thermoscientific

# Antibody verification by protein immunoprecipitation and mass spectrometry (IP-MS) characterizes antibody performance and identifies protein-protein interactions

Gregory K. Potts[1], Bhavin Patel[2], Leigh Foster[2], John C. Rogers[2]; Abbvie, Abbott Park, IL, [2]Thermo Fisher Scientific, Rockford, IL

## ABSTRACT

Antibodies have been adopted as investigative tools to empower research and diagnostic applications in academia and industry. In this role, antibodies have been utilized to enrich protein targets for the detection and quantification of proteins and their post-translational modification (PTMs) from complex samples. While hundreds of thousands of antibodies are commercially available, many of these antibodies are poorly characterized.[1,2] This lack of characterization places a burden on researchers due to delayed project timelines, increased costs, and potentially flawed research conclusions.

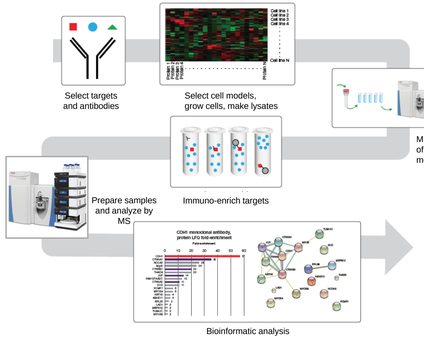
To verify antibody quality and performance, we have created a comprehensive workflow to assess antibody specificity using immunoprecipitation combined with mass spectrometry (IP-MS). In preliminary experiments, we performed deep proteome analyses of 12 cultured cancer cell lines using a bottom-up MS workflow to analyze unfractionated and fractionated peptide samples. Each unfractionated digest identified 3,800-4,500 unique protein groups, and each fractionated digest identified 7,500-9,000 protein groups. These protein expression profiles were incorporated into a library to facilitate the selection of cell lysates which expressed protein targets at low to medium abundance for subsequent IP-MS analysis. An initial screen of over 650 antibodies to nearly 100 key cancer signaling proteins determined that over 70% of antibodies which were previously validated for immunocapture could be used to bind and identify their intended targets by IP-MS, and over 45% of screened antibodies not previously validated for IP applications successfully pulled down their intended targets.

Utilizing MS-based label free quantification (LFQ), we developed a system to generate a fold-enrichment score to better visualize an antibody's selectivity for its intended target compared to the non-specifically bound proteins identified in each IP experiment. Beyond simply determining the presence or absence of an antibody's target following IP, we show that our IP-MS approach is uniquely capable of calculating a fold-enrichment score for interacting proteins or potential off-target proteins. For example, in multiple CDH1, CTNNB1, and TP53 IP-MS experiments, screened antibodies enriched their targets by several orders of magnitude versus background and bound to known protein interactors as determined using the STRING database and GO term enrichment. To demonstrate the efficacy of these antibodies, we used a subset to simultaneously immunocapture twelve proteins in the Akt/mTOR pathway, and then quantified the proteins and their phosphorylation in three hIGF-1 stimulated and unstimulated cell lines using MS-based targeted quantification.

## INTRODUCTION

A number of different approaches have been suggested to help verify antibody quality.[3-5] To help characterize antibody performance and assess the binding specificity of Invitrogen™ antibodies produced by Thermo Fisher Scientific, here we describe a new approach to antibody target verification. Through the use of optimized sample preparation reagents and methods, high resolution MS instrumentation, and a novel data analysis pipeline, we have created a comprehensive workflow to assess an antibody's specificity for its intended target using IP-MS (Fig. 1). The benefits of this IP-MS approach include: identification of the antibody target(s), isoforms, and modifications, quantitative assessment of antibody selectivity by calculation fold-enrichment of targets and off-target proteins, and identification of interacting proteins.

**Figure 1. IP-MS sample preparation workflow and data analysis pipeline.**




## MATERIALS AND METHODS

**Cell Culture and Deep Proteome Analysis**

12 cell lines were purchased from ATCC, including: HCT116, A549, MCF7, HepG2, LNCaP, NIH3T3, BT-549, SK-MEL-5, Hs 578T, SR, HeLa, and HEK293. All media and cell growth products were purchased from Thermo Fisher Scientific, including trypsin (Cat. No. 25200-056) and HBSS (Cat. No. 14175-079), and all media was supplemented with 10% FBS (Cat. No. 16000-036), 1X penicillin-streptomycin (Cat. No. 15140-163), and insulin (Cat. No. 12585014), if needed. For targeted MS of 12 AKT-mTOR proteins, cells were starved in 0.1% charcoal stripped FBS for 24 hours before stimulation with 100ng/ml of hIGF-1 for 15 minutes. Cells were grown to ~80% confluency and at passage 12–18 before lysis with IP Lysis Buffer (Cat. No. 87788) and 1:100 Halt™ Protease and Phosphatase Inhibitor Cocktail (Cat. No. 78445). Protein concentration was determined with the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Cat. No. 23225) using a Multiskan™ GO instrument for measurement.

Following MS sample preparation using the Thermo Scientific™ Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (Cat. No. 84840), 100 µg of digested peptide samples were fractionated with the Thermo Scientific™ Pierce™ High pH Reverse-Phase Peptide Fractionation Kit (Cat. No. 84868) using a custom fractionation profile and analyzed by LC-MS. MS data were searched using Thermo Scientific™ Proteome Discoverer™ 1.4 software. Cell lines were selected for IP if they expressed protein targets at low to moderate abundance.

**Immunoprecipitation and MS Sample Preparation**

The Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Protein A/G, Cat. No. 90409) was used to screen and validate antibodies for over 650 protein targets from 500µg cell lysate. Validated antibodies were biotinylated with the Thermo Scientific™ Pierce Antibody Biotinylation Kit for IP. The Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Streptavidin, Cat. No. 90408) was used to perform the single or multiplex IPs for target enrichment. IP samples were processed by an in-solution digestion method where IP eluates were reconstituted in 6M Urea, 50mM TEAB, pH 8.5 followed by reduction, alkylation and trypsin digestion overnight at 37°C. The digested samples were acidified with TFA before MS analysis.

**Liquid Chromatography and Mass Spectrometry**

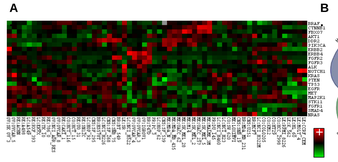
Tryptic digest samples were desalted on-line using the Thermo Scientific™ Acclaim™ PepMap 100 C18 Trap Column. For discovery MS, the IP samples were analyzed by nanoLC-MS/MS using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System and Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer. For targeted MS, the samples were analyzed using the UltiMate 3000 RSLCnano System and the Thermo Scientific™ TSQ™ Vantage™ Mass Spectrometer (SRM mode) or the Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (PRM mode).

**IP-MS Data Analysis**

Samples with detectable targets were searched using MaxQuant 1.5.3.51 to obtain relative quantification of peptides and proteins and compare these protein abundances from replicate IP samples to unfractionated and fractionated proteome lysate samples. Label-free quantification (LFQ) was calculated using a minimum threshold of 2 unique peptides for quantification.

Protein LFQ values were used to generate scatterplots to characterize the specificity of antibodies used in IP. LFQ values were plotted to compare the relative abundances of proteins identified in a "test" IP to those proteins identified in a negative control IP for an unrelated target. Fold-enrichment and scatterplot calculations were generated using a custom web application to streamline the data analysis and generation of graphs for IP verification. LFQ values for replicate IPs were utilized to further filter the data for those proteins which were observed reproducibly across replicates. A 30% CV cutoff was used to filter proteins which were not reproducibly identified or quantified across replicate IP samples. The remaining quantified proteins were analyzed using the STRING database (http://string-db.org) to identify known protein interactions described in the literature. For targeted MS data analysis, Thermo Scientific™ Pinpoint software and Skyline software (University of Washington) were used to measure limit of quantitation (LOQ) from the calibration curve and target analyte concentration from unknown samples.

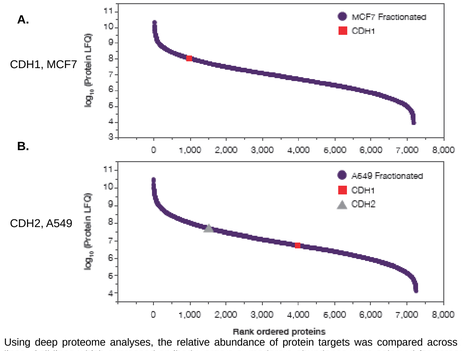
**Figure 2. Selection of cell lines for antibody verification by IP-MS.**




The protein expression profile of multiple cell lines was examined to help aid in the selection of cell lines which expressed protein targets screening antibodies by IP-MS. RNA expression Z-scores from the NCI60 cell line panel were hierarchically clustered for 22 genes in the Ion Ampliseq Colon and Lung Cancer panel to compare expression profiles across cell lines. (Fig. 2A) To further explore the overlap in expressed proteins, the proteomes of 12 cancer cell lines were prepared by offline fractionation and analyzed by MS. The overlap and target proteins was compared across cell lines (Fig. 2B), and targets which were expressed at low to moderate abundance were selected for subsequent screening by IP-MS.

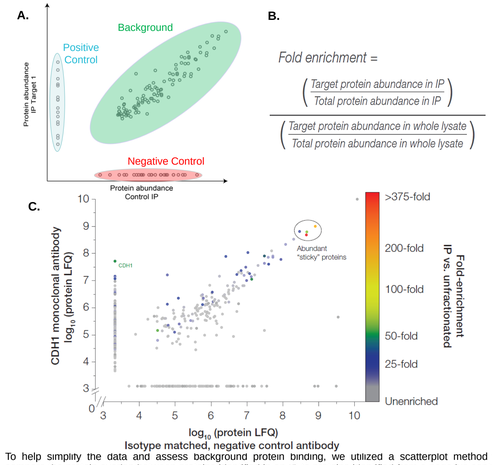
## RESULTS

**Figure 3. Cell model selection for CDH1 and CDH2 antibody screening by IP-MS.**



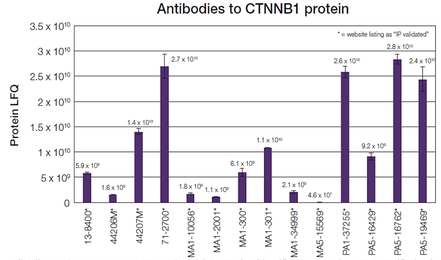

Using deep proteome analyses, the relative abundance of protein targets was compared across 12 cell lines. Cell lines which expressed antibody targets at moderate abundance were selected for screening by IP-MS. For example, MCF7 and A549 were used as the cell models of choice for screening CDH1 and CDH2 antibodies, respectively, since these cell lines expressed CDH1 and CDH2 in the mid-range of their protein abundance curves (Figs. 3A and 3B).

**Figure 4. CDH1 fold-enrichment and protein scatterplot to assess antibody selectivity**



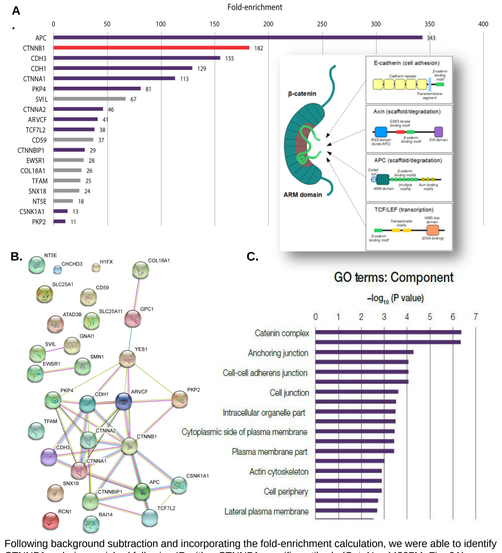

To help simplify the data and assess background protein binding, we utilized a scatterplot method to compare the protein overlap between proteins identified in an IP to proteins identified from a negative control IP (Fig. 4A). The fold-enrichment was calculated for proteins which were unique for the target IP present on the y-axis (Fig. 4B). Implementing these with real data, we were able to remove abundant, "contaminant" proteins from a CDH1 dataset and visualize the enrichment of remaining proteins, including CDH1 and known interactors (Fig. 4C).

**Figure 5. Relative abundance of CTNNB1 determined by IP-MS across 14 CTNNB1 antibodies**




Antibodies to CTNNB1 were screened and the proteins identified by IP-MS were quantified using MaxQuant LFQ.

**Figure 6. CTNNB1 and known CTNNB1 interactors were enriched using a CTNNB1 antibody (Cat. No. 44207M).**




Following background subtraction and incorporating the fold-enrichment calculation, we were able to identify CTNNB1 as being enriched following IP with a CTNNB1 specific antibody (Cat. No. 44207M, Fig. 6A). Interacting proteins were also enriched as annotated by the STRING database (Fig. 6B). Several of these annotated interactors have CTNNB1 binding domains (Fig. 6A, inset), and GO term enrichment displayed overrepresentation of proteins mapping to the catenin complex (Fig. 6C).

**Figure 7. Multiplex IP of 11 Phosphorylated and 12 Total AKT-mTOR Pathway Proteins and nanoLC-MS/MS Analysis.**

| Target | 11 plex phospho assay | | 12 plex total assay | |
|---|---|---|---|---|
| | No. of Unique Peptides | | | |
| | - IGF | + IGF | - IGF | + IGF |
| AKT1 | - | 9 | 25 | 30 |
| AKT2 | - | 4 | 24 | 26 |
| mTOR | 48 | 56 | 25 | 28 |
| IGF1R | 1 | 3 | 32 | 35 |
| IR | N/A | N/A | 29 | 26 |
| PRAS40 | 5 | 7 | 9 | 10 |
| p70S6K | 9 | 14 | 11 | 12 |
| TSC2 | 5 | 10 | 42 | 45 |
| PTEN | - | 1 | 5 | 9 |
| GSK3α | 7 | 6 | 19 | 21 |
| GSK3β | 13 | 10 | 23 | 23 |
| IRS1 | 4 | 11 | 45 | 54 |
| PIK3R1 | - | - | - | 22 |
| PIK3CA | - | - | - | 2 |
| PIK3CB | - | - | - | 6 |
| PIK3R2 | - | - | - | 22 |

11 phosphorylated and 12 total AKT-mTOR pathway protein targets were enriched simultaneously from unstimulated and hIGF-1 stimulated MCF7 lysates with biotinylated antibodies and Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Streptavidin). nanoLC-MS/MS analysis identified 11 proteins in multiplex phospho assay and 12 proteins for multiplex total assay.

## CONCLUSIONS

- Deep proteome analysis of multiple cell lines was crucial for determining the protein expression profile of each cell line.
- Cell lines which expressed antibody targets at moderate abundance were selected for screening panels of antibodies.
- IP-MS was capable of identifying antibody targets and off-target proteins, including interacting and background proteins.
- Incorporating novel filtering steps into the informatics workflow allowed background proteins to be characterized and removed from datasets.
- Using a scatterplot and fold-enrichment calculations, enrichment of protein targets and interactors can be successfully preserved, increasing confidence in antibody performance.
- Total and phosphorylated mIP-tMS assays allowed simultaneous identification of total AKT-mTOR pathway proteins and phosphorylation sites in unstimulated and hIGF-1 stimulated MCF7 lysate.

## REFERENCES

1. Bordeaux, J., et al., Antibody validation. Biotechniques, 2010. 48(3): p. 197–209.
2. Bjorling, E. and M. Uhlen, Antibodypedia, a portal for sharing antibody and antigen validation data. Mol. Cell. Proteomics, 2008. 7(10): p. 2028–37.
3. Pauly, D. and K. Hanack, How to avoid pitfalls in antibody use. F1000Res, 2015. 4.
4. Marcon, E., et al., Assessment of a method to characterize antibody selectivity and specificity for use in immunoprecipitation. Nature Methods, 2015. 12, 725-731.
5. Uhlen, M., et. Al., A proposal for validation of antibodies. Nature Methods, 2016. doi:10.1038/nmeth.3995

## TRADEMARKS/LICENSING

For Research Use Only. Not for use in diagnostic procedures.
© 2017 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.