Abstract

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

Methods: A549 were cultured with SILAC media containing 10% dialyzed FBS and one of eight different lysine isotope formulations (K000, K202, K040, K602, K341, K440 and K080 [lysine with designated number of 13C, 2H, and 15N, respectively]). Cells cultured with designated number of 13C, 2H, and 15N, respectively. Cells cultured with the different lysine isotope exhibited similar viability and rate of heavy amino acid incorporation for two high-resolution mass spectrometers and two different isotopologs were isolated using LC-MS for identification and quantification of targets.

Results: CED and IP target data was compared across different isotope conditions and overall, NeuCode SILAC methodology was successful in quantifying the majority of AKT/mTOR targets.

Conclusions: Multiplex SILAC quantification of AKT/mTOR targets using NeuCode SILAC labeling protocol enables high-throughput quantification of AKT/mTOR targets.

Materials and Methods

Sample Preparation

4plex (ATCC) were cultured with SILAC media (Thermo Fisher Scientific) containing 10% dialyzed FBS and one of eight different lysine isotope formulations (K000, K202, K040, K602, K341, K440 and K080 [lysine with designated number of 13C, 2H, and 15N, respectively]). Cells with different lysine isotope exhibited similar viability and growth by both RT-PCR and AlamarBlue assay (data not shown). NeuCode amino acids were also sequenced 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 pathway targets. 100 μg cell lysate was used for each 10% protein A/G IP. Samples were processed using an in multiplexed IP. Immunoprecipitated protein targets were quantified using DDA method, two or three IP samples were analyzed using an in multiplexed IP.

Liquid Chromatography and Mass Spectrometry

Prior to MS analysis, protein digest samples were desalted on-line using the Thermo Scientific™ Accurate™ mass 10 T chip Column. Spectra were acquired by LC-MS using a Thermo Scientific™ Exactive™ or Thermo Scientific™ Orbitrap Fusion™ mass spectrometers. A 4-plex, DDA method was used for qualitative analysis.

Results

AKT/mTOR pathway targets were immunoprecipitated from different cell lysates with Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP-Kit (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 identified in IP-enriched samples compared to neat lysate (Figure 4). Protein isoforms and interacting partners were also identified for CED176, PKB1 and PDK1 targets (Figure 6). In addition, relative protein phosphoproteins were identified using a Thermo Scientific™ Exactive™ mass spectrometer.

CONCLUSIONS

Enrichment is necessary for identification and quantification of low abundant signaling pathway proteins, interacting partners & PTMs for MS applications.

Multiplex IP-MS assays in combination with NeuCode metabolite labeling enabled detection and quantification of multiple target AKT/mTOR pathway proteins and their interacting partners in a single LC-MS analysis.

Samples mixed in equal and fixed ratios showed high correlations with expected ratios as measured using the latest version of MaxQuant which can process NeuCode raw files.

Evaluation of high multiplex samples using targeted MS methods (MSMS) is limited by lack of software tools for quantification of multiple isocline clusters.

REFERENCES


TRADEMARKS/LICENSES

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