Applications of Mass Spectrometry Targeted Assays for Quantitative Analysis of Cancer Signaling Proteins

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ABSTRACT

Purpose: The AKT/mTOR pathway plays a central role in tumor progression and drug resistance. Quantitative measurement of alterations in the expression of pathway proteins and post-translational modifications (PTM) is necessary for understanding cancer biology. Highly accurate monitoring of these pathway proteins has not been achieved, due to poor reproducibility, unreliable quantitation, and lack of standardized methods and reagents. To overcome these challenges, the novel Thermo Scientific™ SureQuant™ pathway panels have been applied, which utilize an optimized multiplex immunoprecipitation to targeted mass spectrometry (mIP-tMS) workflow. SureQuant assays can quantitate multiple proteins, PTMs and interacting partners, which creates new possibilities for a broad range of applications, including cancer, drug development, and research into precision medicine.

Methods: The SureQuant total and phospho pathway panels contain two modules: 1) The IP-MS Sample Prep Module includes reagents necessary to immunoenrich AKT pathway, RAS, or TP53 proteins, and perform MS sample preparation in one day 2) The Absolute or Relative Quantitation Modules include a Thermo Scientific™ Pierce™ LC-MS/MS System Suitability Standard, AQUA Ultimate Heavy and/or AQUA Ultimate Light Peptides, and verified MS instrument and data analysis methods. Serum-starved, inhibitor-treated (LY294002/NVP-BEZ235/Rapamycin) HCT116, A549, and MCF7 cells were stimulated with hIGF-1. SureQuant AKT pathway panels (total and phospho) were used to determine the absolute concentration of target peptides using targeted MS analysis. The panels were benchmarked against Western blotting using three unstimulated, hIGF-1 stimulated or inhibited cell lysates, as well as several tissue/xenograft lysates.

Results: Previously, we verified antibodies and target peptides to AKT and RAS pathways using an optimized mIP-tMS workflow. From the standard curve, all target peptides were monitored with <20% CV, 3 orders of magnitude dynamic range, linearity (R^2) >0.97, and accuracy of 80-120% in a complex matrix. Using the SureQuant pathway panels, absolute quantitation of 37 target peptides in unknown samples was achieved with <20% CV across multiple cancer cell lines. The SureQuant pathway analysis workflow allowed absolute quantitation of target peptides from positive control lysate with <15% individual operator %CV and <20% combined %CV using PRM analysis. Kit performance was evaluated through analysis of abundance levels between three different cancer cell lines, A549, HCT116, and MCF7, using the SureQuant AKT Total and Phospho assay showed preferences for certain inhibitors in specific cell lines treated with hIGF-1. The PI3K inhibitor LY294002 functioned the best in HCT116 cells whereas the dual PI3K/Rapamycin inhibitor NVP-BEZ235 worked predominantly in A549 cells. Analysis by mass spectrometry allowed for more accurate and informative data with the determination of fmol levels of protein expression and capability to discriminate between isoforms of many proteins that are unable to procure with western blot analysis. Absolute quantitation of 12 phosphorylated AKT pathway targets was obtained from five patient derived lung tumor xenograft samples. Additionally, all 12 total and 12 phospho AKT pathway targets were quantitated from three different tissue lysates, Lung, Large Intestine, and Breast tumor.

INTRODUCTION

Multiplex Immunoprecipitation to Mass spectrometry (IP-MS) kits from Thermo Fisher Scientific are developed for simultaneous enrichment and quantitation of total abundance and phosphorylation levels of multiple proteins from the AKT/mTOR Signaling Pathway. The immunoenriched, digested samples are spiked with heavy peptide internal standards, which can then be processed using discovery MS (DDA) and targeted MS (PRM) methods for analysis.

Figure 1. SureQuant Multiplex IP to Targeted MS Modules & Kits

IP/MS sample prep module

- 10 reactions (samples)
- Biotinylated antibodies mix
- Positive control lysate
- Streptavidin magnetic beads
- Wash buffers (A and B)
- Trypsin
- Sample prep buffer
- Reduction/alkylation reagents
- 10% TFA
- Low-binding tubes

Absolute quantitation module

- 10 reactions (samples)
- AQUA heavy peptide mix
- AQUA light peptide mix
- 6 protein digest matrix
- 7 x 5 system suitability standard
- Peptide Diluent
- Instrument method with Skyline doc
- Low-binding tubes

Relative quantitation module

- 10 reactions (samples)
- AQUA heavy peptide mix
- 7 x 5 system suitability standard
- Peptide Diluent
- Instrument method with Skyline doc
- Low-binding tubes

AKT pathway: total targets	AKT pathway: phospho targets
AKT1/AKT2/ AKT3	AKT1/AKT2 pSer473/pSer474
PTEN	PTEN pSer380
IRS1	IRS1 pSer312
IGF1R	IGF1R pTyr1162/1163
GSK3a	GSK3a pSer21
GSK3b	GSK3b pSer9
PRAS40 (AKT1S1)	PRAS40 pThr246
mTOR	mTOR pSer2448
P70S6K	P70S6K pThr389
TSC2	TSC2 pSer939

Figure 2. Experimental Workflow for Multiplex IP-MS Assays

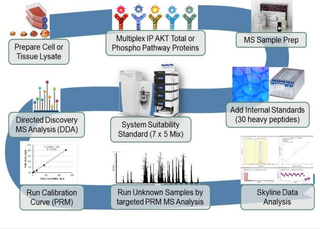


Figure 3. AKT Pathway hIGF-1 Stimulation

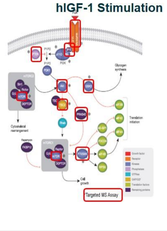


Figure 4. MS Sample Prep Workflow Optimization for Immuno-enriched (IP) Samples

Traditional MS Sample Prep Workflow for IP

SureQuant™ Target Assay MS Sample Prep Workflow for IP

MATERIALS AND METHODS

Cell Lines and Tissue Lysate: A549, HCT116 and MCF7 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. Breast, Lung, and Large Intestine Tumor and Normal Adjacent Tissue were purchased from BioVT. Cells and tissue samples were lysed with Thermo Scientific™ Pierce ™ IP Lysis buffer (PN#87788) supplemented with 1X Thermo Scientific™ Halt Protease and Phosphatase inhibitor cocktail (PN#78440).

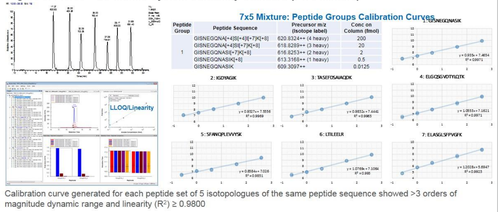
Multiplex Immunoprecipitation to MS Sample Preparation and MS Quantitation: The SureQuant IP and MS Sample Preparation Modules for AKT Pathway (PN# A40081, A40086, A40091), were used to immunoenrich relevant protein targets. The SureQuant Absolute Quantitation Modules for AKT Pathway (PN# A40083, A40093)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

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 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 separation 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. Verified instrument acquisition methods were used, as well as inclusion lists relevant to each Absolute Quantitation Module.

MS Data Analysis: Discovery MS data were analyzed with Thermo Scientific™ Proteome Discoverer™ to assess percent sequence coverage, unique peptides, areas/intensities of identified peptides, and PTMs. For targeted MS data analysis, 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

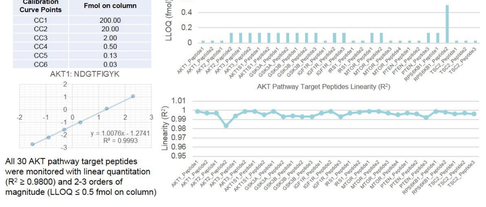
Figure 5. Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mixture)



Calibration curve generated for each peptide set of 5 isotopologues of the same peptide sequence showed >3 orders of magnitude dynamic range and linearity (R^2) ≥ 0.9800

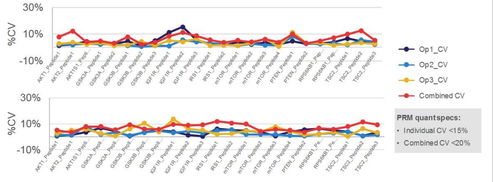
Figure 6. Determination of Quantitation Limits and Linearity of AQUA Light Peptides for SureQuant Pathway Kits

Calibration Curve Points	Fmol on column
CC1	200.00
CC2	20.00
CC3	2.00
CC4	0.50
CC5	0.13
CC6	0.03



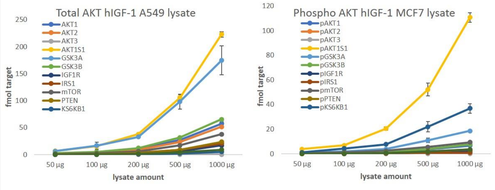
All 30 AKT pathway target peptides were monitored with linear quantitation (R^2 ≥ 0.9800) and 2-3 orders of magnitude (LLOQ ≤ 0.5 fmol on column)

Figure 7. Precision of AKT/mTOR Signaling Pathway Proteins Using SureQuant™ Targeted MS Kits



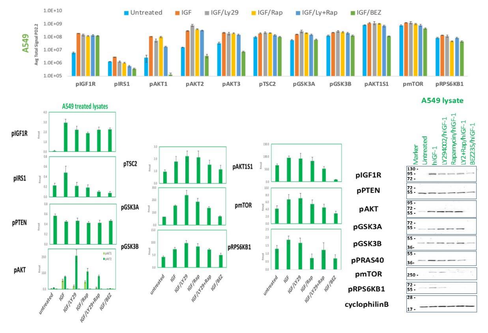
AKT SureQuant Pathway Mass Spec Assay kits allowed absolute quantitation of target peptides from positive control lysate with <15% individual CV and <20% combined CV using PRM analysis.

Figure 8. Quantitation of Total and Phospho AKT Pathway Proteins using Different Positive Control Lysate Amounts



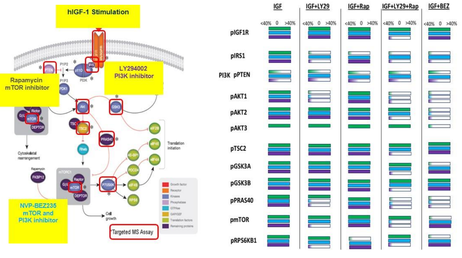
Total and phospho AKT PRM pathway analysis allowed absolute quantitation of all target peptides from 50 µg to 1000 µg of positive control lysate. Overall linear correlation between lysate amount and target quantitation was observed.

Figure 9. Absolute or Relative Quantitation of AKT/mTOR Signaling Pathway Proteins Using SureQuant



AKT/mTOR pathway proteins were enriched through multiplex immunoprecipitation using the SureQuant AKT Pathway or AKT Phospho Pathway Mass Spec Assay kit. Analyses were performed on a Q Exactive HF Orbitrap mass spectrometer using directed discovery (DDA) and targeted MS (PRM) acquisition methods. 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 positive control lysate. Western Blot data showed differential expression for phosphorylated AKT pathway proteins with hIGF-1 stimulation and inhibitor treatments

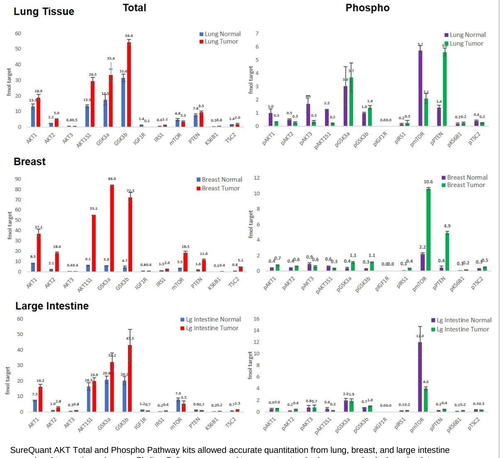
Figure 10. Summary of Quantitative Changes in Phospho AKT Pathway following Targeted Inhibition



Calculated concentration (fmol) values were used to summarize data where an increase in phosphorylation was designated by at least a 40% increase compared to untreated. Inhibition 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:

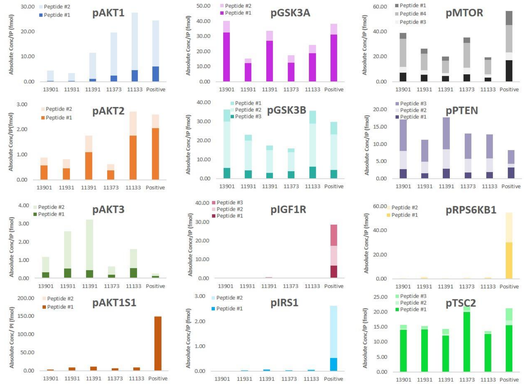
- The PI3K inhibitor (LY294002) was more effective than the mTOR inhibitor (Rapamycin) with effectiveness HCT116 > MCF7 > A549
- Combined LY294002 + Rapamycin treatment was most effective in HCT116 cells with effectiveness HCT116 > A549 > MCF7
- Dual inhibitor (NVP-BEZ235) worked best in A549 cells compared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

Figure 11. Quantitation of Total and Phospho AKT Pathway Proteins in Lung, Breast, and Large Intestine Normal and Tumor Tissue Samples.



SureQuant AKT Total and Phospho Pathway kits allowed accurate quantitation from lung, breast, and large intestine normal and tumor tissue lysates. Skyline Software was used to generate standard curves and calculate absolute concentrations of unknown samples.

Figure 12. 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 Q Exactive HF-X and EASY-nLC 1200 system. A 50cm EASY-Spray column (ES803) in direct-injection setup was used for all LC-MS data acquisition and Skyline Software was used to generate standard curves (data not shown) and calculate absolute concentrations of unknown samples.

CONCLUSIONS

- **Pierce™ LC-MS/MS System Suitability Standard (7 x 5 mixture) achieves appropriate linearity and dynamic range to assess system performance prior to acquisition of unknown samples.**
- **SureQuant Multiplex IP-MS and Absolute Quantitation Modules for AKT pathway proteins allowed simultaneous absolute quantitation of multiple total and phospho AKT pathway proteins in treated cell lines and tumor samples with high accuracy and precision (CV <20%).**
- **Analysis of abundance levels between three different cancer cell lines using the SureQuant AKT Total and Phospho kits revealed preferences for certain inhibitors in specific cell lines, with PI3K inhibitor LY294002 demonstrating highest efficacy 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, albeit the level of precision and dynamic range achieved with the SureQuant kit is difficult or impossible to achieve with Western Blot analysis.**
- **SureQuant AKT pathway kits are amenable to diverse sample sources and allowed identification of target proteins from cell lysate, tissues and patient derived xenograft tissue samples.**
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