Applications of SureQuant™ Pathway Panels for Quantitative Analysis of Cancer Signaling Proteins

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ABSTRACT

Purpose: To develop a robust method for quantitation of AKT pathway proteins and benchmark against western blotting.

Methods: The SureQuant™ AKT pathway (total or phospho) panels include a multiplex immunoprecipitation (IP) and mass spectrometry (MS) sample prep module (antibodies, lysate, buffers), absolute or relative quantitation modules (AQUA Ultimate peptides standards), and instrument method /Skyline software templates. Serum-starved, inhibitor-treated (LY294002/Rapamycin/ NVP-BEZ235) A549, MCF7, and HCT116 cells were stimulated with hIGF-1 and prepared for MS analysis using the SureQuant™ AKT total and phospho multiplex pathway modules in order to determine the absolute concentration of pathway peptides using targeted MS analysis. The panels were benchmarked against Western blotting (WB) using cell lysates as well as tissue/xenograft lysates.

Results: The SureQuant™ multiplex pathway modules achieved absolute quantitation of multiple total and phosphorylated targets across unstimulated, hIGF-1 stimulated and inhibited + hIGF-1 stimulated cell lysates as well as tissue/xenograft lysates. Benchmarking of SureQuant™ AKT Pathway Targeted MS assays relative to WB showed overall good correlation between orthogonal techniques. Any discrepancies between two techniques may be result from differences in the antibodies used for each assay.

INTRODUCTION

The AKT/mTOR pathway utilizes multiple mechanisms for cells to regulate survival, proliferation, and motility. Therefore, this signaling pathway plays a central role in tumor progression and drug resistance[1]. Attempts to monitor pathway monitoring proteins with high accuracy suffer from many challenges due to poor reproducibility, unreliable quantitation, and a lack of standardized methods and reagents[2]. To overcome these challenges, the novel SureQuant™ pathway panels have been developed and applied, utilizing an optimized multiplex immunoprecipitation to targeted mass spectrometry (IP-MS) workflow. Multiplexed, targeted IP-MS assays can quantitate multiple proteins, PTMs, and interacting partners, introducing new possibilities for a broad range of applications in precision medicine, including cancer diagnosis and prognosis, and drug development.

Figure 1. Experimental Workflow for Multiplex IP-MS Assays

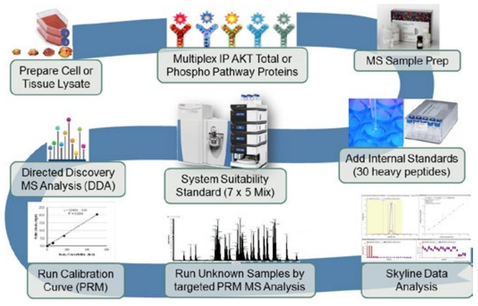
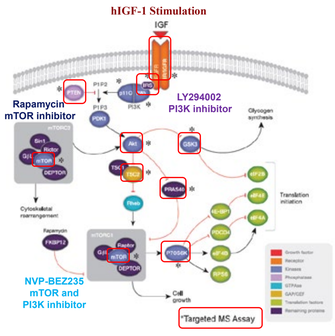


Figure 2. AKT Pathway



MATERIALS AND METHODS

Cell Culture

Three cancer cell lines were used A549 (Lung, ATCC PN#CCL-185), MCF7 (Breast, ATTC PN#HTB-22), and HCT116 (Colon, ATCC PN#CCL-247). Cells were grown according to ATCC instructions to approximately 80% confluency and serum starved with 0.1% charcoal striped FBS supplemented media for approximately 24 hours prior to treatment. Inhibitors were added to cells in the media prior to hIGF-1 stimulation: 50µM LY294002, PI3K inhibitor, for 15 mins (CST PN#9901S), 10 nM Rapamycin, mTOR inhibitor, for 1 hour (CST#9904S), LY294002 and Rapamycin in combination, or 500 nM NVP-BEZ235, dual PI3K and mTOR inhibitor, for 2 hours (CST PN#131015). After inhibition.12.8 nM hIGF-1 was added for 15 minutes (Thermo Fisher Scientific PN#PHG0071) or cells were left untreated. Subsequent to treatments, cells were scraped to harvest and lysed in IP Lysis Buffer (Thermo Fisher Scientific PN#8778) with added 1C HALT protease and phosphatase inhibitor (Thermo Fisher Scientific PN#78440) added. Protein concentration of lysates was determined with BCA assay (Thermo Fisher Scientific PN#23225).

Western Blot Benchmarking

Antibodies for AKT 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™ or myECL imager and density was calculated using iBright iCloud app or myImage Analysis Software.

Multiplex Immunoprecipitation to MS Sample Preparation and MS Quantitation

1 mg (Phospho) or 500µg (Total) cell lysate was used per IP. The SureQuant™ IP and MS Sample Preparation Modules for AKT Total and Phospho Pathways (PN# A40081, A40086) were used to immuno-enrich pathway protein targets. Abslolute quantitation was determined using the SureQuant ™ AKT Pathway Absolute Quantitation Module (PN#A40083) or SureQuant™ AKT Pathway (Phospho) Absolute Quantitation Module (PN#A40088).

Liquid Chromatography and Mass Spectrometry

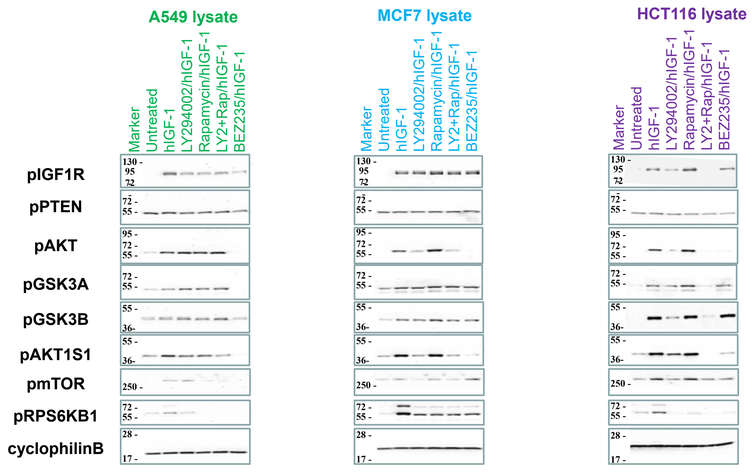
Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mixture) (PN# A40010) was used to assess dynamic range and sensitivity (LLOQ) of nanoLC-MS system prior to running calibration curves or unknown samples. Enriched and trypsin digested samples were desalted on-line using the Thermo Scientific™ Acclaim™ PepMap 100 C18 Trap Column (PN#164564) followed by seperation using the Thermo Scientific™ Easy Spray C18 column (PN#ES800). Unless stated for discovery MS and targeted PRM-MS analysis, the samples were acquired using the Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System and Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer and the verified instrument acquisition methods as well as inclusion lists relevant to each Absolute Quantitation Module.

MS Data Analysis

Discovery MS data were analyzed with Thermo Scientific™ Proteome Discoverer™ 2.2 software to assess unique peptides, total peptide area, PTMs and PCA plot analysis. For targeted MS data analysis, Skyline software (University of Washington) were used to measure limit of quantitation (LLOQ) from the calibration curve and target analyte absolute concentration from unknown samples.

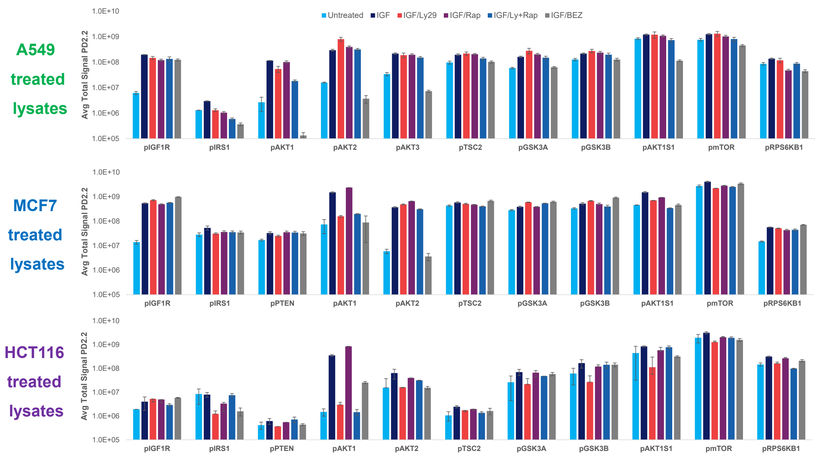
RESULTS

Figure 3. Western Blot Data of Phospho AKT Pathway Targets for Three Cell Lines with Various Treatments



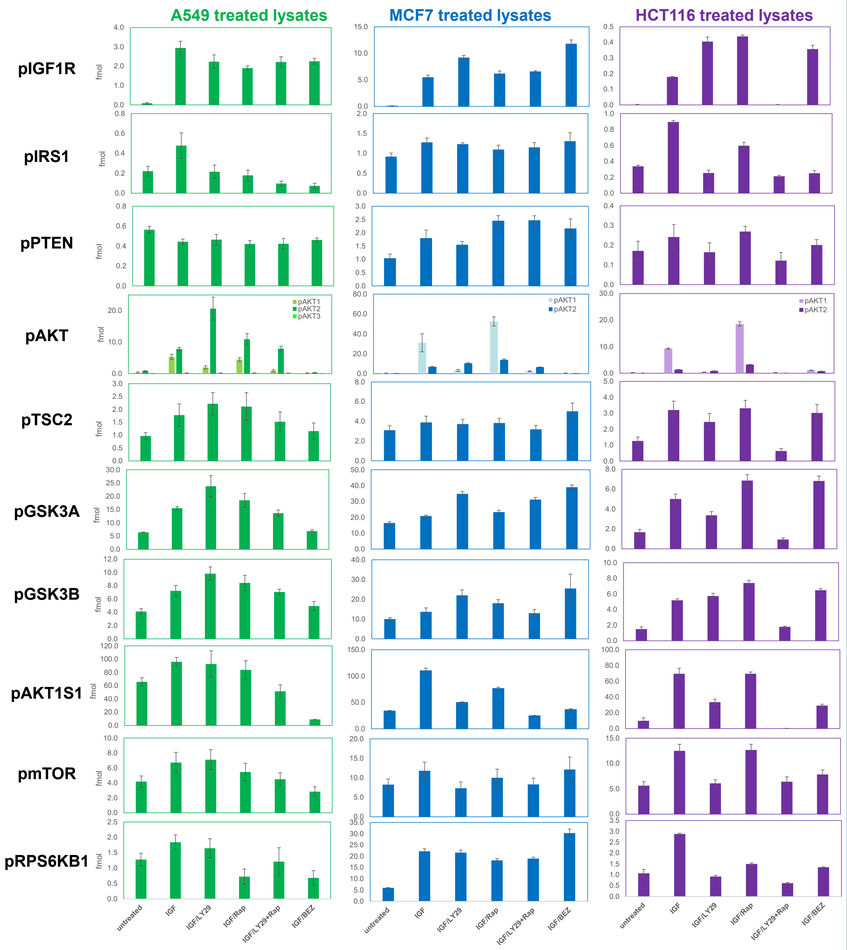
Western Blot data showed differential expression for phosphorylated AKT pathway proteins with hIGF-1 stimulation and inhibitor treatments

Figure 4. DDA Analysis Using the SureQuant™ AKT Phospho Pathway Kit



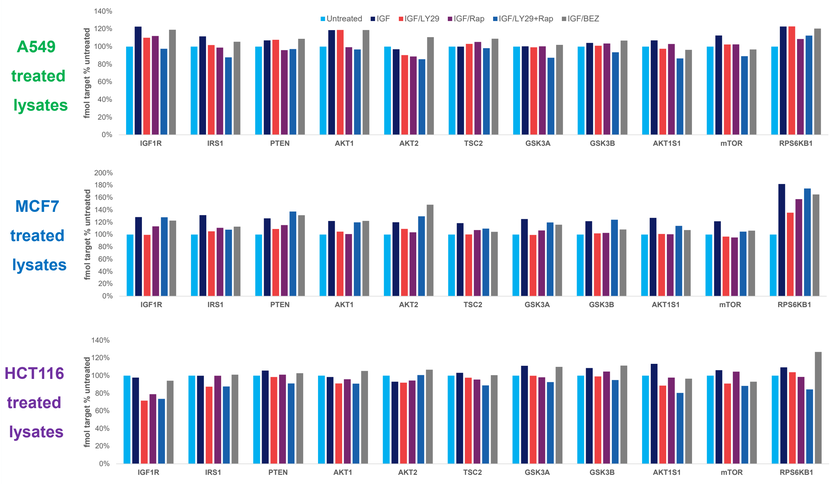
Directed discovery MS (DDA) data demonstrated upregulation in most AKT Phospho targets upon hIGF-1 stimulation. Cell line dependent and/or specific inhibitor treatment changes in phospho targets were also observed.

Figure 5. PRM Analysis Using the SureQuant AKT Phospho Pathway Kit for Absolute Quantitation



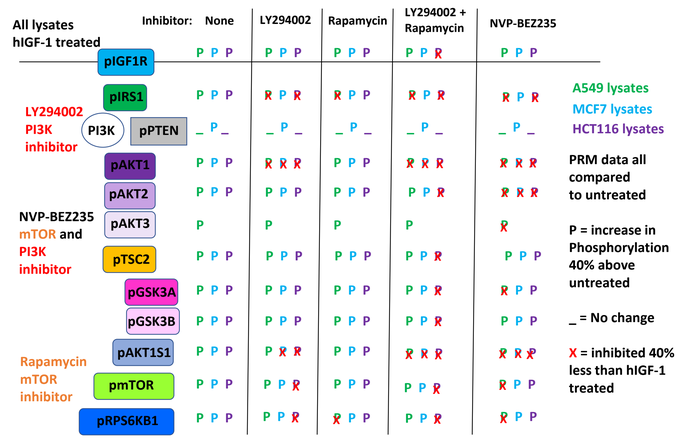
Targeted MS (PRM) data illustrated upregulation in most AKT Phospho targets upon hIGF-1 stimulation. Cell line dependent and/or treatment specific changes in phospho targets were also observed.

Figure 6. PRM Analysis using the SureQuant™ AKT Total Pathway Kit for Absolute Quantitation



Targeted MS (PRM) data compared to untreated revealed no significant changes in total AKT pathway targets with hIGF-1 stimulation and different inhibitor treatments.

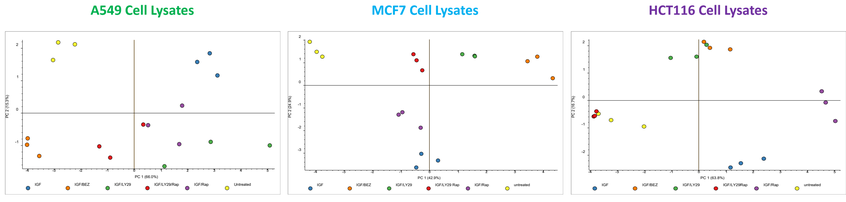
Figure 7. Summary of Expression Changes in Phospho AKT Pathway Targets



Calculated concentration (fmol) values were used to summarize data where an increase in phosphorylation (**P**) was designated by at least a 40% increase compared to untreated. Inhibition (**X**) was designated if below 40% hIGF-1 treated value. All pathway phosphorylated proteins showed increase in abundance with hIGF-1 treatment across all three cell lines, whereas inhibitors functioned differently among three cell lines:

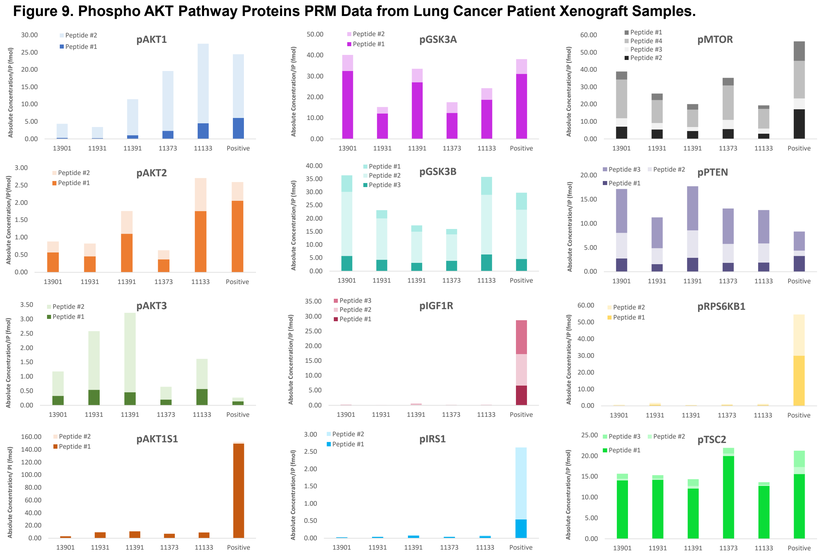
- The PI3K inhibitor (LY294002) was more effective than the mTOR inhibitor (Rapamycin) with effectiveness HCT116 > MCF7 > A549
- Combined LY294002 + Rapamycin treatment was most effective in HCT116 cells with effectiveness HCT116 > A549 > MCF7
- Dual inhibitor (NVP-BEZ235) worked best in A549 cells compared to LY294002 and Rapamycin together with effectiveness A549 > HCT116 > MCF7

Figure 8. Expression Changes of AKT Proteins PCA Plots



PD2.2 Data PCA plots from DDA data of ratio between untreated / treated of all targets showed distinct patterns between cells lines.

Figure 9. Phospho AKT Pathway Proteins PRM Data from Lung Cancer Patient Xenograft Samples.



Accurate quantitation was obtained from five patient derived lung tumor xenograft samples using the SureQuant™ AKT Phospho Pathway kit. Patient derived xenograft samples were lysed in 2% SDS before multiplex IP enrichment followed by Targeted MS (PRM) analysis using the Thermo Scientific™ Q Exactive™ HFX and Easy-1200 nLC system. ES803 50cm column with single column set up was used for all LC-MS data acquisition and Skyline Software was used to generate standard curves (data not shown) and calculate absolute concentration from unknown samples.

CONCLUSIONS

- **Analysis of abundance levels between three different cancer cell lines using the SureQuant™ AKT Total and Phospho kits demonstrated preferences for certain inhibitors in specific cell lines, with PI3K inhibitor LY294002 functioning the best in HCT116 cells, whereas the dual PI3K/Rapamycin inhibitor NVP-BEZ235 was most effective in A549 cells.**
- **Orthogonal evaluation between PRM assays and Western Blot analysis of three cancer cell lines treated with hIGF-1 and various inhibitors showed similar trends in the protein expression changes.**
- **SureQuant™ assay kits using targeted PRM-MS analysis allowed for more accurate and precise quantitation pathway targets and the capability to distinguish between target isoforms (AKT); this level of accuracy and quantitation is difficult or impossible to achieve with Western Blot analysis.**
- **SureQuant™ AKT pathway kits allowed identification of target proteins from cell lysate and patient derived xenograft tissue samples.**

REFERENCES

1. Logue JS, Morrison DK. Complexity in the signaling network: insights from the use of targeted inhibitors in cancer therapy. Genes Dev. 2012 Apr 1; 26(7):641-50.

2. Carr SA, Abbatiello SE, Ackermann BL et al. Targeted Peptide Measurements in Biology and Medicine: Best Practices for Mass Spectrometry-based Assay Development Using a Fit-for-Purpose Approach. Mol Cell Proteomics. 2014 Mar; 13(3):907-17.

TRADEMARKS/LICENSING

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