Demonstration of Orthogonal Complementary Enrichment Methods for Enhanced Phosphopeptide Profiling of Drug-Treated Gastric Carcinoma Cells

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Introduction

The post-translational modification of proteins by phosphorylation has been shown to regulate many aspects of cellular function from growth and differentiation to basic metabolism. Here we show that two phosphopeptide enrichment strategies (IMAC and Motif Antibody) are highly complementary with an overlap of ~6% in the number of unique, non-redundant phosphopeptide identifications. In conjunction with using label-free quantitation methods, we show effects of two classes of kinase inhibitor on down stream substrates in the human gastric carcinoma cell line, MKN-45. Following treatment of cells with the c-Met RTK inhibitor SU11274 or the PKC family inhibitor staurosporine, phosphopeptides were isolated using a combination of 14 serine and threonine phospho specific motif antibodies, a Phospho-Tyrosine specific rabbit monoclonal antibody or an IMAC enrichment using Fe^{3+} charged agarose beads. The results show over 20,000 unique phosphopeptides were identified with these methods.

Methods

The gastric cancer cell line MKN-45 was treated with vehicle, SU11274 or staurosporine for 2 hours. Tryptic and LysC peptides were generated from control and treated cellular extracts. Phosphopeptide enrichments were performed using a combination of motif antibodies and Fe^{3+} charged NTA agarose beads. Purified peptides were analyzed by LC-MS/MS using an Orbitrap Velos™ (Thermo Fisher Scientific Inc.); phospho-serine/threonine and tyrosine peptides were identified using SEQUEST (The Scripps Research Institute, La Jolla, CA). Label-free quantification was used to perform relative quantification for all identified phosphopeptides. Quantitative label-free quantification was performed using Skyline Software (MacCoss Lab Software, University of Washington, Seattle, Washington); results were analyzed using TIBCO Spotfire DecisionSite software (TIBCO Software, Inc., Palo Alto, CA).

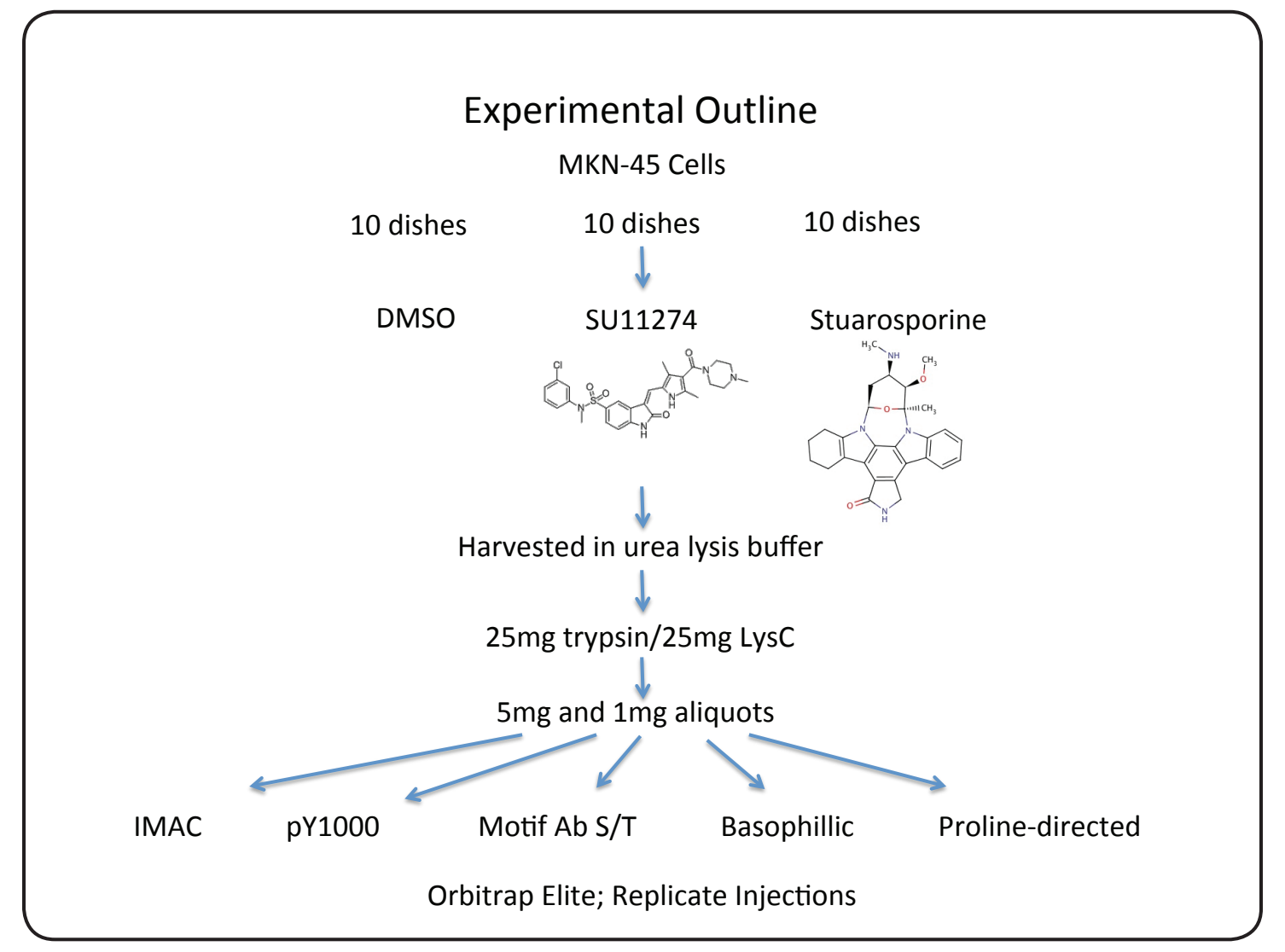


Figure 1: Study Design. A total of $1X10^8$ MKN-45 cells per experiment were treated as described above. The cells were harvested in urea lysis buffer, reduced, alkylated and digested with LysC or trypsin using standard methods (Rush et al.) prior to immunoaffinity enrichment.

Phospho-Motif Antibodies				
Motif Antibody	**Kinase Family**	**Motif**	**Antibody #**	**Type**
Akt Substrate	AGC	RXX(s/t)	9614	Baso, ST Mix
Akt Substrate	AGC	RXRXX(s/t)	10001	Baso, ST Mix
PKA Substrate	AGC	(K/R)(K/R)X(s/t)	9624	Baso, ST Mix
PKC Substrate	AGC	(K/R)XsX(K/R)	2261	Baso, ST Mix
PKD Substrate	AGC	LXRXP(s/t)	4381	Baso, ST Mix
CDK Substrate	CMGC	(K/R)sPX(K/R)	9477	Baso, ST Mix
AMPK	CAMKL	LxRXX(s/t)	5759	Baso, ST Mix
pY1000	Tyrosine	xYX	8954	pY
ATM/ATR Substrate	Atypical	(s/t)QG, sQ	6966, 9607	ST Mix
CK2 Substrate	CK1	t(D/E)X(D/E)	8738	ST Mix
MAPK Substrate	MAPK	PXsP	2325	Proline, ST Mix
tP Motif	Proline Based	tP, tPP	8134	Proline, ST Mix
tPE Motif	Proline Based	tPE, tP	3004	Proline, ST Mix
PLK Binding motif	Proline Based	StP	5243	Proline, ST Mix
tXR Motif	Proline Based	tXR, tPR	8139	Proline, ST Mix
14-3-3	Proline Based	(R/K)XXsXP	9442	Proline, ST Mix

Figure 2: Motif Ab List. The following substrate motif antibodies were used to enrich for phosphopeptides. A combination of related motif antibodies were utilized to enrich for similar motif TYPES as outlined above (pY, S/T Mix, Basophilic & Proline-Directed).

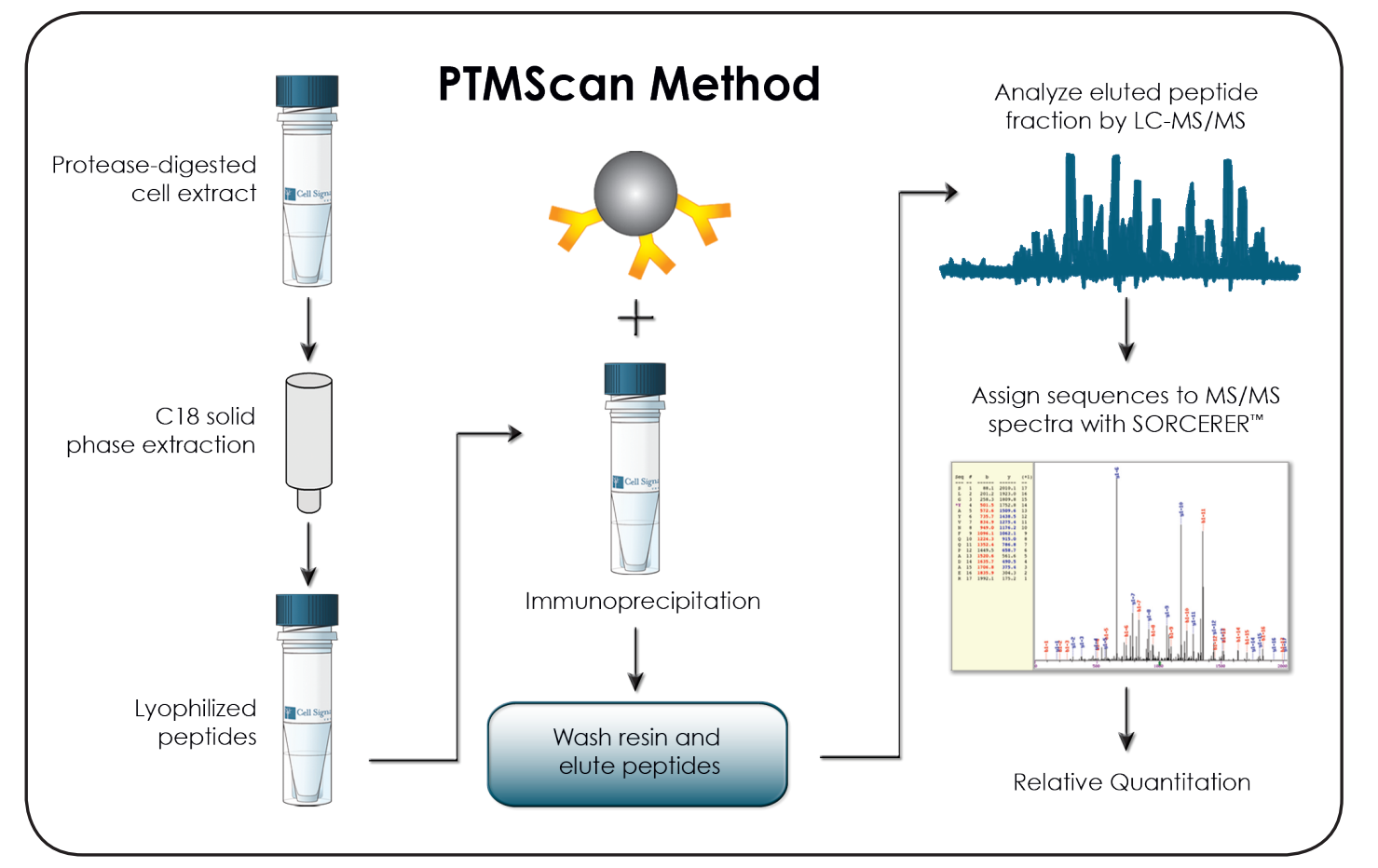
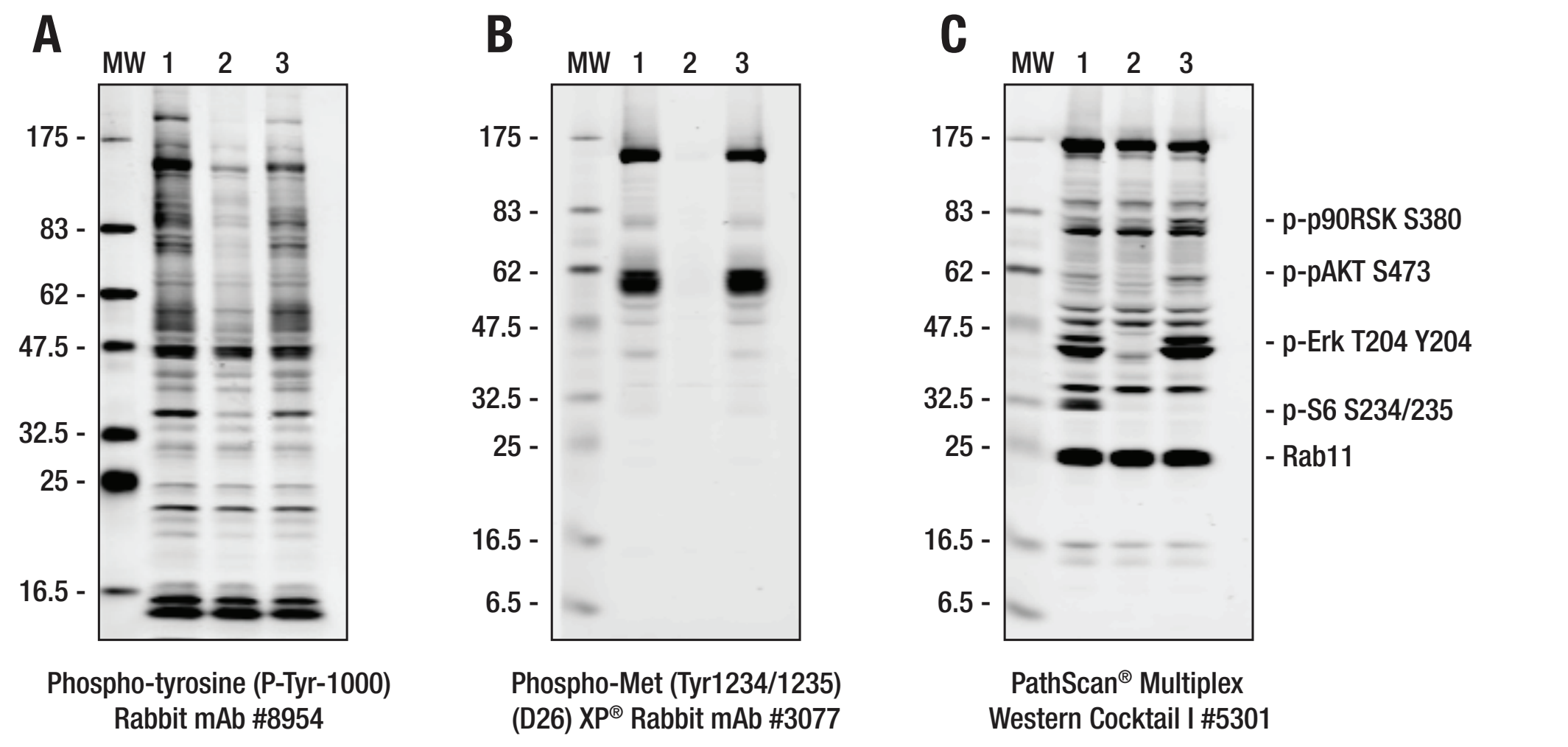


Figure 3: PTMScan Method. The following method was used for immunoaffinity LC-MS/MS analysis. Following peptide enrichment and LC-MS/MS, label-free quantification is performed using Skyline Software (MacCoss Lab Software, University of Washington, Seattle, Washington).

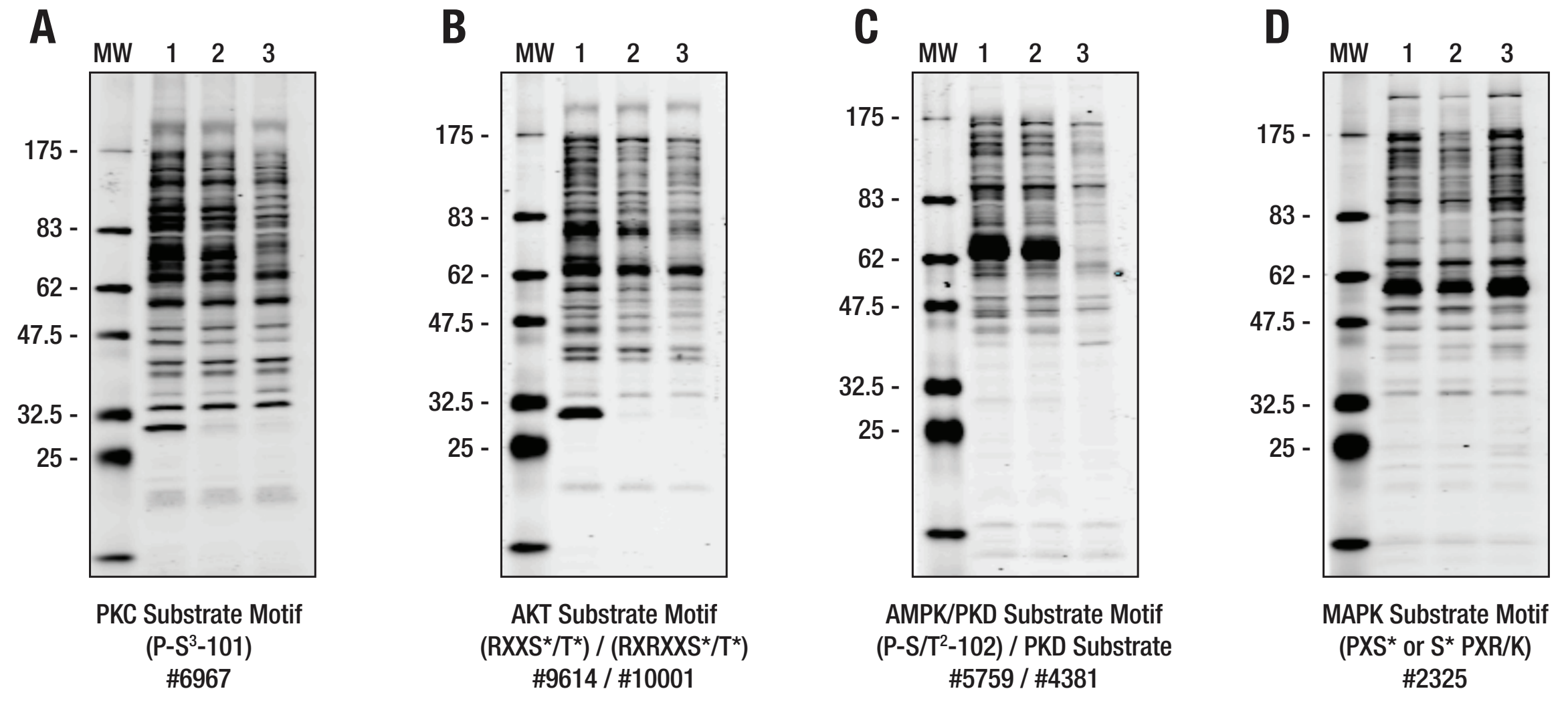


Phospho-tyrosine (P-Tyr-1000) Rabbit mAb #8954

Phospho-Met (Tyr1234/1235) (D26) XP® Rabbit mAb #3077

PathScan® Multiplex Western Cocktail I #5301

Figure 4: Control Western Blots. A total of 20 µg of protein per condition was used for western blot analysis (lane 1- DMSO, lane 2- SU11274, lane 3- staurosporine). The following western blots are illustrated above: (**A**) Phospho-tyrosine antibody; (**B**) Phospho-Met Y1234/1235; (**C**) PathScan® multiplex western cocktail.



PKC Substrate Motif (P-S^3-101) #6967

AKT Substrate Motif (RXXS*/T*) / (RXRXXS*/T*) #9614 / #10001

AMPK/PKD Substrate Motif (P-S/T^2-102) / PKD Substrate #5759 / #4381

MAPK Substrate Motif (PXS* or S* PXR/K) #2325

Figure 5: Motif Antibody Western Blots. Protein extracts were probed with the following kinase substrate motif antibodies: (**A**) PKC, (**B**) Akt, (**C**) AMPK and PKD, (**D**) MAPK.

			Strict		
Enrichment	**Sample**	**Treatment**	**Number Phosphopeptides**	**Reverse**	**False Discovery Rate**
Pan-tyrosine	MKN-45	DMSO	6316	44	1.4%
Pan-tyrosine	MKN-45	DMSO	6445	38	1.2%
Pan-tyrosine	MKN-45	SU11274	3018	22	1.5%
Pan-tyrosine	MKN-45	SU11274	2976	22	1.5%
Pan-tyrosine	MKN-45	Staurosporine	5817	53	1.8%
Pan-tyrosine	MKN-45	Staurosporine	5846	31	1.1%
Basophillic	MKN-45	DMSO	2167	25	2.3%
Basophillic	MKN-45	DMSO	2233	19	1.7%
Basophillic	MKN-45	SU11274	1886	32	3.4%
Basophillic	MKN-45	SU11274	1912	19	2.0%
Basophillic	MKN-45	Staurosporine	2053	34	3.3%
Basophillic	MKN-45	Staurosporine	1988	24	2.4%
S/T Mix	MKN-45	DMSO	4024	78	3.8%
S/T Mix	MKN-45	DMSO	4057	93	4.5%
S/T Mix	MKN-45	SU11274	3685	107	5.6%
S/T Mix	MKN-45	SU11274	3710	106	5.6%
S/T Mix	MKN-45	Staurosporine	4159	90	4.2%
S/T Mix	MKN-45	Staurosporine	4154	93	4.4%
IMAC	MKN-45	DMSO	11812	275	4.6%
IMAC	MKN-45	DMSO	12574	265	4.1%
IMAC	MKN-45	SU11274	11753	232	3.9%
IMAC	MKN-45	SU11274	12031	253	4.1%
IMAC	MKN-45	Staurosporine	11688	252	4.2%
IMAC	MKN-45	Staurosporine	12124	254	4.1%
Proline Mix	MKN-45	DMSO	3436	17	1.0%
Proline Mix	MKN-46	DMSO	3500	16	0.9%
Proline Mix	MKN-47	SU11274	2657	14	1.1%
Proline Mix	MKN-48	SU11274	2579	17	1.3%
Proline Mix	MKN-49	Staurosporine	2398	16	1.3%
Proline Mix	MKN-50	Staurosporine	2332	20	1.7%

Figure 6: Qualitative MS/MS Summary. MS/MS spectra were evaluated using SEQUEST 3G and the SORCERER 2 platform from Sage-N Research (v4.0, Milpitas CA).

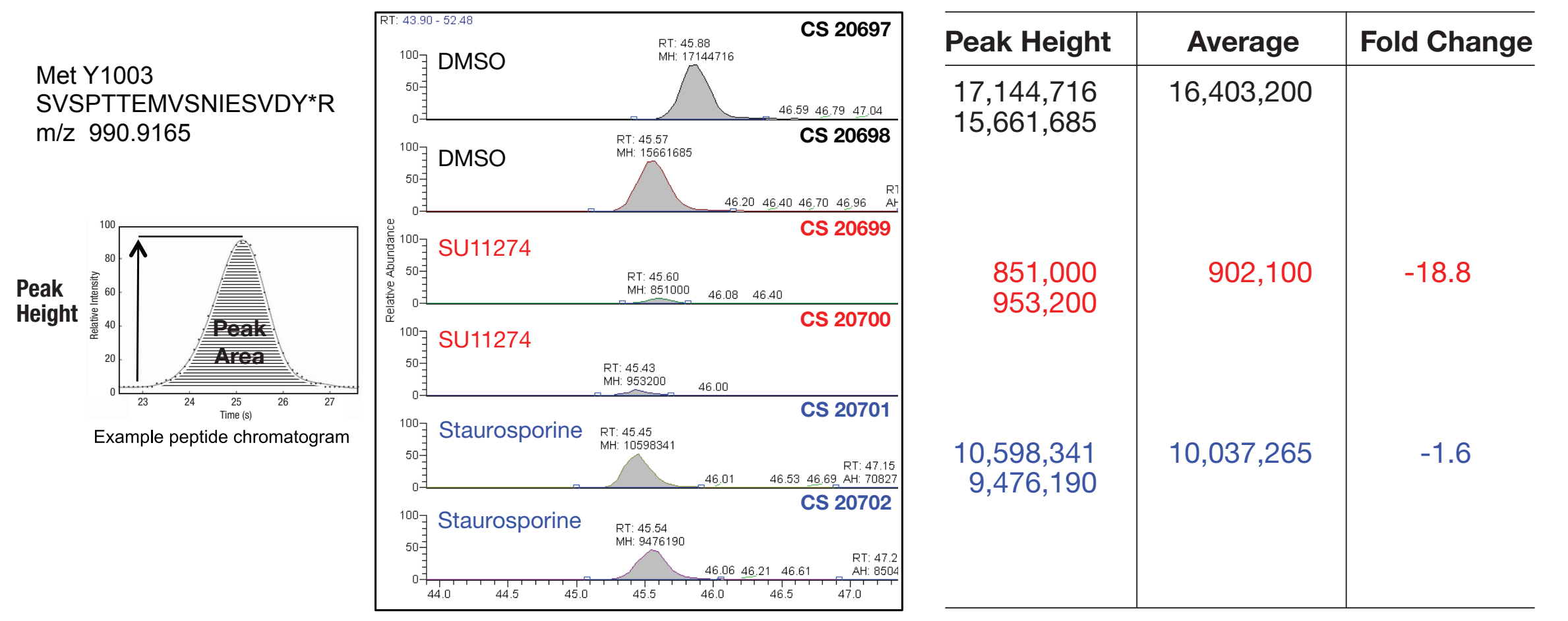


Figure 7: Label-Free Quantitation. Phosphopeptides were quantified based on MS1 peak height or area measurements, and expressed as ratios of treated as compared to DMSO control. Peak integration was performed using either XCalibur or Skyline software.

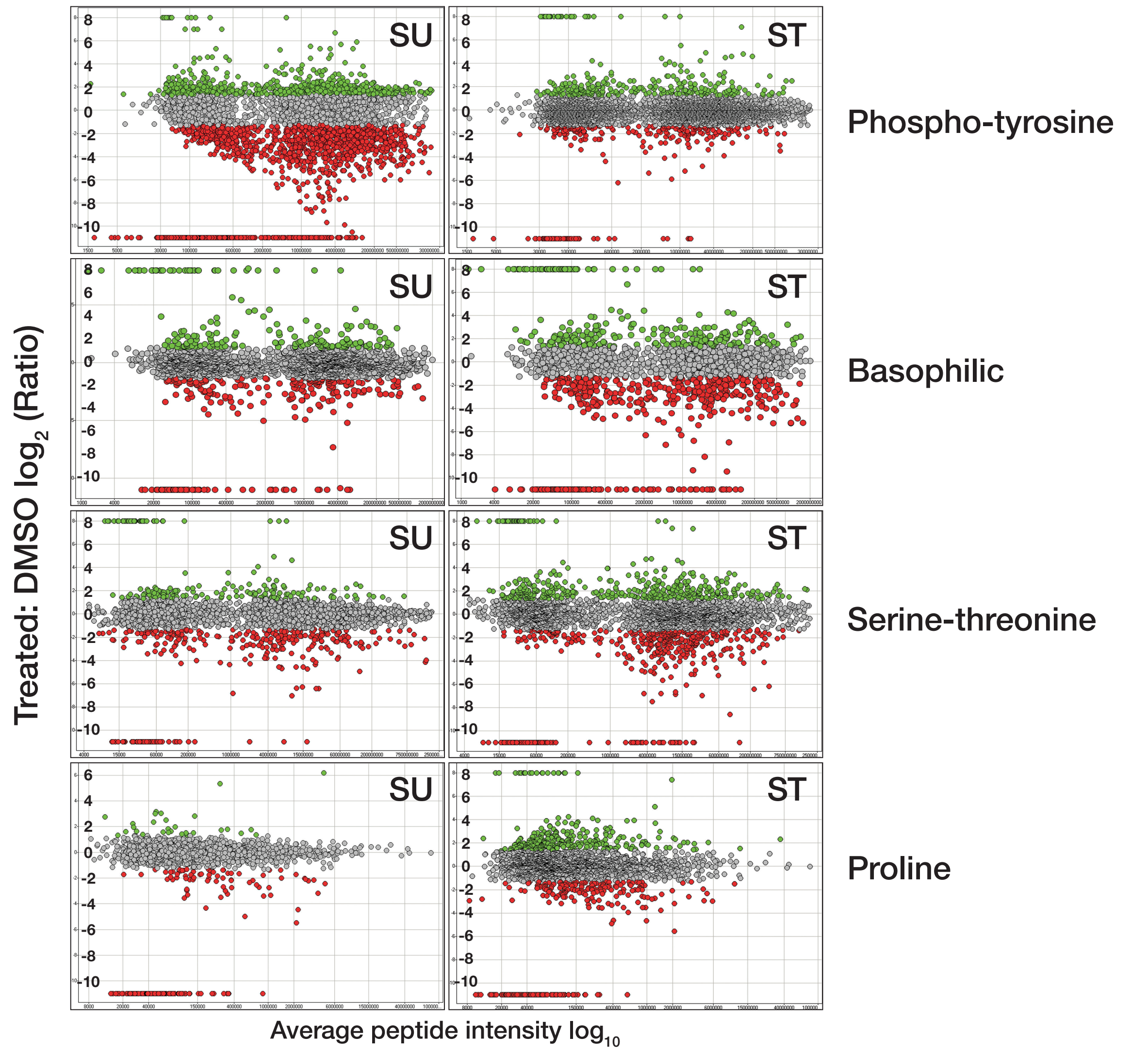


Figure 8: Relative Quantitation for SU11274 and Staurosporine Treated MKN-45 Cells. Scatter plots are illustrated for the relative quantitation of SU11274 and Staurosporine-treated MKN-45 cells (versus DMSO control) from each of the motif phosphopeptide enrichment strategies (y-axis is log^2 (ratio) and x-axis is average peptide intensity across all conditions).

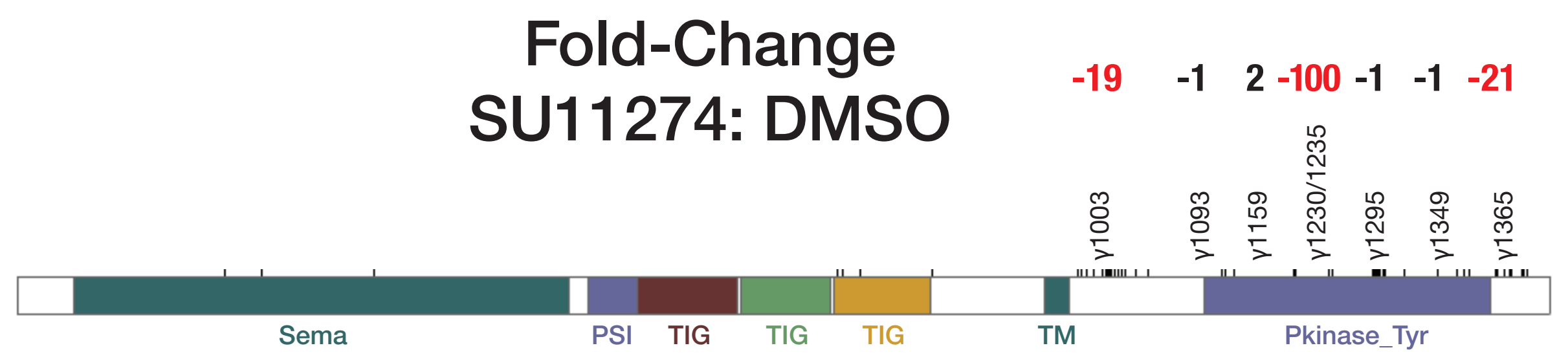


Figure 9: Relative Quantitation of Phosphorylation Sites on Met. The relative quantitation of tyrosine phosphorylation on the receptor tyrosine kinase, Met. Fold-change values are provided for SU11274 treatment versus DMSO.

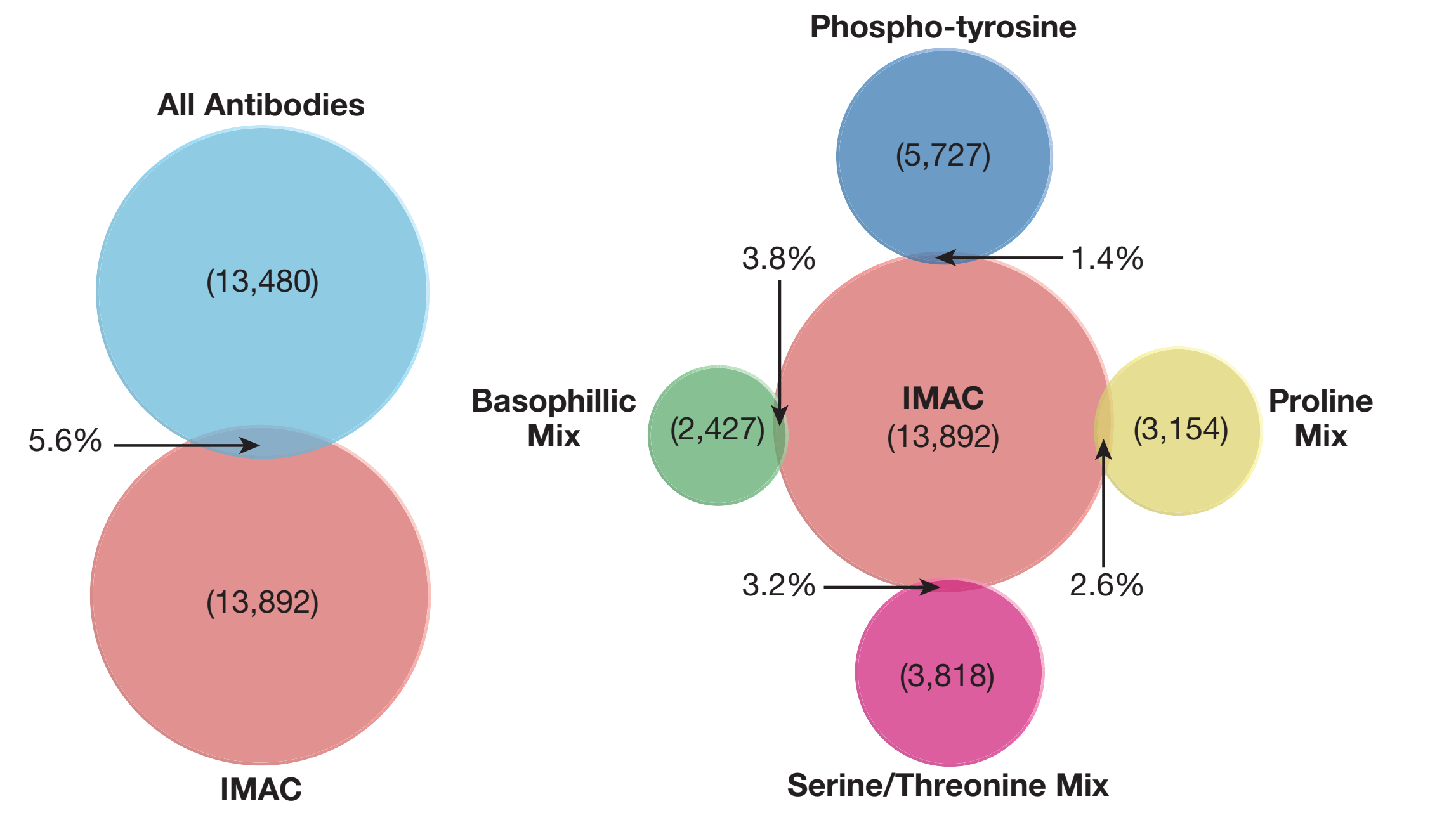


Figure 10: Qualitative Comparison of IMAC and Motif Antibody Analaysis. A total of 27,372 unique phosphopeptides were isolated from the combined IMAC and motif antibody enrichment strategies. The Venn intersection between the different methods is shown. The non-redundant number of phosphopeptides for each enrichment strategy are in brackets. The percent shared between each method is shown at the intersection.

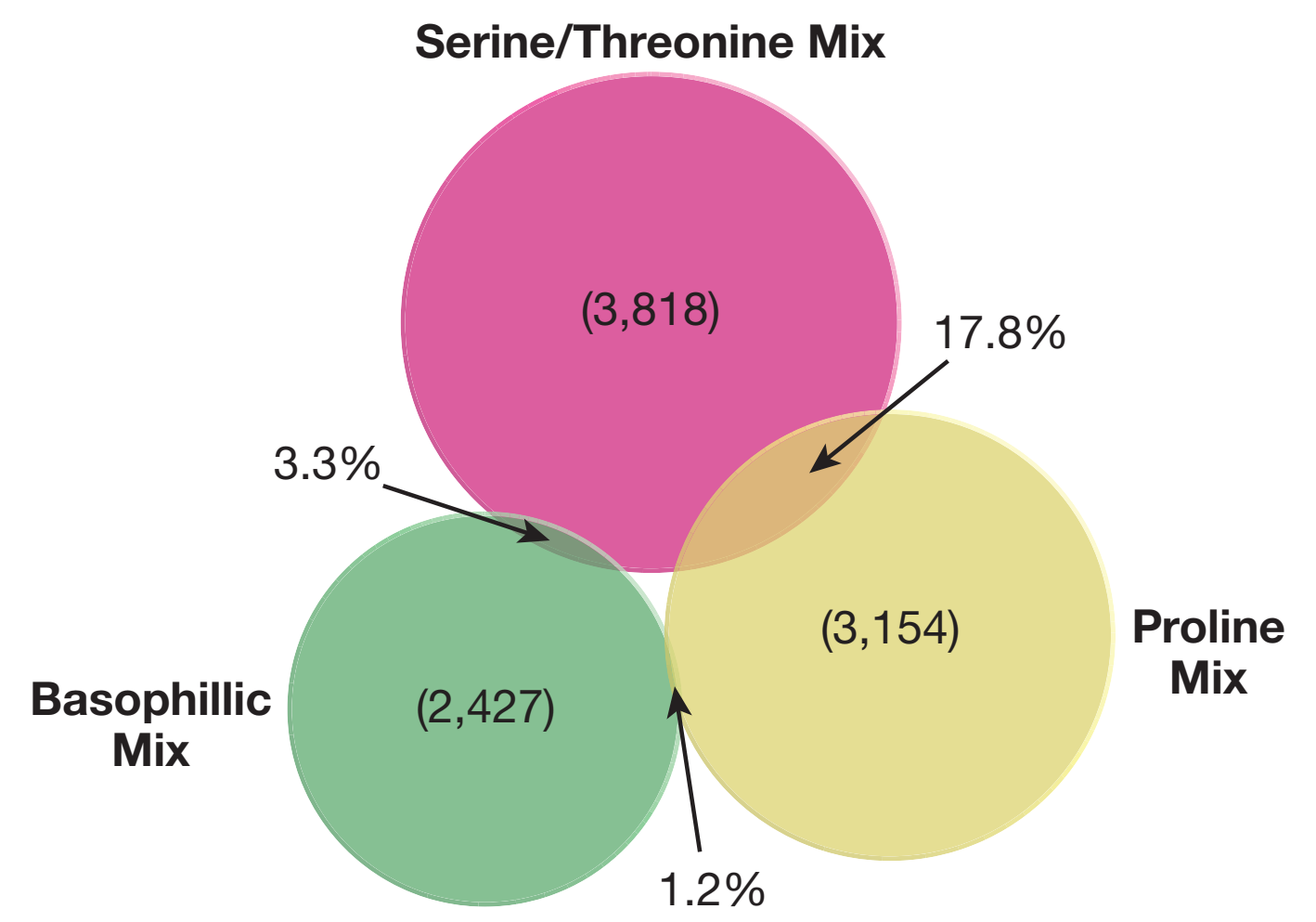


Figure 11: Overlap of Motif Antibody Enrichment Strategies. The shared percentage of unique phosphopeptides is shown. The total number per each enrichment strategy is in brackets.

Normalized Fold Change						
SU11274: DMSO	**Staurosporine: DMSO**	**Protein Name**	**Site**	**-7/+ 7**	**Peptide**	**Upstream Kinase**
-5.0	-4.6	EphA2	897%	RVSIRLPsTSGSEGV	LPS*T*SGSEGVPFR	Akt1
-13.6	-2.1	FOXO1A	%319	TFRPRTSsNASTISG	TSS*NASTISGR	Akt1
-158.0	-7.2	FOXO4	%32	QSRPRSCtWPLPRPE	SCT*WPLPRPEIANQPSEPPEVEPDLGEK	Akt1
-3.4	1.8	QIK	%358	DGRQRRPsTIAEQTV	RPS*TIAEQTVAK	Akt1, Akt2
-13.3	-29.4	S6	%235, %236, %240	IAKRRRLsSLRASTS	RLS*S*LRAS*TSK	Akt1, Akt2, P70S6KB, PKACA, PKCA, PKCD
-7.0	-24.5	S6	%236, %240	AKRRRLSsLRASTSK	RLSS*LRAS*TSK	Akt1, Akt2, P70S6KB, PKACA, PKCA, PKCD
2.6	1.1	BRAF	%365	GQRDRSSsAPNVHIN	SSS*APNVHINTIEPVNIDDLIR	Akt1, Akt3
-7.0	-9.4	GSK3B	9%	SGRPRTTsFAESCKP	TTS*FAESCKPVQQPSAFGSMK	Akt1, AurA, CAMK2B, GSK3B, KHS1, PKACA, PKCA
-5.3	N.D.	GSK3B	%9, %21	SGRPRTTsFAESCKP	TTS*FAESCKPVQQPS*AFGSMK	Akt1, AurA, CAMK2B, GSK3B, KHS1, PKACA, PKCA
-21.3	-3.0	PEA-15	%116	KDIIRQPsEEEIIKL	DIIRQPS*EEEIIK	Akt1, CAMK2A, CK2A1
-2.1	-2.9	GSK3A	%21	SGRARTSsFAEPGGG	TSS*FAEPGGGGGGGGPGGSASGPGGTGGGK	Akt1, CAMK2B, PKACA, PKCA, PKCB
-10.3	-1.8	RANBP3	126%	VKRERTSsLTQFPPS	TSS*LTQFPPSQSEER	Akt1, ERK1, RSK2, p90RSK
2.7	2.5	eIF4B	%422	RERSRTGsESSQTGT	TGS*ESSQTGTSTTSSR	Akt1, p70S6K, p90RSK
4.8	2.5	eIF4B	%422, %425	RERSRTGsESSQTGT	TGS*ESS*QTGTSTTSSR	Akt1, p70S6K, p90RSK
-4.5	-20.0	rictor	%1135	NRRIRTLtEPSVDFN	TLT*EPSVDFNHSDDFTPISTVQK	Akt1, P70S6KB, SGK1, p70S6K
-2.0	-2.4	rictor	%1135, %1138	NRRIRTLtEPSVDFN	TLT*EPS*VDFNHSDDFTPISTVQK	Akt1, P70S6KB, SGK1, p70S6K
-8.5	-2.1	PFKFB2	%483, %493	IRRPRNYsVGSRPLK	NYS*VGSRPLKPLS*PLR	Akt1, PKACA, PPP2CA, RSK2, p70S6K
-1.4	N.D.	NDRG2	%330, %332, %338	TRLSRSRtASLTSAA	SRT*AS*LTSAAS*VDGNR	Akt1, PKCT, SGK1, p70S6K, p90RSK
-1.7	-2.7	NDRG2	%332	LSRSRTAsLTSAASV	TAS*LTSAASVDGNR	Akt1, PKCT, SGK1, p70S6K, p90RSK
-1.4	3.9	Huntingtin	%419	GGRSRSGsIVELIAG	SGS*IVELIAGGGSSCSPVLSR	Akt1, PPP2CA, PPP3CA, SGK1
-2.5	N.D.	NDRG2	%346, %348	VDGNRSRsRTLSQSS	S*RT*LSQSSESGTLSSGPPGHTMEVSC	Akt1, SGK1
-1.1	-11.9	NDRG2	%348	GNRSRSRtLSQSSES	T*LSQSSESGTLSSGPPGHTMEVSC	Akt1, SGK1
1.4	-4.1	NDRG2	%348, %353	GNRSRSRtLSQSSES	T*LSQSS*ESGTLSSGPPGHTM#EVSC	Akt1, SGK1
-1.1	-7.3	NDRG2	%348, %357	GNRSRSRtLSQSSES	T*LSQSSESGT*LSSGPPGHTMEVSC	Akt1, SGK1
-10.5	-9.7	AS160	642%	QFRRRAHtFSHPPSS	AHT*FSHPPSSTK	Akt1; Akt1
-1.2	-27.5	GBF1	%1337	GKIHRSAtDADVVNS	SAT*DADVVNSGWLVVGK	AMPKA1
-2.3	-4.1	TBC1D1	%596	AFRRRANtLSHFPIE	ANT*LSHFPIECQEPPQPAR	AMPKA1, Akt1
1.5	3.5	mTOR	%2446, %2450	NKRSRTRtDSYSAGQ	T*DSYS*AGQSVEILDGVELGEPAHK	AMPKA1, Akt1, p70S6K
-1.3	2.6	PFKFB3	%461, %467	NPLMRRNsVTPLASP	RNS*VTPLAS*PEPTK	AMPKA1, Akt1, PKACA, PKCA
-6.5	-1.9	PFKFB2	%466	PVRMRRNsFTPLSSS	RNS*FTPLSSSNTIR	AMPKA1, Akt1, PKACA, PPP2CA, RSK2, p70S6K

Figure 12: Basophilic Motif Antibody Enrichment. A subset of phosphopeptides conforming to the basophilic Akt substrate motif, RXRXX(s/t) or RXX(s/t) is illustrated from a subset of protein-peptide identifications generated from the Basophilic Motif Antibody enrichment.

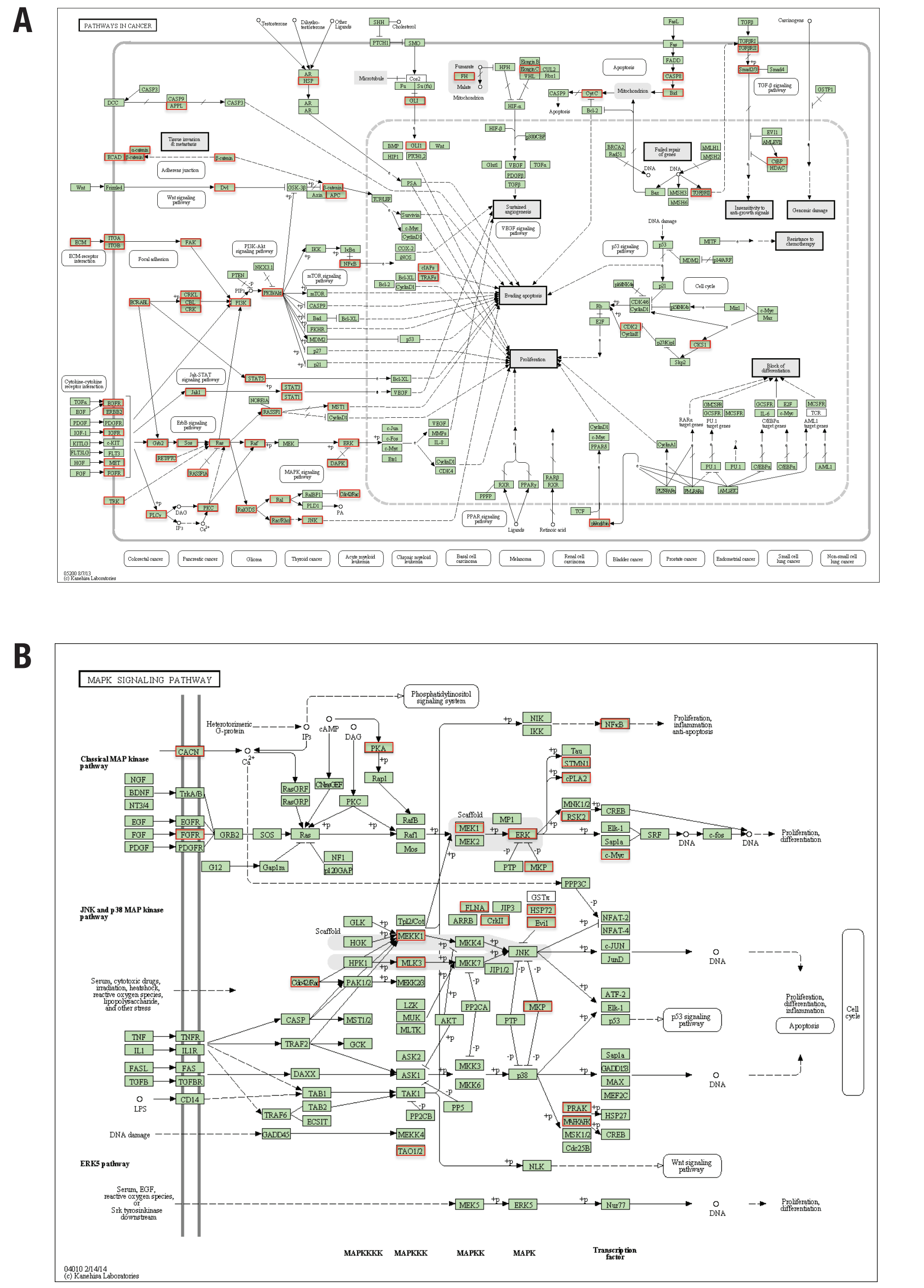


Figure 13: KEGG Pathway Analysis. The tyrosine kinase pathway (**A**), the MAPK kinase pathway (**B**) were annotated from a subset of the data generated in this study. Red outline denotes proteins identified in this study. The pathways are derived from the pathway analysis tool DAVID, from NIAID, http://david.abcc.ncifcrf.gov/

Conclusion

The enrichment strategies used here demonstrate the high degree of complementarity between metal based IMAC and substrate motif antibodies in the identification of phosphopeptides.

Substrate motif antibodies can be used in western blotting to pre-screen samples for qualitative evaluation of response to treatment prior to PhosphoScan®.

References

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2. Soderblom, E.J. et al. (2011) *Anal. Chem.* 83, 3758–3764.

Presentation Posters, Case Studies and Publications

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