Quantitative Analysis of Ras and AKT Signaling Pathways using a SureQuant Targeted MS Workflow

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ABSTRACT

Purpose: To assess SureQuant acquisition methods for multiplex quantitation of Ras and AKT signaling pathways.

Methods: Signaling proteins from three different cancer cell lines were quantified with and without IP enrichment using a multiplexed PRM and SureQuant internal standard (IS)-triggered method, respectively.

Results: The SureQuant IP enrichment method enabled detection of lower abundant signaling protein targets but one is limited to the targets where an IP-verified antibody is available. The SureQuant acquisition requires an internal standard peptide for triggering method and enabled quantitation of a majority of targets without enrichment; however, some of the lower abundant signaling protein targets were not detected.

INTRODUCTION

The RAS/MAPK and AKT/mTOR pathways represent key mechanisms for cells to regulate cell survival, proliferation, and motility[1]. The cross-talk between two pathways plays a central role in tumor progression and anti-cancer drug resistance. The quantitation of pathway protein expression and modifications are critical for characterization of disease, monitoring cancer progression and determining treatment response. A major limitation in the quantitation of pathway proteins is the lack of rigorously validated methods/reagents and a reliance on semi-quantitative results from Western blotting. We have utilized a novel SureQuant internal standard (IS)-triggered method[2] applying a pool of reference internal standards to quantitate abundance of pathway proteins in a single MS run. This turnkey workflow allows reliable targeted quantitation for routine pathway analysis.

MATERIALS AND METHODS

Cell Line Lysates: A549, HCT116, MCF7 and HeLaS3 cells were grown in Ham's F-12K media, McCoy's 5A Media, and DMEM Media, respectively, with 10% FBS/1xPenStrep to ~70-80% confluency. Cells were serum starved with 0.1% charcoal stripped FBS for 24 hours before stimulation with 100 ng/mL of IGF for 15 minutes. Cell samples 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).

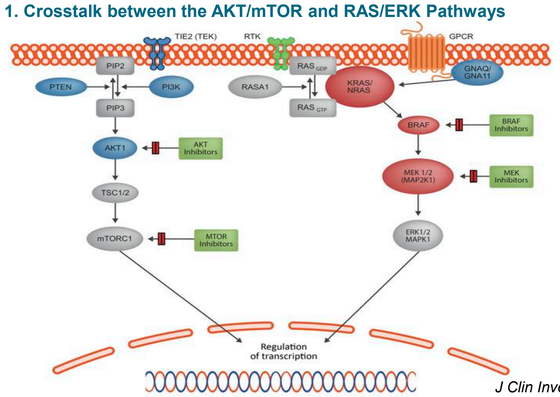
Multiplex Immunoprecipitation to MS Sample Preparation and MS Quantitation: The Thermo Scientific™ SureQuant™ IP and MS Sample Preparation Modules for AKT Pathway (PN# A40081, A40086, A40091), were used to immunoenrich relevant protein targets. The Thermo Scientific™ SureQuant™ Absolute Quantitation Modules for AKT Pathway (PN# A40083, A40093) was used to generate calibration curves and determine concentrations of target peptides from unknown samples.
Verified antibodies and peptides for RAS/MAPK pathway proteins were used for IP to MS quantitation.

EasyPep MS Sample Prep: The same cancer cell lysates prepared in IP lysis buffer (A549, HCT116, and MCF7) as stated above were precipitated with acetone followed by Thermo Scientific™ EasyPep™ MS sample prep (PN# A45734). 50 fmol of internal standard peptide mixtures for each pathway targets was spiked-in to clean digest.

LC-MS Analysis: Thermo Scientific™ Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mixture) (PN# A40010) was used to assess dynamic range and sensitivity (LLOQ) of the nanoLC-MS system prior to running calibration curves or unknown samples. IP-enriched and trypsin digested samples were then desalted on-line using the Thermo Scientific™ Acclaim™ PepMap™ 100 C18 Trap Column (PN#164564) followed by seperation using a Thermo Scientific™ EASY-Spray™ C18 column (PN#ES800). For discovery MS and targeted PRM-MS analysis, the samples were analyzed using the Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System and Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer.
LC-MS analysis of SureQuant method was performed with a Thermo Scientific™ EASY-nLC™ 1200 coupled to Thermo Scientific™ Orbitrap Exploris™ 480. For both survey and SureQuant analysis, 60 min gradients, at 300nL/min were used.

MS Data Analysis: Discovery MS data was analyzed with Thermo Scientific™ Proteome Discoverer™ software to assess percent sequence coverage, unique peptides, peptide areas/intensities, and PTMs. For targeted PRM or SureQuant data analysis, Skyline software (University of Washington) was used to measure light/heavy ratios and calculate concentrations from unknown samples.

Figure 1. Crosstalk between the AKT/mTOR and RAS/ERK Pathways



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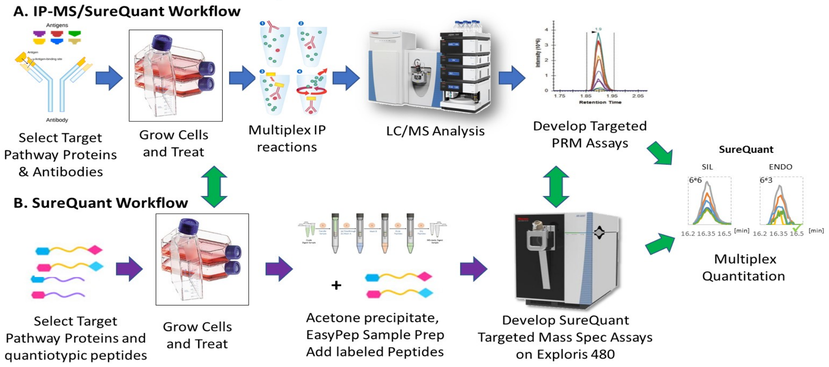
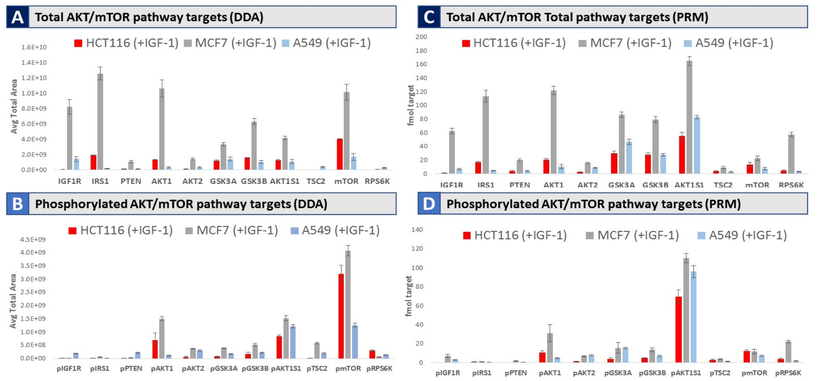
RESULTS

Figure 2. Pathway Analysis using SureQuant Workflow



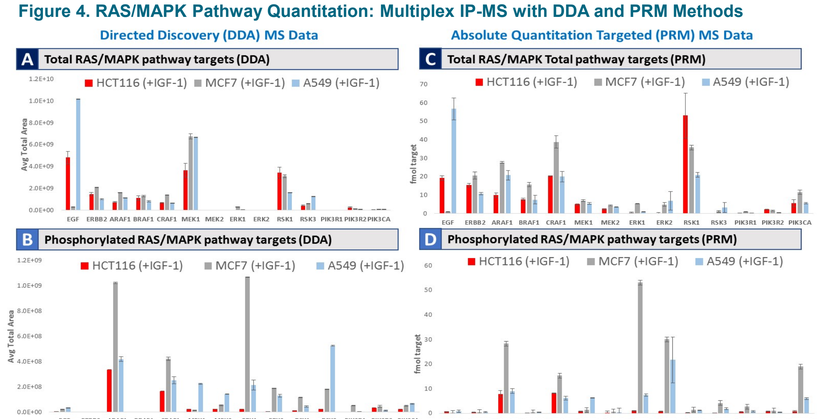
A. IP-MS/SureQuant uses multiple antibodies to enrichment and clean up. B. SureQuant acquisition method uses EasyPep Maxi to clean and digest then adds heavy labeled peptides both leading to Multiplex quantitation.

Figure 3. AKT/mTOR Pathway Quantitation: Multiplex IP-MS with DDA and PRM Methods



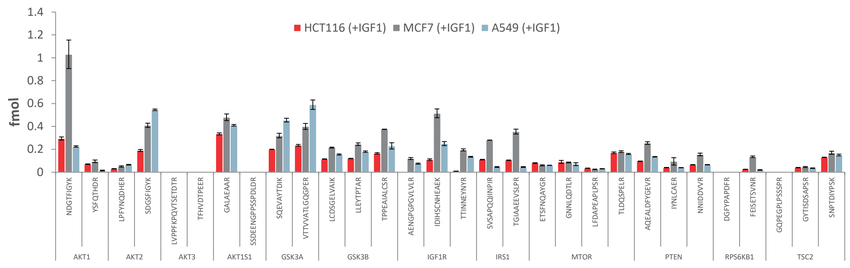
AKT/mTOR pathway proteins were enriched through multiplex IP using the SureQuant AKT Pathway total or phospho assay kit. DDA and PRM data were analyzed in Proteome Discoverer and Skyline software, respectively. PRM analysis using the calibration curve allowed absolute quantitation of each target peptide from stated lysate.

Figure 4. RAS/MAPK Pathway Quantitation: Multiplex IP-MS with DDA and PRM Methods



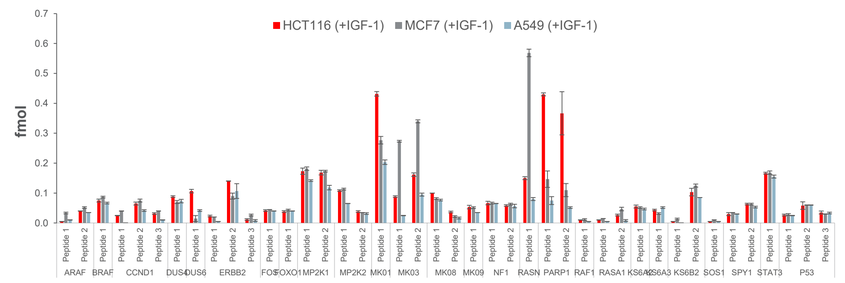
RAS/MAPK pathway proteins were enriched through multiplex IP using the SureQuant RAS/MAPK Pathway total or phospho assays. DDA and PRM data were analyzed in Proteome Discoverer and Skyline software, respectively. PRM analysis using the heavy light ratios allow for quantitation of each target peptide from stated lysate.

Figure 5. AKT/mTOR Pathway Quantitation: MS Sample Prep with SureQuant Acquisition Method



AKT pathway target proteins were quantitated from unenriched digested samples with SureQuant acquisition method (60K/116 ms; 30-min gradient). 50fmol IS was injected in 500ng of each cancer cell line digest (n=3).

Figure 6. RAS/MAPK Pathway Quantitation: MS Sample Prep with SureQuant Acquisition Method



RAS pathway target proteins were quantified from unenriched digested samples with SureQuant acquisition method (60K/116 ms; 60-min gradient). 50fmol IS was injected in 500ng of each cancer cell line digest (n=3).

CONCLUSIONS

- Using immuno-enrichment with an internal peptide calibration curve, identification and quantitation of multiple low-abundance protein targets and PTMs is achievable.
- SureQuant targeted MS assays/kits containing verified antibodies, positive control lysate, peptides, buffers, instrument methods, and Skyline data analysis templates provide a complete workflow solution for simultaneous quantitation of total and phospho AKT and RAS pathway proteins.
- A novel workflow utilizing the heavy peptides spiked-in to EasyPep MS sample prep digest followed by SureQuant acquisition method allows simultaneous quantitation of multiple total proteins from AKT/mTOR and RAS/MAPK pathways.

REFERENCES

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2. Gallien, S., Kim, S. Y., & Domon, B. (2015). Large-Scale Targeted Proteomics Using Internal Standard Triggered-Parallel Reaction Monitoring (IS-PRM). Molecular & cellular proteomics : MCP, 14(6), 1630–1644.

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TRADEMARKS/LICENSING

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