Automated Immunoaffinity Enrichment for Phosphotyrosine Peptides Using Protein A and Streptavidin IMCStips®

Sunil Hwang*¹, Todd Mullis², Michael Walla², Matthew Manter¹, Jingyun Lee³, Christina M Furdui³, L. Andrew Lee¹

¹ Integrated Micro-Chromatography Systems, Inc, Columbia, SC; ² University of South Carolina, Columbia, SC; ³ Wake Forest School of Medicine, Winston-Salem, NC



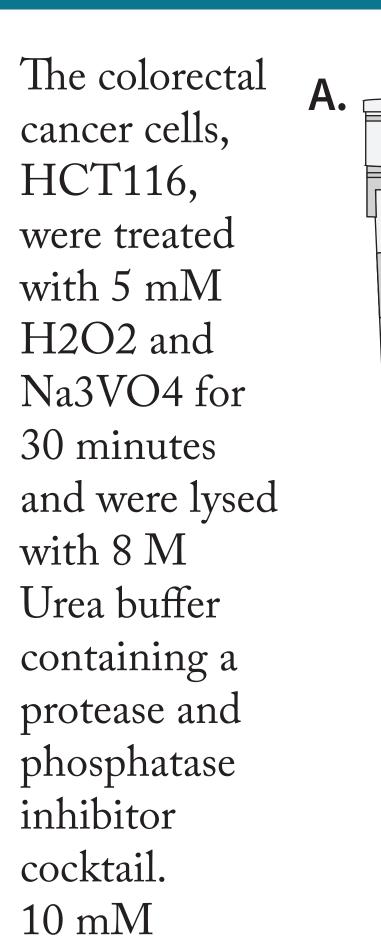
ABSTRACT

- Established automated Protein A and Streptavidin immunoaffinity enrichment protocol for highthroughput sample preparation
- Identified over 1000 phosphotyrosine peptides using anti-phosphotyrosine antibodies and Protein A / Streptavidin IMCStips
- Achieved over 94% specificity of phosphotyrosine peptide enrichment using Hamilton® Microlab STAR workstation and IMCStips

INTRODUCTION

Signal transduction of protein phosphorylation is a key mechanism for survival, maintenance, and death of eukaryotic cells (1-4). The global enrichment of phosphoserine and phosphothreonine peptides has been successfully optimized using immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO2) using IMCStips with an automated liquid handling system. However, enrichment of phosphotyrosine (p-Tyr) peptides are less efficient with these methods due to the low stoichiometry of p-Tyr peptides to global phosphorylation event (5-11). Here, we introduce anti-phosphotyrosine antibody enrichment using a dispersive pipette extraction technology in IMCStips. The method uses an automatic liquid handling system to enrich p-Tyr peptides for high-throughput and reproducible sample preparation.

METHODS



dithiothreitol

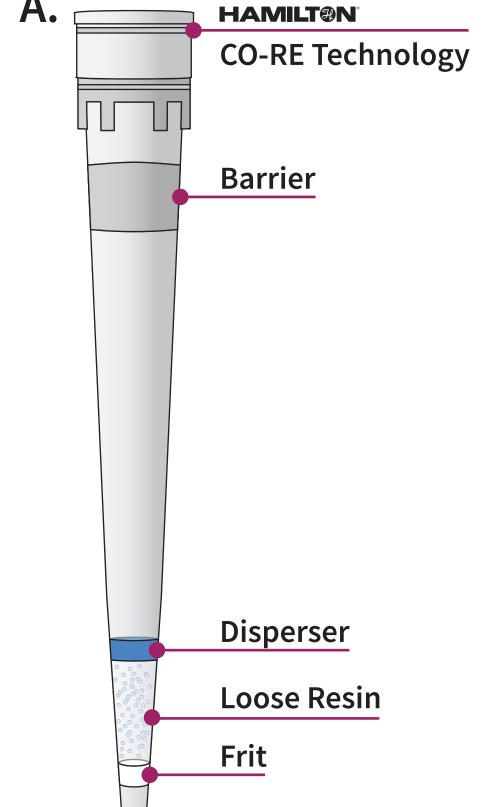




Figure 1. (a) IMCStips Diagram (b) Hamilton® Microlab STAR workstation

was added at 56 °C for 30 minutes to reduce disulfide bonds, then 50 mM iodoacetamide was added for alkylation of thiols for 30 minutes in the dark followed by overnight tryptic digestion at 37 °C. The p-Tyr-100 and p-Tyr-1000 anti-phosphotyrosine antibodies with and without biotin-tags were purchased from Cell Signaling Technology and 4G10 anti-phosphotyrosine antibodies with and without biotin-tags were purchased from MilliporeSigma. For automated sample processing, we developed a method for the Hamilton® Microlab STAR workstation using IMCStips (Figure 1).

The p-Tyr peptides were eluted with 0.1% formic acid, water and dried down completely. Once dried, pellets were stored at -80 °C and resuspended in 20 µL of 3% acetonitrile, 0.5% formic acid, which was then injected to LC-MS/MS.

To measure enriched p-Tyr peptides quantity, we used UPLC TSQ Endura triple quadrupole mass spectrometry with optimized conditions (Data not shown here). For global phosphopeptide identification, we used the Q ExactiveTM HF mass spectrometer coupled with the Ultimate 3000 nano-UHPLC system for the mixed antibody enriched samples. 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.

For evaluating two individual antibodies, samples were resuspended in 10 µL 20 mM citric acid and 2% ACN, and 4 µL was injected. Analysis was performed using 90 minutes gradient 3-30% ACN with 0.1% formic acid on a 25 cm x 75 um HSS T3 C18 column at 55 °C and 0.4 µL/min on nano-ACQUITY UPLC system (Waters) followed by Thermo Fusion Lumos mass spectrometry analysis with 120k MS1

and 15k MS2 HCD fragmentation settings.

Spectra were searched using Sequest HT algorithm within Proteome Discoverer v2.2 (Thermo Scientific) against 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.

We used Venny and Venn diagram webtools to find unique and common components of the data (12, 13) and STRING (version 10.5) and DAVID (version 6.8) webtools for the pathway analysis, functional annotation, and data mining (14, 15).

RESULTS

Optimization of Hamilton® Microlab STAR workstation



Figure 2. Deck layout of the Hamilton® Microlab STAR workstation for the phosphotyrosine immunoaffinity enrichment

Automatic sample preparation using the Hamilton® Microlab STAR workstation was optimized for 300 μL tips, each containing 5 μL protein A or streptavidin resins. The deck layout of the STAR system for the immunoaffinity enrichment and reverse phase desalting is shown

In Figure 2. The entire automatic affinity capture process took less than 50 minutes and required minimal hands-on time, followed by the optional peptide desalting for LC-MS/MS analysis (Table

Briefly, the anti-phosphotyrosine antibodies in tryptic digests were incubated overnight at 4°C on a rotator and the antibody complexes were captured using the Hamilton® Microlab STAR workstation as listed below. We optimized liquid classes for the immunoaffinity enrichment of the Hamilton® Microlab STAR workstation and the settings are listed below (Table 2).

#	Procedure	Buffer	Buffer, μL	Aspiration, μL	Cycle#
1	Condition	TBS	600	300	3
2	Equilibration	TBS	600	300	3
3	Binding	TBS	400	300	20
4	Washing 1	TBS	600	300	3
5	Washing 2	Water	600	300	3
6	Elution	0.5% FA	400	300	5
7	RP - Condition	100% ACN	600	300	3
8	RP – Equilibration	1% TFA	600	300	3
9	RP – Binding	1% TFA	600	300	10
10	RP – Washing 1	1% TFA	600	300	3
11	RP – Washing 2	0.5% FA	600	300	3
12	Elution	50% ACN, 0.5% FA	400	300	5

Table 1. Workflow of phosphotyrosine peptide immunoaffinity enrichment and cleanup using Hamilton® Microlab

	Aspiration (μl/sec)	Dispense (μl/sec)	Swap speed (mm/ sec)	Settling time (sec)
Protein A	100	5	2	1
Streptavidin	100	5	2	1
Desalting	100	10	2	1

Table 2. Liquid classes of the Hamilton® Microlab STAR workstation for immunoaffinity enrichment and desalting using 300 µL tips

Phosphotyrosine immunoaffinity enrichment using Protein A or Streptavidin IMCStips®

In order to test global high-throughput immunoaffinity enrichment for phosphotyrosine peptides, we used 1 mg HCT116 colorectal cancer cell digests and three anti-phosphotyrosine antibodies. In each experiment, 20 µL of three non-biotinylated or biotinylated anti-phosphotyrosine antibodies were added to 1 mg HCT116 cell digests in 400 µL 1x Tris-buffered saline (TBS), pH 7.4 and

incubated on rotator overnight at 4 °C. Antibody and peptide mixtures were isolated using Protein A IMCStips and p-Tyr peptides were eluted using 0.1% formic acid in water. The co-eluted antibodies then went through a cleaning process using reverse phase IMCStips and were dried down completely. Biotinylated anti-phosphotyrosine antibodies and p-Tyr peptide immune-complexes were isolated using streptavidin IMCStips. The specificity of the phosphotyrosine peptide enrichment from all identified phosphopeptides using Protein A and Streptavidin tips were 94.1% and 94.6%, respectively (Figure 3 A) The overlap of identified phosphotyrosine protein using two different tips was 78.4% (Figure 3 B).

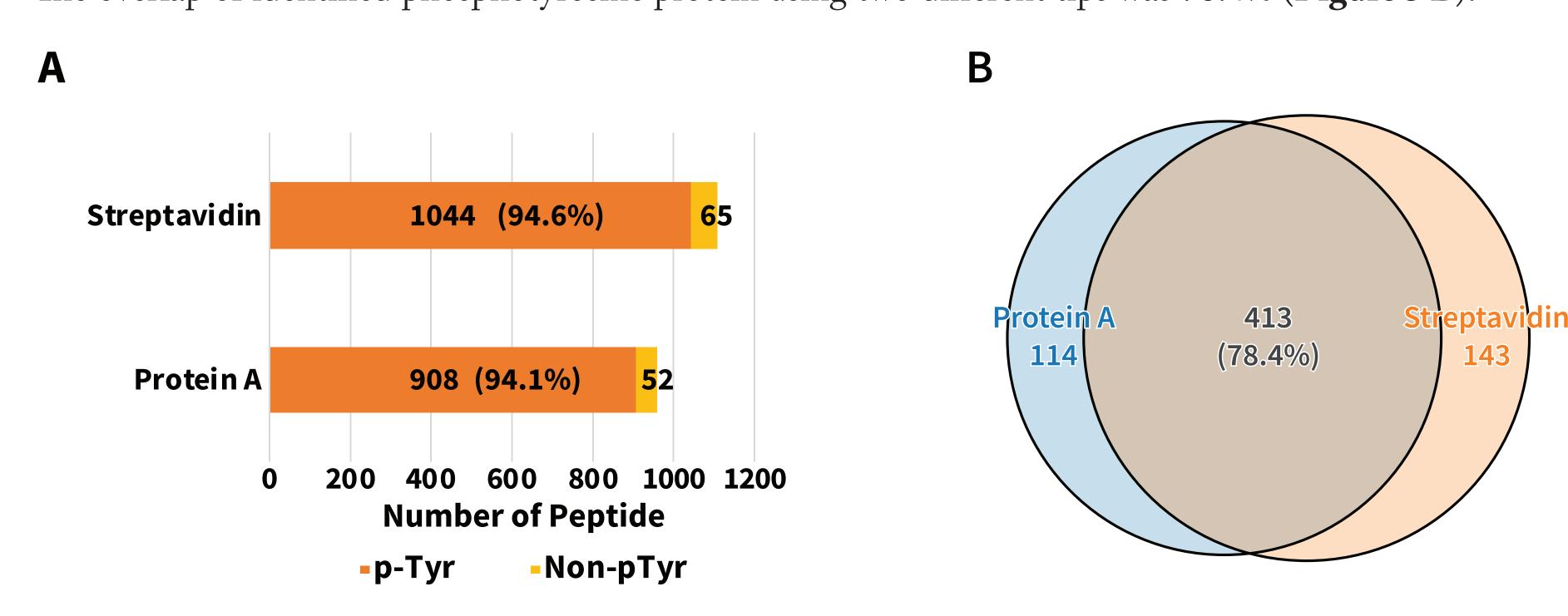


Figure 3. Specificity of the phosphotyrosine enrichment from identified phosphopeptides (A) and overlap of the phosphotyrosine proteins (B) To test the specificity of anti-phosphotyrosine antibodies, we used 20 μL of p-Tyr-1000 (Cell Signaling Technology), 4G10 (MilliporeSigma), and 1:1 mix of these two antibodies and compared the overlap of the identified phosphotyrosine containing proteins. Only 55.5% of phosphotyrosine containing proteins

were in common between p-Tyr-1000 and 4G10 antibody enrichments (Figure 4).

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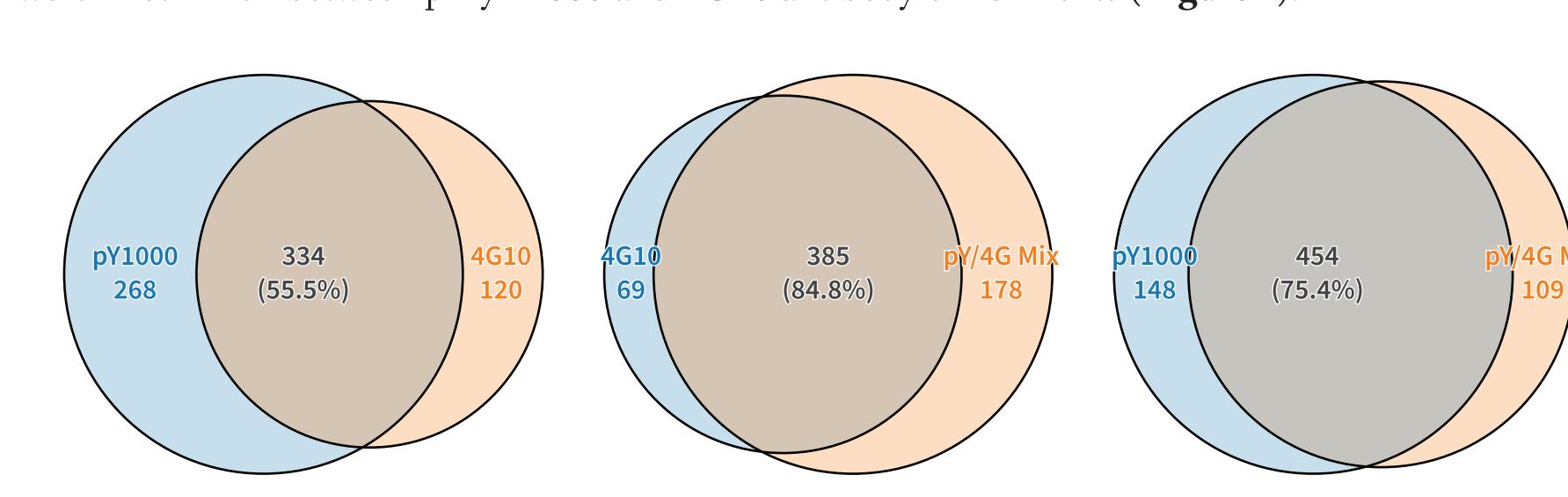


Figure 4. Overlap of the identified phosphotyrosine proteins between two different antibodies

Comprehensive phosphoproteome analysis

To capture comprehensive signaling of the phosphoserine, phosphothreonine, and phosphotyrosine peptides, we utilized the immobilized metal affinity chromatography, PolyTiTM (Tymora Analytical) and ZrO, IMCStips, from flow-throughs of the immunoaffinity enrichment (See other poster: # ThP 638, Comparison of Different IMAC Resins for Automated Phosphopeptide Enrichments for Deep-Profiling of HCT 116 Cell Line). While there was no difference of the enrichment efficiency of these two resins from fresh digests, PolyTiTM IMCStips identified > 1.7 times more phosphopeptides than ZrO₂ tips (Figure 5).

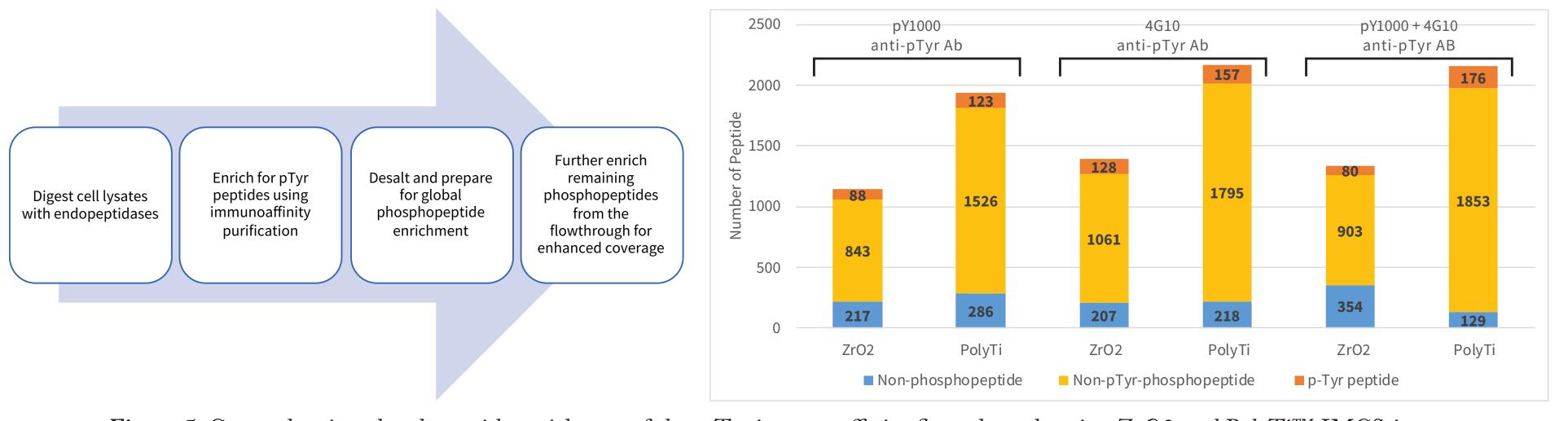


Figure 5. Comprehensive phosphopeptide enrichment of the p-Tyr immunoaffinity flow-throughs using ZrO2 and $PolyTi^{TM}$ IMCStips

Pathway analysis, functional annotation, and data mining

We used webtools such as STRING and DAVID for data mining of the identified phosphotyrosine peptides with relative quantification by number of peptide spectral matches (PSMs). By filtering data using PSMs, we defined the total network into a focused regulated phosphotyrosine signaling network using STRING software (Figure 6 A and B). We used DAVID for summary of functional annotation, disease, categories, gene ontology, pathways, protein domains, and clustering of the data (Figure 6 C).

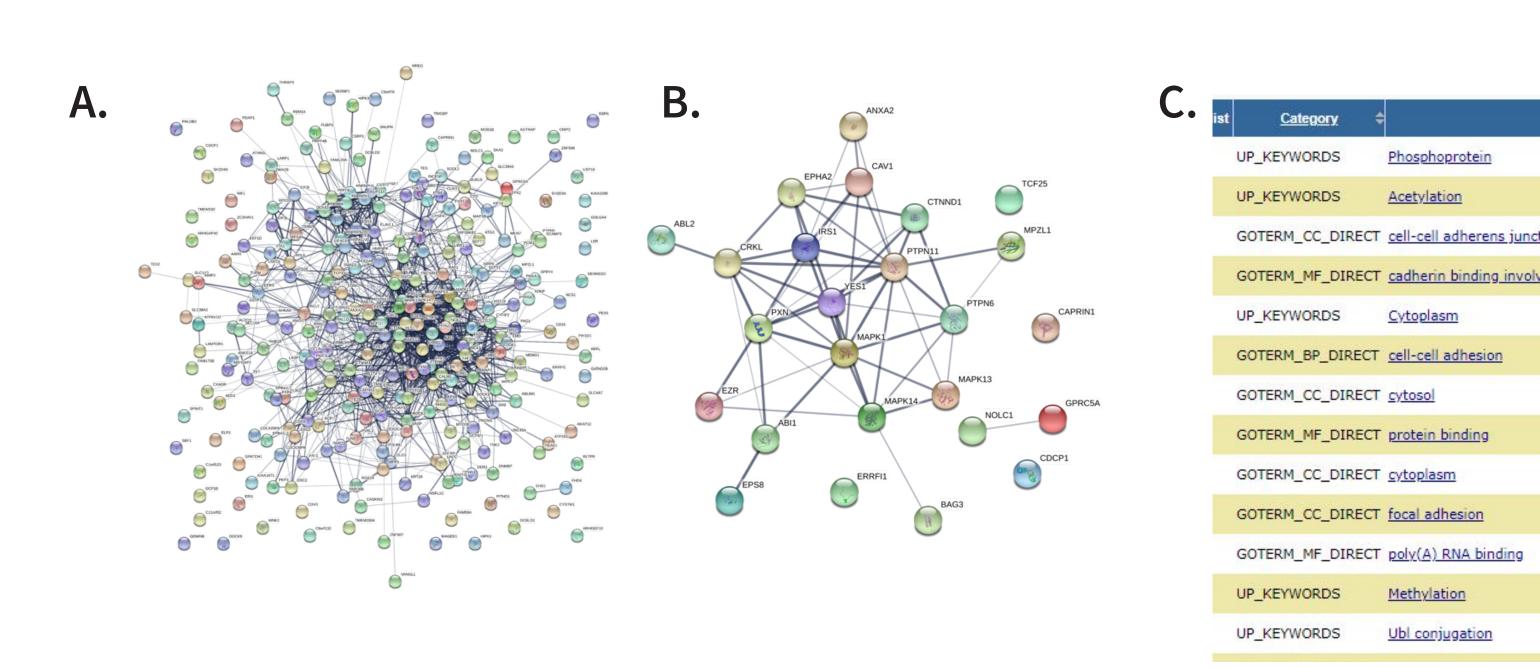


Figure 6. Functional protein association networks using STRING (A, B) and functional annotation using DAVID (C)

CONCLUSIONS

With optimized p-Tyr peptide enrichment workflow on the Hamilton® Microlab STAR workstation, we were able to identify over 1,000 phosphotyrosine peptides with over 90% specificity within one hour of processing time.

A future workflow will be created to further reduce processing time, which will also contain a buffer dispense, on-plate immunocapturing, IMAC, and combined RP desalting cleanup in on single process.

Protein A and streptavidin IMCStips for phosphotyrosine peptide immuno-enrichment using automatic liquid handling system increases throughput and reproducibility for biopharmaceutical research.

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Abbreviations

- ACN: Acetonitrile, FA: Formic acid, IMAC: Immobilized metal affinity chromatography, p-Tyr: Phosphotyrosine, PSMs: peptide spectral matches, RP: Reverse phase, TBS: Tris-buffered saline, TFA:
- Trifluoroacetic acid.

*Contact: Sunil Hwang, Ph.D. – Sunil.Hwang@imcstips.com Artwork and layout created by Sarah Woods and Robert Herring.

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