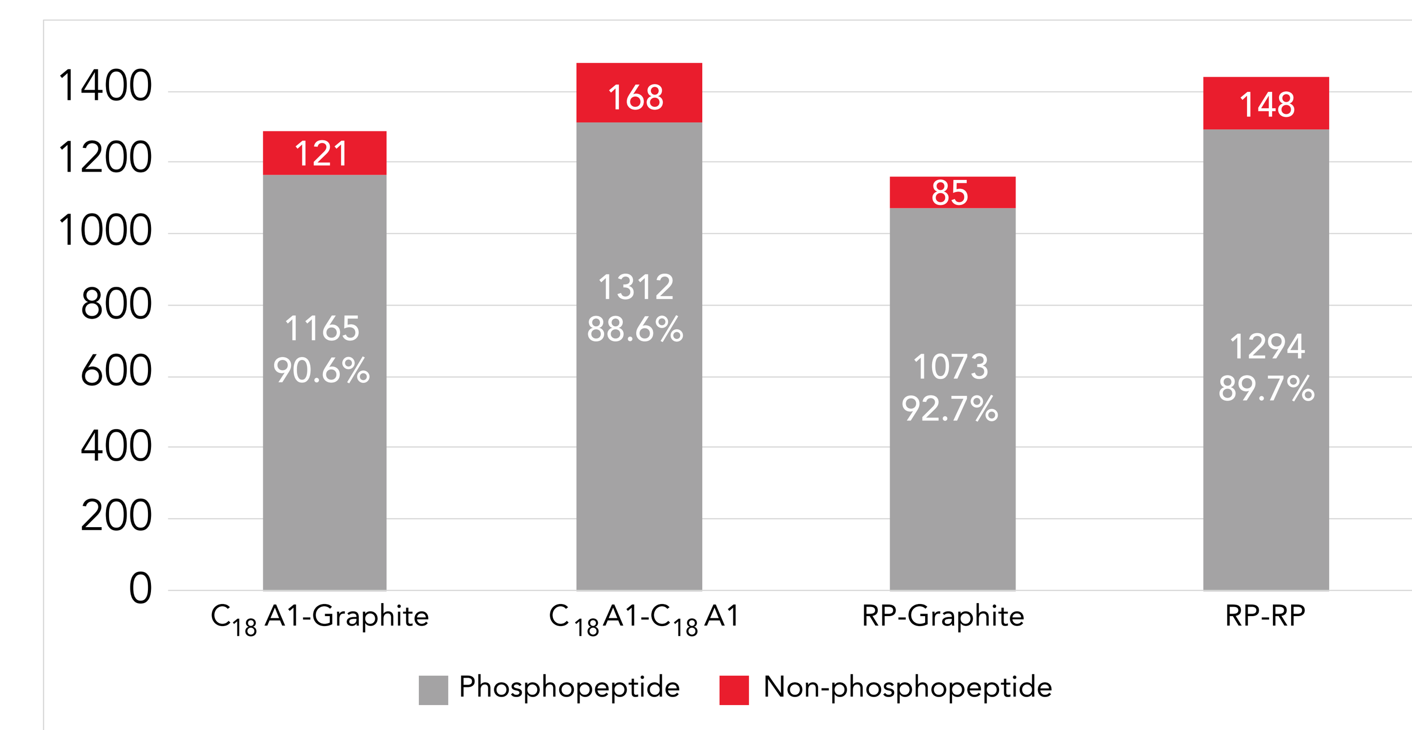

Figure 4. Phosphopeptide identification using TiO_2 IMCStips enrichement and Q-Exactive LC-MS/MS. Pre-enrichment desalting: 50 mg C_{18}A1 and RP spin columns, Phosphopeptide enrichment: 10mg TiO_2 IMCS 1 mL IMCS tips, post-enrichment clean-up: Thermo graphite spin column, 50 mg C_{18}A1 and RP IMCS tips.

We further tested volatile base elution buffers to increase the efficiency and eliminate the desalting step followed by TiO2 phosphopeptide enrichment (*Figure 5*).

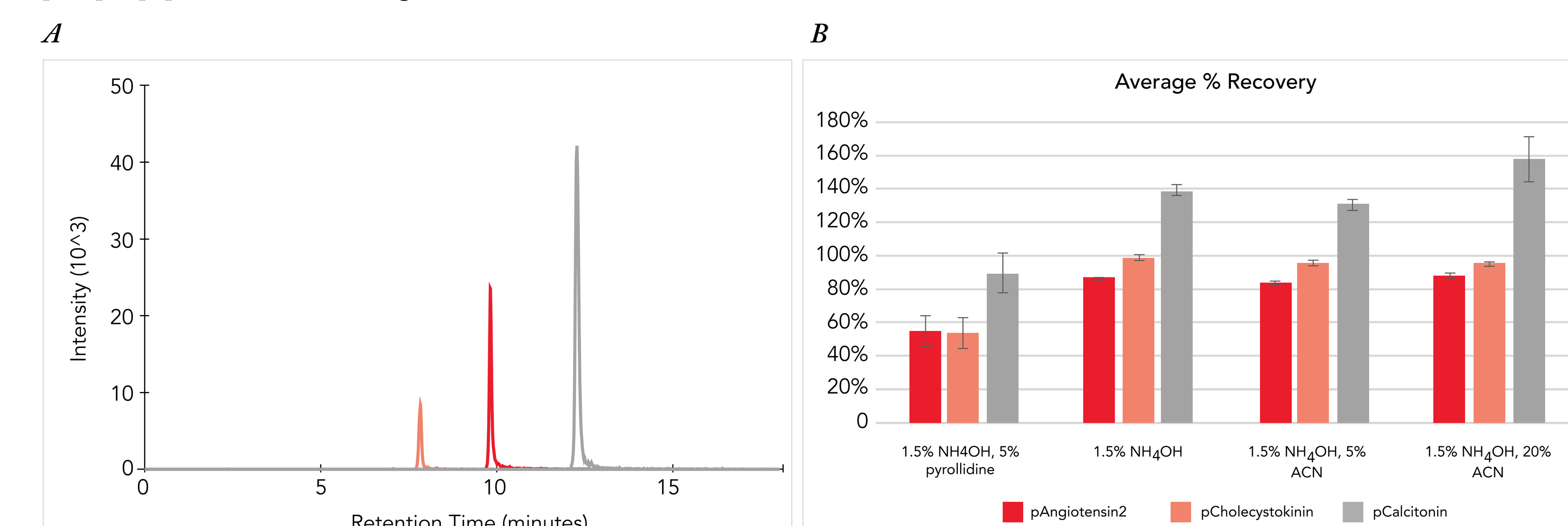

Figure 5. Optimization of the elution buffers. A) Extracted ion chromatogram and automatic integration of the area under the curve for the phosphopeptides using Skyline software, B) 10 pmols of three phosphopeptides were spiked into the sample prior to the TiO_2 enrichment. The elutes were dried completely and resuspended with 2% ACN, 5% Formic acid for TSQ analysis.

CONCLUSIONS

Dispersive pipette extraction for phosphopeptide enrichment using an automatic liquid handling system increases reproducibility and specificity for biopharmaceutical research. IMCStips containing TiO_2 showed nearly 90% recovery of the phosphopeptide standards even in the presence of alpha and beta casein peptides. For the global proteomics, over 1,300 phosphopeptides with 88.6% specificity were enriched from HEK293T cell lysates. The enrichment process involved minimal hands-on time and the entire process was executed on the robotic system without additional fractionations. The ability to automate such complex sample preparations would lead to reduced error and noise on the sample, and the key signal of the cellular phosphorylation event in a limited number of biological replicate will be elucidated with less time and effort.

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Abbreviations:
ACN: Acetonitrile; AUC: Area under the curve; C_{18}A1: C_{18} 100 Å resin; C_{18}A3: C_{18} 300 Å resin; C_{18}N: C_{18} spin column from vendor N; C_{18}T: C_{18} spin column from vendor T; RP: wettable polystyrene cross linked with divinylbenzene; F.A.: Formic acid; GCB: Graphitized carbon black; TFA: Trifluoroacetic acid.

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