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Targeted Mass Spectrometry Assay Kits for Absolute Quantitation of Signaling Pathway Proteins

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ABSTRACT

Purpose: Many genetic mutations in cancer cells alter protein expression of AKT, RAS and TP53 pathway components. Quantitative measurements of alterations in the expression of pathway proteins and post-translational modifications (PTM) are necessary for classifying disease states, monitoring cancer progression and determining treatment response. Major limitations for quantitation of these proteins are a lack of rigorously verified methods and reagents, and reliance on Western blotting. To address these bottlenecks, we have optimized a multiplex immunoprecipitation (IP) to targeted mass spectrometry (MS) workflow to develop the Thermo Scientific™ SureQuant™ pathway panels, thereby achieving simultaneous enrichment and absolute quantitation of total abundance and phosphorylation levels of multiple proteins from the AKT pathway, along with RAS and TP53 levels.

Methods: The SureQuant total and phospho pathway panels contain two modules: 1) The IP and 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 Pierce™ LC-MS/MS System Suitability Standard, AQUA UltimateHeavy and/or AQUA UltimateLight Peptides, and verified MS instrument methods and Skyline software analysis templates. A calibration curve is generated using constant amounts of heavy peptide and variable amounts of light peptide (0.03-200 fmol). IP-enriched, digested samples spiked with heavy peptides are analyzed using targeted MS (nanoLC-PRM/MS) and Skyline software.

Results: We verified antibodies and target peptides to AKT and RAS pathways using an optimized IP-MS workflow. From the standard curve, all target peptides were monitored with <20% CV, 2-3 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 all target peptides was achieved in multiple cancer cell lysate samples with <20% CV between replicates.

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 pathway, along with RAS and TP53 levels. 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. Thermo Scientific™ 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

Positive control cell lysates

- A549 (hIGF-1 stimulated)
- MCF7 (hIGF-1 stimulated)
- HCT116 (hIGF-1 stimulated)
- BT549

Absolute quantitation module

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

Relative quantitation module

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

AKT pathway: total targets	AKT pathway: phospho targets
AKT1/AKT2/AKT3	AKT1/AKT2
PTEN	pSer473/pSer474
IRS1	PTEN pSer380
IGF1R	IRS1 pSer312
GSK3A	IGF1R pTyr1162/1163
GSK3B	GSK3A pSer21
PRAS40 (AKT1S1)	GSK3B pSer9
mTOR	PRAS40 pThr246
P70S6K (RPS6KB1)	mTOR pSer2448
TSC2	P70S6K pThr389
	TSC2 pSer939

AKT Proteins
Total AKT1/AKT2/AKT3
Phospho AKT1/AKT2/AKT3 (pSer473/pSer474/Ser472)

TP53 Proteins
Total TP53
Phospho TP53 (pSer15)

RAS Proteins
KRAS
HRAS
NRAS

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. BT-549 cells were grown in RPMI-1640 with 0.023 IU insulin. Subsequent to IGF stimulation, 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). Lung and breast tumor tissues were obtained from BioIVT. The tissue lysates were prepared using the IP-Lysis buffer after PBS washes and homogenization. Protein concentration of cell lysates and tissue lysates were determined with BCA assay (Thermo Fisher Scientific PN#23225).

Multiplex Immunoprecipitation to MS Sample Preparation and MS Quantitation

The SureQuant IP and MS Sample Preparation Modules for AKT Pathway (PN# A40081, A40086, A40091), TP53 (PN# A40101) and RAS proteins (PN# A40096) were used to immunoenrich relevant protein targets from 500 or 1000 µg positive control cell lysate followed by MS sample preparation to generate digested peptides. The SureQuant Absolute Quantitation Modules for AKT Pathway (PN# A40083, A40093), TP53 (PN# A40103) and RAS proteins (PN# A40098) were was used to generate calibration curves and determine concentrations of target peptides from unknown samples.

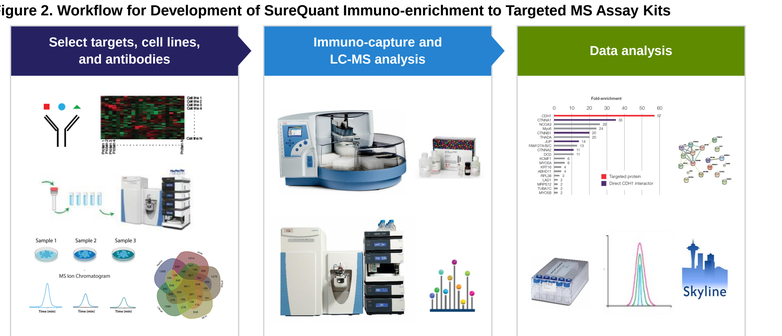
Liquid Chromatography and Mass Spectrometry

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

MS Data Analysis

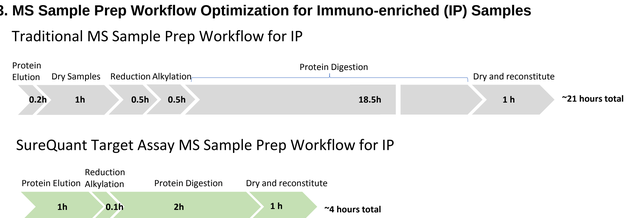
Discovery MS data were analyzed with Thermo Scientific™ Proteome Discoverer™ to assess percent sequence coverage, unique peptides, areas/intensities of identified peptides, and PTMs. Proteome Discoverer software searches were executed using a custom Uniprot human protein database. All peptide peak areas were calculated for each pathway target to assess relative abundance across different cell lines. For targeted MS data analysis, Skyline software (University of Washington) and Excel templates were used to measure limit of quantitation (LLOQ) from the calibration curve and target analyte concentration from unknown samples.

Figure 2. Workflow for Development of SureQuant Immuno-enrichment to Targeted MS Assay Kits



mRNA and deep proteome data were used to identify abundance level of specific target in relevant cell line(s). Multiple antibodies for a given target were screened by IP-MS analysis to verify capture efficiency and specificity of each antibody followed by selection of antibody with the best capture efficiency. Directed discovery data from IP-MS verified antibody is used to identify quantotypic peptides and develop targeted PRM assay using AQUA Peptides.

Figure 3. MS Sample Prep Workflow Optimization for Immuno-enriched (IP) Samples



RESULTS

Figure 4. Procedure Overview for SureQuant Targeted Mass Spec Assay Kits

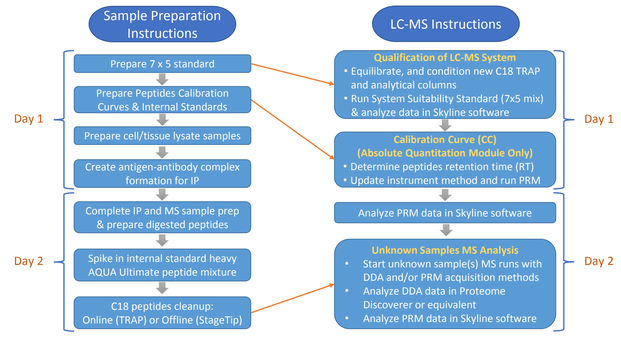
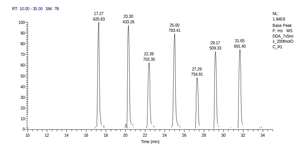
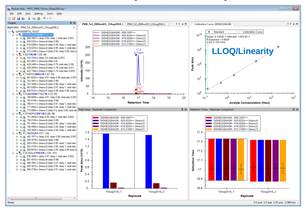


Figure 5. Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mixture)

Representative Chromatogram of 7 x 5 Mixture

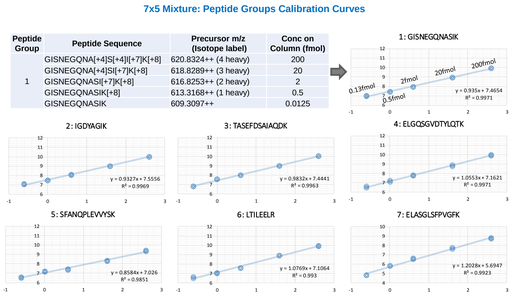


7x5 Mixture: Skyline Software Analysis



7x5 Mixture: Peptide Groups Calibration Curves

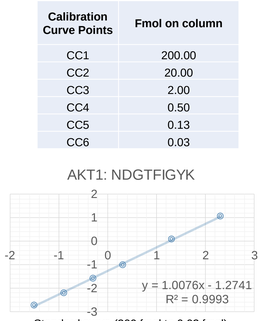
Peptide Group	Peptide Sequence	Precursor m/z (Isotope label)	Conc on Column (fmol)
1	GISNEGQNA[+4]S[+4]I[+7]K[+8]	620.8324++ (4 heavy)	200
	GISNEGQNA[+4]S[+7]K[+8]	618.8289++ (3 heavy)	20
	GISNEGQNASI[+7]K[+8]	616.8253++ (2 heavy)	2
	GISNEGQNASIK[+8]	613.3168++ (1 heavy)	0.5
	GISNEGQNASIK	609.3097++	0.0125



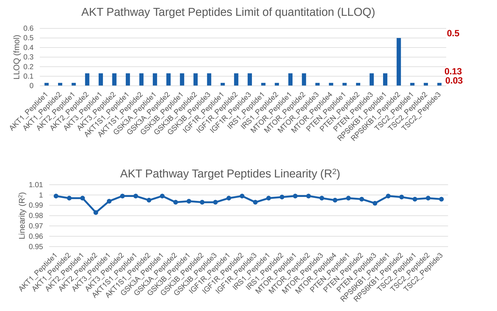
Calibration curve generated for each peptide set of 5 isotopologues of the same peptide sequence showed >3 orders of magnitude dynamic range and linearity (R^2) ≥ 0.9800

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

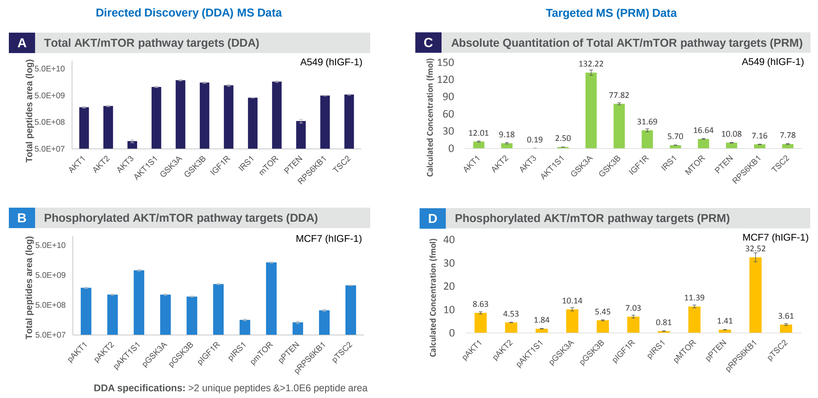


Standard curve (200 fmol to 0.03 fmol)



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)

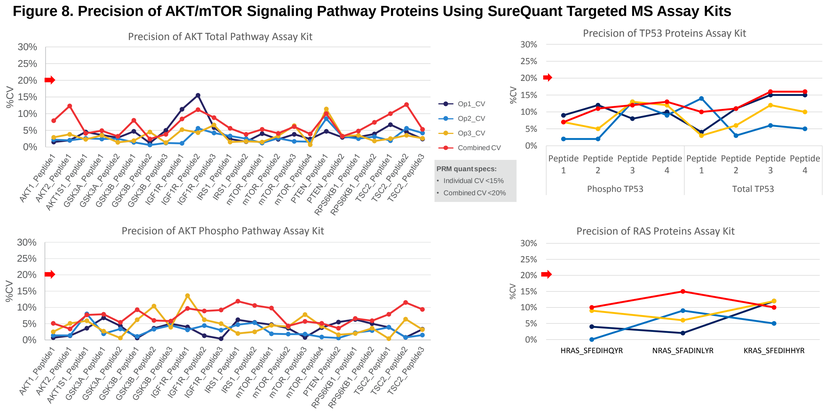
Figure 7. Absolute or Relative Quantitation of AKT/mTOR Signaling Pathway Proteins Using SureQuant Targeted Mass Spec Assay Kits



DDA specifications: >2 unique peptides &>1.0E6 peptide area

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 Thermo Scientific™ 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.

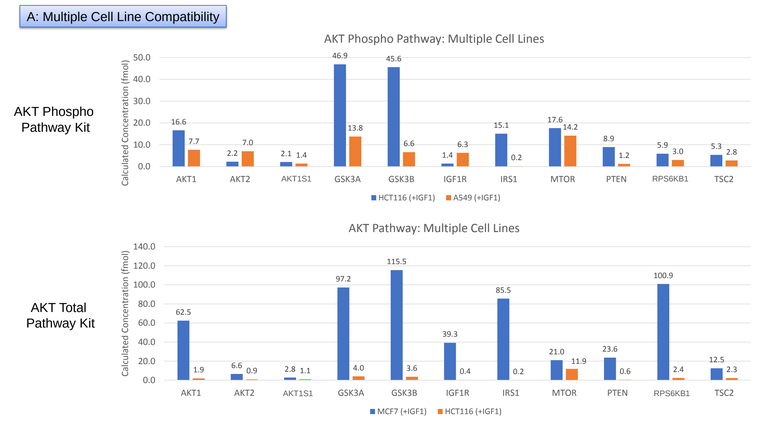
Figure 8. Precision of AKT/mTOR Signaling Pathway Proteins Using SureQuant Targeted MS Assay Kits



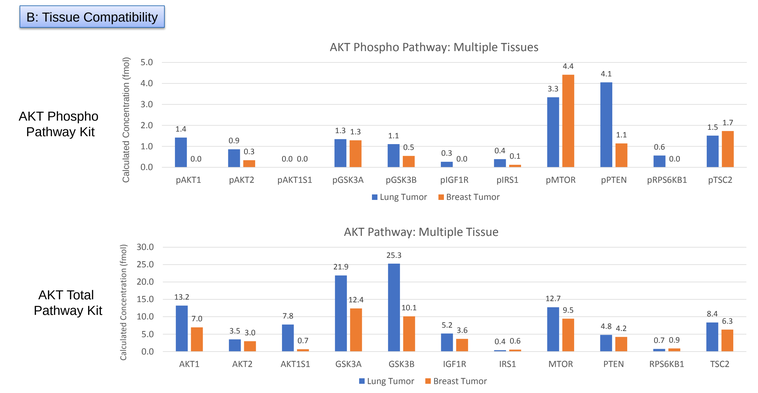
All SureQuant Pathway Mass Spec Assay kits allowed absolute quantitation of target peptides from positive control lysate with <15% individual CV and <20% combined CV using PRM analysis.

Figure 9. Multiple Cell Lines and Tissue Lysate Compatibility for SureQuant AKT Pathway IP-MS Assay Kits

A: Multiple Cell Line Compatibility

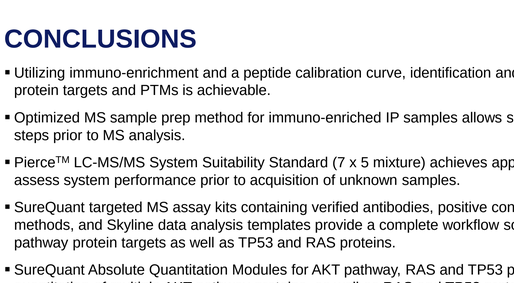


B: Tissue Compatibility



AKT/mTOR pathway proteins were enriched from three cancer cell lines (IGF1-treated MCF7, A549 or HCT116) (A) and two tumor tissues (Breast tumor or Lung tumor) (B) using the SureQuant AKT Pathway or AKT Phospho Pathway Targeted Mass Spec Assay kits (IP and MS sample prep module and Absolute Quantitation module). Data was acquired on a Thermo Scientific™ QExactive™ HF-Orbitrap™ mass spectrometer using the directed discovery (DDA, data not shown) 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 all 11 total and phosphorylated AKT pathway targets across all cell lines. 10 of 11 phospho AKT pathway targets were quantitated in lung tumor samples and 7 of 11 phospho AKT pathway targets were quantitated in breast tumor samples. All 11 total AKT pathway targets were quantitated across lung and breast tumor samples.

Figure 10. Multiple Cell Lines and Tissue Lysate Compatibility for SureQuant RAS Proteins IP-MS Assay Kit



RAS proteins were enriched from two cancer cell lines (IGF1-treated HCT116 or HEK293) and two tumor tissues (Breast or Lung) using the SureQuant RAS Proteins Targeted Mass Spec Assay kits (IP and MS sample prep module and Absolute Quantitation module). Acquisition was performed on a Thermo Scientific™ Q_Exactive™ HF Orbitrap™ mass spectrometer using targeted MS (PRM) acquisition method. PRM analysis using the calibration curve allowed absolute quantitation of all 3 RAS isoforms across all lysates.

CONCLUSIONS

- Utilizing immuno-enrichment and a peptide calibration curve, identification and quantitation of multiple low-abundance protein targets and PTMs is achievable.
- Optimized MS sample prep method for immuno-enriched IP samples allows single-day sample prep with fewer handling steps prior to MS analysis.
- 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 targeted MS assay kits containing verified antibodies, positive control lysate, peptides, buffers, instrument methods, and Skyline data analysis templates provide a complete workflow solution for quantitation of AKT-mTOR pathway protein targets as well as TP53 and RAS proteins.
- SureQuant Absolute Quantitation Modules for AKT pathway, RAS and TP53 proteins allowed simultaneous absolute quantitation of multiple AKT pathway proteins, as well as RAS and TP53 proteins, in treated cell lines and tumor samples with high accuracy and precision (CV <20%).

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