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Quantitative Analysis of Two Cancer Signaling Pathways Using Multiplex Immunoprecipitation and Targeted Mass Spectrometry

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ABSTRACT

Introduction: The objective of this study was to determine the efficacy of multiplex IP to targeted MS (mIP-tMS) technique for measurement of the total and phosphorylated AKT/mTOR & RAS/ERK pathway targets and to evaluate whether mIP-tMS assays are as effective as the current Western Blot (WB) techniques.

Methods: Serum starved and LY294002 (PI3K inhibitor) treated HCT116 and A549 cells were stimulated with hIGF-1. Antibodies to targets in the AKT/mTOR and RAS/ERK pathways were selected for verification of antibody specificity by IP-MS. mIP-tMS assays were developed and validated for absolute quantitation of protein targets in these pathways and benchmarked with Western blot using unstimulated, hIGF-1 stimulated and LY294002 treated samples for two cell lysates.

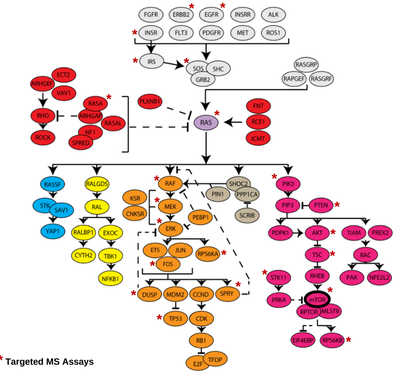
Preliminary Data: Previously, we demonstrated that an optimized IP-MS workflow for Protein A/G and Streptavidin magnetic beads increases target protein abundance with low non-specific background. In this study, we validated antibodies to several AKT/mTOR and RAS/ERK pathway protein targets using an optimized IP-MS workflow. mIP-tMS assays allowed for absolute quantitation of multiple total and phosphorylated protein targets from both pathways in low to sub-nanogram concentrations across unstimulated, hIGF-1 stimulated and LY294002 treated samples for two cell lysates. The benchmarking of mIP-tMS assays indicated target dependant correlation for quantitation of total and phosphorylated protein targets relative to Western blot. Targets with low correlation between techniques may be caused by differences in the specificity of antibodies used for each assay technique.

Novel Aspects: Utilization of mIP-tMS assays for simultaneous quantitation of AKT/mTOR and RAS/ERK pathway targets and benchmark against Western blot.

INTRODUCTION

The AKT/mTOR and RAS/ERK pathways represent key mechanisms for cells to regulate survival, proliferation, and motility[1]. These two pathways extensively engage in cross-talk in order to both positively and negatively regulate each other[2]. Some major bottlenecks in the accurate quantitation of pathway proteins are the lack of rigorously validated reagents and a reliance on semi-quantitative results from immunoassays. Immunoprecipitation coupled with mass spectrometry (IP-MS) enables assessment of antibody specificity and identification of these low-abundant pathway targets[3-5]. Multiplexed IP coupled with targeted MS (mIP-tMS) can quantitate multiple proteins of interest, PTMs and interacting partners in a single MS run.

Figure 1. AKT/mTOR and RAS/ERK Pathways.

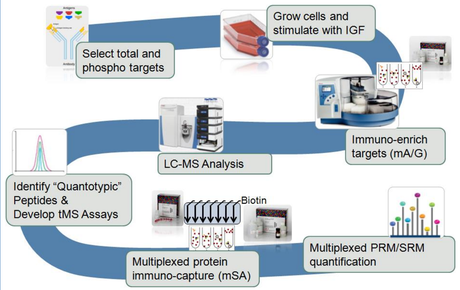


* **Targeted MS Assays**

https://www.addgene.org/cancer/ras-pathway/
Dominic Esposito and Frederick National Laboratory for Cancer Research

MATERIALS AND METHODS

Figure 2. Experimental Workflow for mIP-tMS Assay



Cell Culture

A549 and HCT116 cells were grown in Hamm's F-12K media, and McCoy's 5A Media, respectively, with 10% FBS/1xPenStrep to ~70-80% confluency. A549 or HCT116 cells were serum starved for 24 hours prior to the following treatments: untreated, stimulated (15 min hIGF-1 (100ng/mL; Cell Signaling Technology PN#8917SF)), inhibited then stimulated (1 hour LY294002 (50 µM; Cell Signaling Technology PN#9901S) plus 15 min hIGF-1). Subsequent to treatments, cells were lysed with IP-Lysis buffer (Thermo Fisher Scientific PN#87788) supplemented with 1X HALT Protease and Phosphatase inhibitor cocktail (Thermo Fisher Scientific PN#78440). Protein concentration of lysates was determined with BCA assay (Thermo Fisher Scientific PN#23225).

Western Blot Benchmarking

Antibodies for total and phospho pathway targets were verified for Western blot application. 20 µg of each cell lysate was separated by SDS-PAGE and Western blot was performed. Images were scanned using the iBright imager and density was calculated using iBright iCloud app.

Multiplex Immunoprecipitation and MS Sample Preparation

The Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit (Protein A/G) was used to screen and validate antibodies for total and phosphorylated AKT/mTOR and RAS/ERK pathway targets from 500µg cell lysate. Validated antibodies were biotinylated with the Thermo Scientific™ Pierce™ Antibody Biotinylation Kit for IP. The Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit (Streptavidin) was used to perform single or multiplex IPs for target enrichment. IP samples were processed by an in-solution digestion method in which IP eluates were reconstituted with 6M Urea, 50mM TEAB(pH 8.5) followed by reduction, alkylation and trypsin digestion overnight at 37°C. The digested samples were acidified with TFA. AQUA heavy peptides were spiked into each sample before MS analysis.

Liquid Chromatography and Mass Spectrometry

Prior to MS analysis, tryptic digest samples were desalted on-line using the Thermo Scientific™ Acclaim™ PepMap 100 C18 Trap Column followed by chromatography separation using the Thermo Scientific™ EASY-Spray™ C18 Analytical Column (PN# ES800) and Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System. Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer was used with data-dependent acquisition (DDA) method for discovery MS and Parallel Reaction Monitoring (PRM) method for targeted MS. Discovery MS data was acquired using a top10 DDA method dynamically choosing the most abundant precursor ions from the survey scan (375–1600 Th) with 120,000 resolution (at 200*m/z*), target AGC of 3e6, maximum IT of 50ms. Resolution for HCD spectra was set to 30,000 at *m/z* 200 and Normalized collision energy was 27 eV. For PRM method, peptide scheduling window widths were set at 3 min. PRM acquisition was performed using a resolution of 30,000, isolation windows of 2 Th, target AGC of 2e5, and maximum IT of 115 ms. Fragmentation was performed with a normalized collision energy of 27 eV.

MS Data Analysis

Discovery MS data were analyzed with Thermo Scientific™ Proteome Discoverer™ 2.1 software to assess unique peptides, Top3 peptides Area, and PTMs. For targeted MS data analysis, Thermo Scientific™ Pinpoint™ software and Skyline software (University of Washington) were used to measure limit of quantitation (LOQ) from the calibration curve and target analyte concentration from unknown samples.

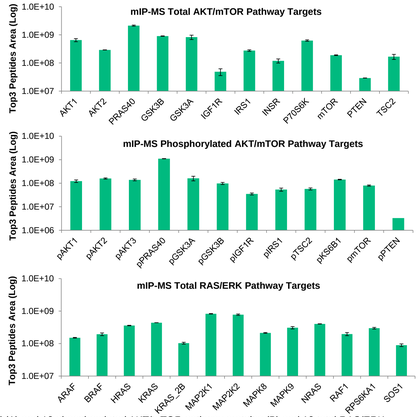
RESULTS

Figure 3. Enrichment of AKT-mTOR and RAS/ERK Pathway Targets.

IP Antibody	Target	# of Unique Peptides		Relevant Phospho peptide ID
		Neat	Enriched-IP	
Phospho AKT	AKT1	-	20	Ser473
	AKT2	-	14	Ser474
	AKT3	-	13	-
AKT1	AKT1	2	12	-
	AKT2	2	11	-
PRAS40	PRAS40	2	8	Thr246
Phospho PRAS40	PRAS40	-	6	Thr246
Phospho mTOR	mTOR	15	82	Thr2446, Ser2448
	RICTOR	-	2	-
	SIN1	-	3	-
	Gbl	-	4	-
Pan Ras	HRAS	-	15	-
	KRAS	-	13	-
	NRAS	2	14	-
PIK3R2	PIK3R2	2	32	Ser262; Ser263
	PIK3CA	-	29	-
	PIK3CB	-	30	-
RAF1	RAF1	2	42	Ser259; Ser621
RSK1	RPS6KA1	5	60	Ser221; Ser363

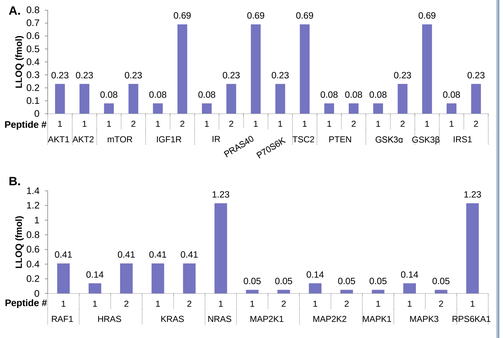
AKT/mTOR & RAS/ERK pathway proteins were immunoprecipitated from hIGF-1 stimulated HCT116 lysate with Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kits (Protein A/G or Streptavidin) for MS analysis. Significantly more unique peptides were identified in IP enriched samples compared to neat lysate. Protein isoforms and interacting partners were identified for AKT, mTOR, RAS and PI3K targets. Relevant phosphorylation sites were detected for AKT1, AKT2, mTOR, PRAS40, PIK3R2, RAF1 and RSK1 targets. Candidate quantitative peptides were selected for targeted MS assay development.

Figure 4. Multiplex IP-MS for Total and Phosphorylated AKT/mTOR Pathway and Total RAS/ERK Pathway Targets.



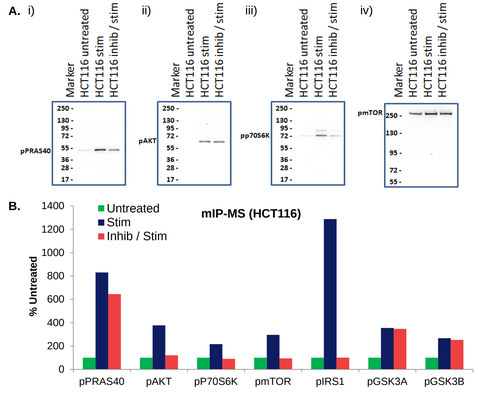
12 total (A) and 12 phosphorylated AKT/mTOR pathway proteins (B) and 12 total RAS/ERK pathway proteins (C) were enriched from hIGF-1 stimulated HCT116 or A549 lysates with biotinylated antibodies using Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Streptavidin). nanoLC-MS/MS analysis for each assay resulted in identification of targeted proteins for AKT/mTOR and RAS/ERK pathways with >2 unique peptides and <20%CV for top 3 peptides area.

Figure 5. PRM Quantitation Limits of Peptides for AKT-mTOR and RAS/ERK Pathway Proteins.



Multiple peptides for 12 AKT/mTOR pathway proteins (A) and 9 RAS/ERK pathway proteins (B) were monitored with linear quantitation (R^2>0.996) and >3 orders of magnitude by nanoLC-PRM/MS. ULOQ for each peptide was 500fmol on column. PRM and SRM methods provided equivalent levels of sensitivity (data not shown).

Figure 6. Comparison of Western Blotting and mIP-tMS Assays for Quantitation of Phosphorylated AKT/mTOR Pathway Proteins



Overall good correlation was observed between Western blot (A) and mIP-tMS assays (B) for seven phosphorylated AKT/mTOR pathway targets. Downregulation in pAKT, pP70S6K, pmTOR, pP70S6K and pIRS1 proteins were seen in HCT116 lysate after treatment with PI3K inhibitor/h-IGF1 stimulation.

Figure 7. Summary of Protein Expression Changes for AKT/mTOR Pathway and RAS/ERK Pathway Targets in Two h-IGF1 Stimulated Cell Lines Without and With Drug Treatment.

Cell Line (Condition)	AKT/mTOR Pathway		RAS/ERK Pathway	
	Total	Phosphorylated	Total	Phosphorylated
HCT116 (Stim)	No Change	↑P	Target dependent changes	No Change
HCT116 (Inhib)		↓P except GSK3		
A549 (Stim)	No Change	↑P except mTOR	↑T	↑P
A549 (Inhib)		↑P except IRS1		↓P

No changes in total AKT/mTOR pathway protein expressions were observed in both cell lines. Multiple phosphorylated AKT/mTOR pathway proteins showed upregulation upon hIGF-1 stimulation and downregulation in PI3K inhibited/h-IGF1 stimulated HCT116 cells. Upregulation in phosphorylated AKT/mTOR pathway proteins observed in A549 cells after hIGF-1 stimulation and PI3K inhibited/h-IGF1 stimulated cells. Total RAS/ERK pathway proteins showed increase in protein expression. Multiple phosphorylated RAS/ERK pathway proteins showed upregulation upon hIGF-1 stimulation and downregulation in PI3K inhibited/h-IGF1 stimulated A549 cells.

CONCLUSIONS

- Enrichment is necessary for identification and quantitation of low abundant signaling pathway proteins, interacting partners & PTMs for MS applications.
- Immunoprecipitation using MS-Compatible Magnetic IP Kits (Protein A/G and Streptavidin) for MS applications resulted in a higher yield of AKT/mTOR and RAS/ERK pathway proteins and fewer non-specific binding proteins.
- IP to MS analysis of total and phosphorylated AKT and RAS pathway proteins enabled identification of multiple isoforms, relevant protein interactions and phosphorylation sites.
- Total and phosphorylated mIP-tMS assays allowed simultaneous quantitation of multiple total and phosphorylated AKT/mTOR and RAS/ERK pathway proteins in the low to sub-fmol range from unstimulated, hIGF-1 stimulated and PI3K inhibited/h-IGF1 stimulated A549 and HCT116 cell lines.
- Elucidating specific pathway differences between A549 and HCT116 cells might lead to targeted treatment for either lung or colon cancer.
- mIP-tMS assays benchmarked against Western Blot:
  - Overall good correlation observed for AKT/mTOR and RAS/ERK pathway proteins.
  - Variability between techniques for some targets could be due to antibody specificity.

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TRADEMARKS/LICENSING

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