iPRM: Quantifying proteins and PTMs through targeted mass spectrometry

Introduction

Accurate quantitation of proteins and sites of post-translational modification can reveal important clues about the health of a biological system, the dynamics of protein interactions and signaling pathways, and the underlying mechanisms of disease. Traditional methods of targeted protein quantitation have relied on immunoassay techniques, such as ELISA and western blot. Array and bead-based platforms have increased throughput and multiplexing capabilities, but the lack of validation standards for immunoassays can lead to potential issues with sensitivity and specificity.

In recent years, liquid chromatography tandem mass spectrometry (LC-MS/MS) has gained widespread acceptance as a powerful tool for quantitative protein analysis. With an LC-MS/MS method, proteins are enzymatically digested into peptides, and then one or more of these peptides is used as a surrogate for protein quantitation. Targeted tandem mass spectrometry techniques, such as multiple reaction monitoring (MRM) or parallel reaction monitoring (PRM), are then used for quantitation. Because of the exquisite accuracy of mass measurements in the mass spec, the quantitation is highly selective. Additionally, the technique is capable of high throughput and can be multiplexed to allow the quantitation of many proteins at once. The addition of heavy isotope-labeled synthetic peptides to the sample as internal standards allows accurate and absolute quantitation.

For many applications, combining targeted LC-MS/MS with an immuno-enrichment strategy (immuno-MRM or immuno-PRM) can extend the advantages of the methodology even further by improving the dynamic range and detection limits. This can be especially beneficial for low-level analytes in complex samples where higher concentration proteins and matrix effects can otherwise make their detection problematic.

Cell Signaling Technology (CST) offers an immuno-PRM (iPRM) Assay Service that combines antibody enrichment with the sensitivity and selectivity of LC-MS/MS. While traditional immunoenrichment strategies are typically performed at the protein level, the iPRM workflow captures and enriches at the peptide level as illustrated in **Figure 1**. This has the advantage that variations in protein sequence, such as subtle differences between post-translationally modified forms, or amino acid substitutions that may be buried at the protein level, can be more easily distinguished and captured. Additionally, because the antibody enriches both the target peptide as well as a heavy isotope-labeled synthetic peptide standard spiked in just prior to immunoenrichment, the internal standards are exposed to the same capture conditions as the native peptides ensuring better accuracy for quantitation.

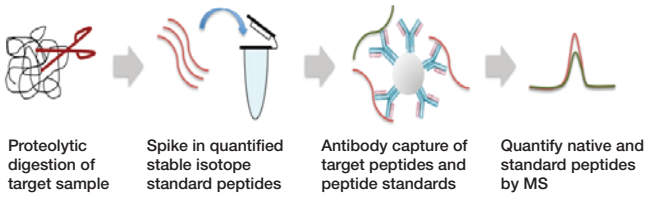

Figure 1. Workflow of the iPRM assay. Proteins in the sample are enzymatically digested into peptides, followed by addition of heavy isotope-labeled synthetic peptide internal standards. Enrichment of selected peptides, along with their heavy isotope-labeled internal standard counterparts, is achieved by antibody capture. Finally, LC-MS/MS is used for targeted detection and quantitation of the enriched peptides.

The advantage of immuno-enrichment prior to mass spectrometry analysis is demonstrated in **Figure 2**. Here, the observed signal-to-noise (S/N) ratio is plotted for different concentrations of a phosphopeptide analyzed using PRM and iPRM. As shown, antibody enrichment allows much lower limits of detection and quantitation and access to protein levels that would otherwise be obscured from detection by matrix effects and other more concentrated proteins.

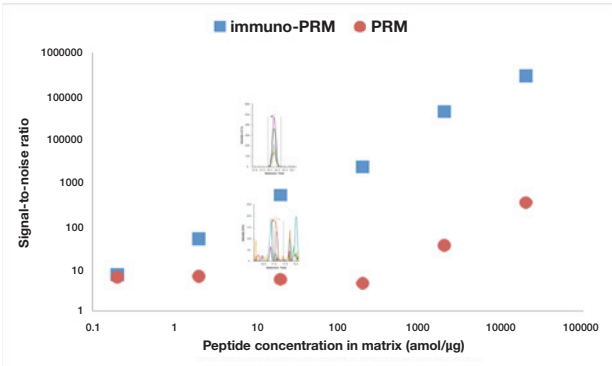

Figure 2. Importance of antibody enrichment. The graph shows the comparison of S/N ratios for various concentrations of heavy-labeled c-Jun phosphopeptide LAS*PELER++ derived from protein JUN. The peptide was spiked into a tryptic digest of an SKBR3 lysate (a human breast cancer cell line) at different concentrations and then analyzed either directly by PRM (red circles) or following immuno-enrichment using the CST antibody #3270 (blue squares). As shown, antibody enrichment using iPRM provides better S/N, enabling much lower limits of detection (LOD) and quantitation (LOQ). Extracted ion chromatograms for the peptide at a concentration of 20 amol/ug are compared in the insets for the iPRM assay (top) and the PRM assay (bottom).

In this application note, the iPRM assay is applied toward the quantitation of proteins from different cell lines in order to demonstrate typical sensitivity, accuracy, precision, and linearity that can be expected. Results are also presented for the iPRM analysis of phosphorylated proteins to demonstrate quantitation of low-level modified proteins in a multiplexed format. For all studies, western blot analysis data are included for comparison.

Methods

Cell lysates, antibodies, and heavy isotope-labeled synthetic peptide internal standards

Cell lysates were from SKBR3 human breast cancer cells, HT29 human colon adenocarcinoma cells, MKN45 gastric cancer cells, and ACHN human renal carcinoma cells.

All heavy isotope-labeled synthetic peptide standards were synthesized using 13C and 15N for heavy atom incorporation and are available from CST.

All antibodies were from CST and were used to capture both native and heavy isotope-labeled synthetic peptide standards from cell lysates.

Preparation of samples

Quantitation of c-Met receptor tyrosine kinase

SKBR3 cells, HT29 cells, and MKN45 cells were grown to 80% confluence, washed with PBS, harvested in urea lysis buffer, sonicated, and centrifuged. Lysates were reduced, alkylated, and digested with trypsin. Digested peptides were purified over C18 columns, and resuspended in IAP buffer, and heavy isotope-labeled synthetic peptide standards were spiked into 100 ug of individual trypsin-digested cell samples.

Multiplexed phosphoprotein study

ACHN cells were treated with Human Interleukin-4 (hIL-4) (CST, #8919) at 40 ng/mL for 20 minutes before harvest. For iPRM assays, ACHN cell proteins were digested with trypsin, then 100 µg of ACHN tryptic peptides were mixed with heavy isotope-labeled synthetic peptides which served as internal standards. A multiplexed immunoprecipitation was performed using each of the CST antibodies listed in **Table 1**.

LC-MS/MS analysis

A Thermo Scientific™ EASY-nLC™ II was connected to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer for PRM. In this mode, a targeted list of peptide precursor ions is sequentially fragmented and subsequently analyzed using high-resolution detection.

Quantitation was achieved by extracting fragment ion data using Skyline Software.[1,2] Peak area ratios (heavy/light) were calculated based on the sum of the most abundant fragment ions from light and heavy peptides.

Western blot analysis

Quantitation of c-Met receptor tyrosine kinase

Twenty micrograms of protein extracts from different cell lines were loaded onto a 4% to 20% SDS-PAGE gel. After transfer to nitrocellulose membrane, c-Met was detected with c-Met antibody (#4560) at 1:1000.

Multiplexed phosphoprotein study

Twenty micrograms of protein extracts from control and treated ACHN cells were loaded onto a 4% to 20% SDS-PAGE gradient gel for each blot. After transfer to nitrocellulose membrane, proteins were probed with phospho-specific antibodies at 1:1000. The same antibodies used for the iPRM assay were also used for the western blot analysis.

Results

Quantitation of c-Met receptor tyrosine kinase

Figure 3 shows development of the iPRM assay for the quantitation of c-Met receptor tyrosine kinase. Three peptides from the c-Met protein were selected for custom antibody generation and assay development based on LC-MS/MS analysis of a tryptic digest of c-Met protein. Heavy and light peptides were synthesized, quantitated, and used in tandem with the custom-developed rabbit monoclonal antibodies to generate standard curves and determine quantitative performance of the assays. **Figure 3** shows the absolute quantitation of the 3 different c-Met peptides. For creation of the assay, a constant amount of light synthetic peptide and varying amounts of heavy isotope synthetic peptide were spiked into SKBR3 peptide background, and the observed ratio of heavy:light was compared to the theoretical ratio. The LLOD ranged from 0.02 to 0.16 fmol/mg and the LLOQ ranged from 0.06 to 0.48 fmol/mg for the different peptides.

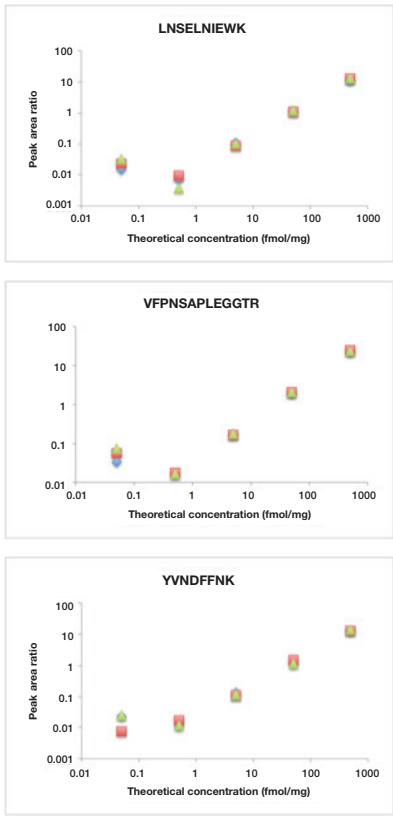

Figure 3. Calibration curves for c-Met peptides. Three different peptides were used to demonstrate quantitation of the c-Met protein. LLOD and LLOQ are as follows: LNSELNIEWK (top) LLOD 0.16 fmol/mg, LLOQ 0.48 fmol/mg; VFPNSAPLEGGTR (middle) LLOD 0.10 fmol/mg, LLOQ 0.30 fmol/mg; YVNDFFNK (bottom) LLOD 0.02 fmol/mg, LLOQ 0.06 fmol/mg.

Figure 4 shows application of the iPRM assay toward the quantitation of c-Met in low-, medium-, and high-expressing cell lines (SKBR3 cells, HT29 cells, and MKN45 cells, respectively). While differences in tryptic digestion efficiency may result in variation in abundance of individual peptides, the quantitative profile obtained with each assay is in agreement with the western blotting data, with error bars reflecting results of triplicate analyses.

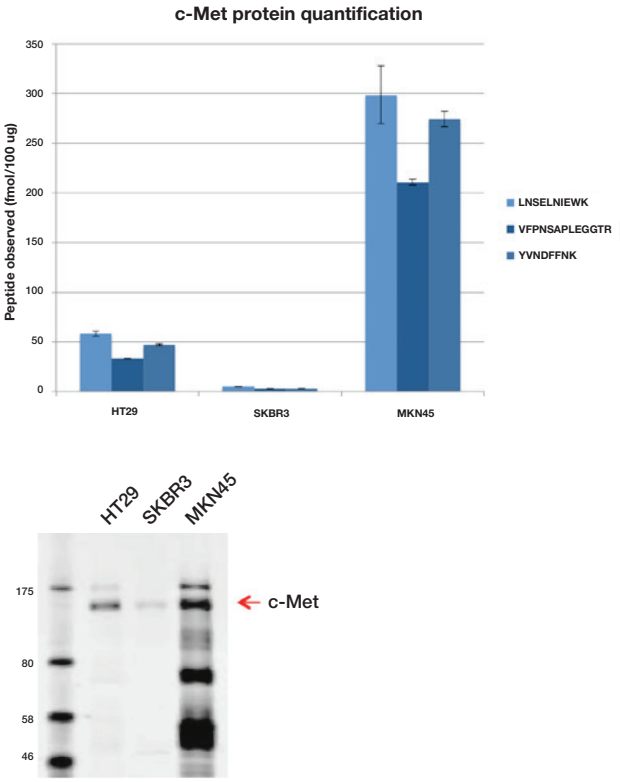

Figure 4. Evaluation of c-Met total protein amount from different cell lines. Low-, medium-, and high-expressing cell lines (HT29, SKBR3, and MKN45, respectively) were evaluated for the total concentration of c-Met protein using the iPRM assay (top) and western blot analysis (bottom). iPRM assay results correlate well with western blot results.

Multiplexed phosphoprotein study

The iPRM assay was used to determine the amounts of ten phosphorylated target proteins in the human renal carcinoma cell line ACHN before and after treatment with interleukin-4 (IL-4). Interleukin proteins are known to regulate communication between cells, including cell growth, differentiation, and motility. The antibodies used in this assay target specific phosphorylation sites within each of these proteins. **Table 1** lists the antibodies, phosphorylation sites, and peptides assayed in this study.

Table 1. CST antibodies, phosphorylation sites, and peptides assayed for multiplexed phosphoprotein study

CST Product Number	Phosphorylation Site	Peptide Assayed
4060	Akt S473	RPHFPQFS*YSASGTA4539
4539	CDK1, CDK2, CDK3 Y15	IGEGTY*GVVYK
2997	PRAS40 T246	LNT*SDFQK
9145	STAT3 Y705	YCRPESQEHPEADPGSAAPY*LK
9314	STAT5A/B Y694	AVDGY*VKPQIK
9364	STAT6 Y641	GY*VPATIK
4370	ERK1 T202/Y204	IADPEHDHTGFLT*EY*VATR
4370	ERK2 T185	IADPEHDHTGFLT*EY*VATR
4370	ERK2 T185/Y187	VADPDHDHTGFLT*EY*VATR
3270	Jun S73	LAS*PELER

As shown in **Figure 5**, IL-4 treatment induced changes in phosphorylation of STAT5, STAT6, and ERK1/2. STAT protein phosphorylation is particularly impacted by addition of IL-4, indicating utilization of the STAT signaling pathways for transmission of this signal. For all targets, the iPRM assay data showed excellent agreement with western blot data.

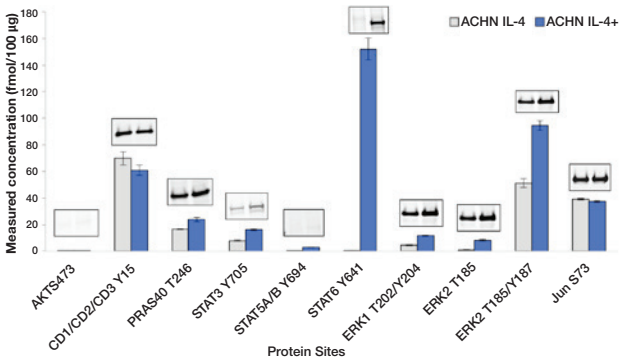

Figure 5. Multiplexed phosphoprotein study. iPRM assay data and corresponding western blot results are shown for 10 different phosphorylated targets in the ACHN cell line before and after addition of IL-4. Peptide abundances measured using the iPRM assay are in good agreement with western blot analysis.

Conclusion

Immuno-parallel reaction monitoring, or iPRM, combines the targeted sample preparation strategies of antibody enrichment with the high sensitivity, specificity, and multiplexing capabilities of targeted LC-MS/MS. CST offers an iPRM Assay Service that can accommodate samples from a wide variety of research, clinical, or preclinical sources, including core needle biopsies, cells, or tissues. Examples of applications that would benefit from iPRM analysis include:

- Quantitation of biomarkers and putative biomarkers in verification and validation assays
- Quantitation of drug on- and off-target effects for many targets simultaneously
- Evaluation of phosphorylation changes in signaling pathways

Because the iPRM assay combines highly specific, validated antibody capture with LC-MS/MS analysis, false positives are virtually eliminated and low-level targets can be accessed that would otherwise be missed with traditional immunoassays or targeted mass spectrometry without immunoenrichment. The CST iPRM Assay Service provides all reagents for all protein targets, including antipeptide antibodies and heavy isotope-labeled synthetic peptide standards. Antipeptide antibodies are available off the shelf and can be evaluated for suitability with iPRM. The goal of the iPRM Assay Service is to provide researchers with the data needed to make better, faster decisions for research, development, and clinical targeted protein quantitation activities.

References

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Technical Support

At CST, providing exceptional customer service and technical support are top priorities. Our scientists work at the bench daily to produce and validate our antibodies, so they have hands-on experience and in-depth knowledge of each antibody's performance. In the process, these same scientists generate valuable reference information that they use to answer your questions and help troubleshoot your experiment by phone or email.

For questions about how to customize your protocol, please contact technical support by emailing support@cellsignal.com, visiting **www.cellsignal.com/support**, or calling 1-877-678-8324.

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