thermoscientific

Multiplex quantitative analysis using NeuCode SILAC metabolic labeling of signaling protein targets

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ABSTRACT

Purpose: The objective of this study was to assess multiplex immunoprecipitation in combination with multiplex quantitation of stable isotope labeling using amino acids in cell culture (SILAC) using neutron encoded amino acids (NeuCode™) for identification and relative quantitation of AKT/mTOR pathway targets.

Methods: A549 were cultured with SILAC media containing 10% dialyzed FBS and one of 6different lysine isotopologs K000, K202, K040, K602, K341 and K080 (KXXX is lysine with designated number of 13C, 2H, and 15N, respectively). Samples were combined for multiplex immunoprecipitation before analysis using Thermo Scientific™ Orbitrap Fusion™ mass spectrometer or the Thermo Scientific™ Orbitrap Fusion™ Lumos™ at 500K or1000K resolution. MaxQuant™ software modified for NeuCode data analysis was used for peptide identification and quantification.

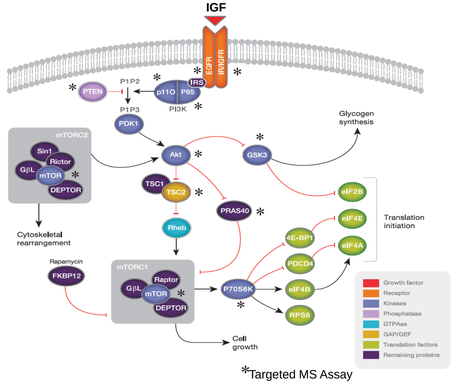
Results: Cells cultured with the different lysine isotopologs exhibited similar viability and rate of heavy amino acid incorporation for two human cell lines. High resolution MS1 scans using Orbitrap technology were used to separate near isobaric lysine isotopolog isotopic clusters at +4 and +8 Da. A modified version of MaxQuant software was used to automatically extract the quantitative signatures.

INTRODUCTION

The IGF1R/AKT/mTOR pathway plays a central role in tumor progression and anti-cancer drug resistance. The quantitative measurement of protein expression and PTMs of the AKT/mTOR pathway is vital to cancer research.[1] A major limitation in the quantitation of AKT/mTOR pathway proteins is the lack of rigorously validated methods/reagents and a reliance on semi-quantitative results from Western blotting. MS is increasingly becoming the detection methodology of choice for protein abundance and PTMs. IP is commonly used upstream of MS as an enrichment tool for low-abundant protein targets.[2,3] In addition to protein identification, IP can be combined with targeted MS to identify proteins of interest and protein-protein interactions (Figure 2).[4]

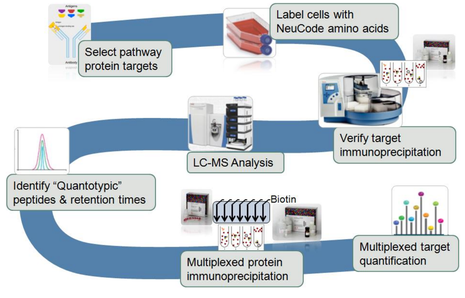
NeuCode is a quantitative proteomics technology that enables higher SILAC multiplexing through the use of near isobaric amino acid isotopologs.[5-6] NeuCode amino acids have the same nominal mass and structure but are labeled with different combinations of 2H, 13C and 15N stable isotopes that can be resolved using high resolution mass spectrometry (Figure 3). Unlike traditional SILAC which compares light versus heavy amino acids, NeuCode technology uses only heavy amino acids for relative quantitation. An advantage of this approach is that sample complexity is not increased upon multiplexing as the labels within an isotope cluster are not fully resolved at low resolution.

Figure 1. IGF1R/AKT/mTOR Signaling Pathway.



MATERIALS AND METHODS

Figure 2. Experimental Workflow for mIP-MS Assay



Sample Preparation

A549 (ATCC) were cultured with SILAC media (Thermo Scientific) containing 10% dialyzed FBS and one of 8 different lysine isotopologs K000, K202, K040, K602, K341 and K080 (lysine with designated number of 13C, 2H, and 15N, respectively). Cells cultured with the different lysine isotopologs exhibited similar viability and growth by both MTT and AlamarBlue assay (data not shown). NeuCode amino acid incorporation was also assessed over time by LC-MS of labeled protein digests before IP enrichment.

Multiplex Immunoprecipitation

Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit (Protein A/G) was used to screen antibodies for total and phosphorylated AKT/mTOR and RAS/ERK pathway targets from 500 µg cell lysate. Successful antibodies which significantly enriched their specific target 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 (see Figure 4 for targets used in multiplexed IPs). IP samples were processed using an in-solution digestion method in which IP eluates were reconstituted with 6M Urea, 50mM TEAB pH 8.5 followed by reduction, alkylation and LysC or trypsin digestion overnight at 37ºC.

Liquid Chromatography and Mass Spectrometry

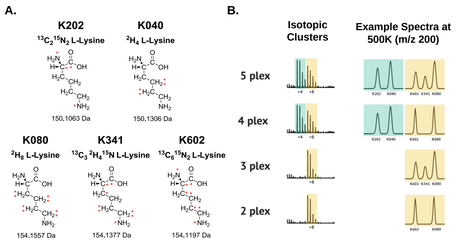
Prior to MS analysis, protein digest samples were desalted on-line using the Thermo Scientific™ Acclaim™ PepMap™ 100 C18 Trap Column. Samples were separated by RP-HPLC using a Thermo Scientific™ Dionex™ UltiMate™ 3000 system connected to a Thermo Scientific™ EASY-Spray™ column, 15 cm x 75 µm over a 1 hr. 2-35% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. Unlabeled samples were analyzed by using a Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer for peptide identification and SRM/PRM assay development.

NeuCode labeled samples were analyzed on the Orbitrap Fusion MS and the Orbitrap Fusion Lumos mass spectrometer operating at an MS1 resolving power of 500K at m/z 200 using top 10 DDA, tSIM or tSIM-DDA top 2 MS2. tSIM experiments were performed using resolution of 500K at m/z200, AGC target of 5e4 and isolation width of 6 amu, max.injection time of 1 sec. For tSIM-DDA method, two MS2 IT CID scans were performed at isolation width of 2 and 1e4 AGC target.

Data Analysis

Spectral data files were analyzed using MaxQuant modified for NeuCode peptide identification and quantification.[6] Carbamidomethylation (+57.021 Da) used as a static modification for cysteine. Different mass modifications for lysine were used as variable modifications for lysine in addition to methionine oxidation (+15.996 Da). Data was searched against a Swiss-Prot® human protein databases with a 1% FDR criteria for peptide and protein matches.

Figure 3. A) Structures and masses of NeuCode amino acids. Stable isotope labels are indicated by red asterisks. B) Different combinations of lysine isotopologs can be used to increase multiplexing from 2-plex to 5-plex at high resolution (500K at m/z 200).



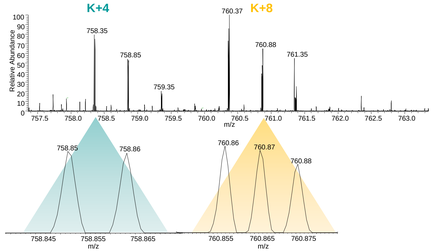
RESULTS

AKT-mTOR pathway targets were immunoprecipitated from different cell lysates with Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kits (Protein A/G or Streptavidin) for MS analysis. Due to relative low abundance, many of these proteins were only able to be identified in the IP-enriched samples as seen by higher numbers of unique peptides were identified in IP-enriched samples compared to neat lysate (Figure 4). Protein isoforms and interacting partners were also identified for CTNNB1, PAK1 and mTOR targets(Figure 7). In addition, relevant phosphorylation sites were detected for CTNNB1, PAK1 and EFGR.

Figure 4. Enrichment is required to identify and quantify medium to low abundant AKT/mTOR pathway target proteins.

IP Antibody	Target	Cell Line	IP Enriched # of Unique Peptides		Relevant Phospho peptide ID
			Neat	Enriched-IP	
AKT1	AKT1	A549	2	12	
	AKT2		2	11	
	AKT3		-	3	
mTOR	mTOR	A549	2	82	
	RICTOR		-	2	
	SIN1		-	3	
	Gbl		-	4	
CTNNB1	CTNNB1	HCT116	11	33	Ser191; Ser551; Ser552; Ser675
	APC		-	35	
	CDH1		6	13	
	CDH3		6	19	
	CTNNA1		21	64	Ser641; Thr654; Thr658
RAS	KRAS	HCT116	-	13	
	HRAS		-	15	
	NRAS		2	14	
PAK1	PAK1	HEK293	3	23	Ser174; Thr230
	GIT1		9	19	
	GIT2		3	7	
RAF1	RAF1	HCT116	2	42	
TSC2	TSC2	A549	-	10	
PIK3CA	PIK3CA	A549	-	7	
	PIK3R1		-	5	
	PIK3R2		-	4	
EGFR	EGFR	A549	18	60	Ser991; Ser1026; Ser1039
RPTOR	RPTOR	HCT116	7	27	

Figure 5. MS spectra of NeuCode 5plex sample mixed at equal ratios. Two lysine isotope clusters (+4, +8) are shown at high resolution (500K at *m/z* 200 on Orbitrap Fusion Lumos MS) can be resolved into 5 distinct peaks for relative quantitation.



By combing various NeuCode lysines, it is possible generate spectra containing multiple isotopic clusters of +4 and +8 Da mass differences that include two or three lysines separated by 27 and 18 mass differences, respectively (Figure 5). For some peptides where the monoistopic peak can not be fully resolved, the M+1 or M+2 peaks can be used for quantitation.

To assess NeuCode quantitation of multiplex IP samples, cells labeled with different NeuCode amino acids were combined and IP-enriched before LC-MS analysis (mIP-MS) using different MS acquisition methods including DDA and targeted SIM methods. As shown in Figure 6, relatively abundant targets such as IQGAP1 could be quantified using DDA, but lower abundant targets such as mTOR benefited from the tSIM method as the DDA method did not provide adequate ion intensity for quantitation.

Figure 6. Comparison mIP-MS using different acquisition methods and NeuCode multiplexing. A) IP target IQGAP1 from 2plex and 4plex NeuCode samples quantified using DDA method. B) IP target mTOR from 4plex NeuCode sample quantified from tSIM method.

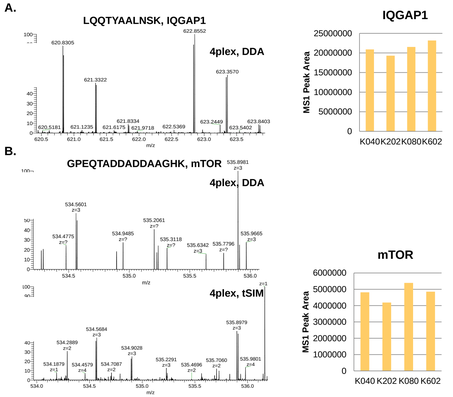
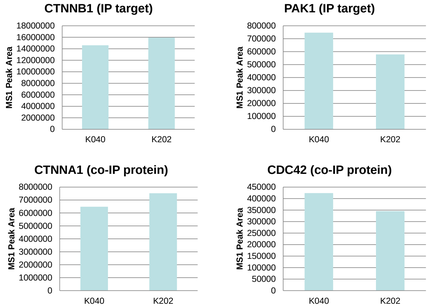


Figure 7. Quantitation of IP and co-IP proteins from 2plex NeuCode samples.



CONCLUSIONS

- Enrichment is necessary for identification and quantitation of low abundant signaling pathway proteins, interacting partners & PTMs for MS applications.
- Multiplex IP-MS assays in combination with NeuCode metabolic labeling enabled detection and quantitation of multiple target AKT/mTOR pathway proteins and their interacting partners in a single LC-MS analysis.
- Samples mixed in equal and fixed ratios show high correlations with expected ratios as measured using latest MaxQuant which can process NeuCode raw files.
- Evaluation of higher multiplex samples using targeted MS methods (tSIM) is limited by lack of software tools for quantification of multiple isotopic clusters.

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