High Throughput Signaling Pathway Analysis Using Multiplex-Immunoprecipitation and Fast LC-PRM

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ABSTRACT

Purpose: High-throughput mass spectrometry-based protein assays were developed, based on multiplex-immunoprecipitation, fast LC separations, and advanced PRM acquisition schemes, to support signaling pathway analysis. They were applied to the monitoring of the main protein components of AKT/mTOR signaling pathway, under"total-" or "phosphorylated-" forms.

Methods: The analyses were performed on a Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer and a Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer operated with several PRM-based acquisition schemes (using instrument programming interface in some cases). Chromatographic separations were carried out using an Evosep One system and a Thermo Scientific™ UltiMate™ 3000 RSLC system equipped for capillary flow. Various gradient lengths and MS acquisition parameter settings were employed to analyze samples of high complexity, *e.g.*, digests of human cell lines, and samples of low complexity obtained through multiplexed immunoprecipitation targeting proteins of AKT/mTOR pathway.

Results: The developed set-ups exhibited the ability to quantify with high sensitivity several dozens of endogenous peptides in one hundred samples within one day under high efficiency acquisition modes. Advanced PRM methods allowed further increases in analytical throughput without compromising the quality of quantification data when combined with multiplexed immunoprecipitation, and minor sensitivity decrease without enrichment.

INTRODUCTION

Signaling pathways play a central role in development and disease. Precise measurements of total form and post-translational modifications (PTM) of signaling proteins (*e.g.*, phosphorylation) are vital to gain insights into mechanisms of diseases as well as to monitor therapeutic responses. Currently, monitoring of signaling pathways still mainly rely on immunoassays, which provide exquisite sensitivity and analytical throughput. However, LC-MS based workflows have significant advantages in terms of specificity, quantification accuracy, and multiplexing capability. Here, we propose to combine multiplex immuno-precipitation of proteins from the AKT/mTOR pathway with fast LC-PRM analyses to measure aberrant activation of this critical signaling pathway in cancer cell line.

MATERIALS AND METHODS

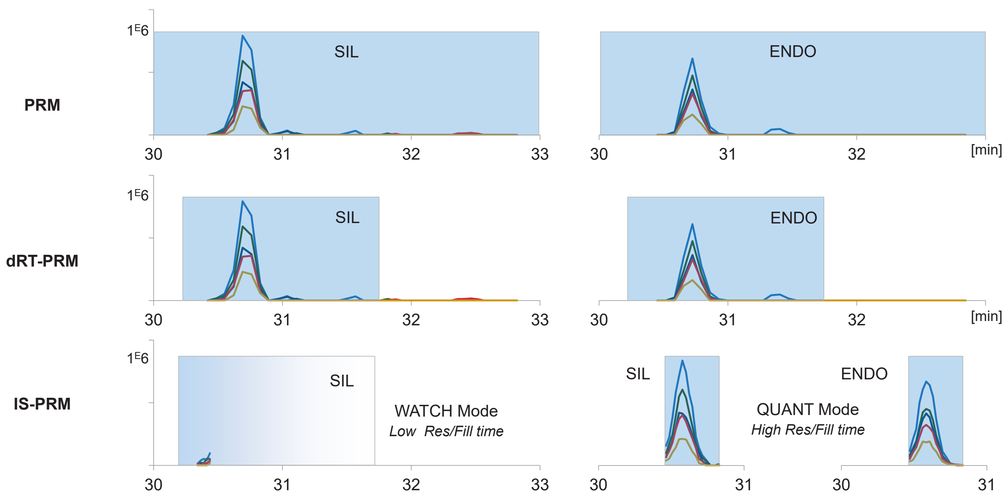
Sample Preparation

Cell Culture: HCT116 cells were grown in McCoy's 5A Media with 10% FBS/1xPenStrep to ~70-80% confluency. HCT116 cells were serum starved in 0.1% charcoal stripped FBS for 24 hours prior to the following treatments: untreated, stimulated (15 min hIGF-1 (100ng/mL; Cell Signaling Technology PN#8917SF)). Subsequent to treatments, 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). Protein concentration of lysates was determined with BCA assay.
Multiplex Immunoprecipitation (mIP): The Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit, Protein A/G (Thermo Fisher Scientific PN#90409) was used to screen and validate antibodies for 13 total and 12 phosphorylated AKT/mTOR pathway targets from 500µg cell lysate. Validated antibodies were biotinylated with the Thermo Scientific™ Pierce™ Antibody Biotinylation Kit for IP (Thermo Fisher Scientific PN#90407). The Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit, Streptavidin (Thermo Fisher Scientific PN#90408) was used to multiplex IPs for target enrichment. IP samples were processed by an in-solution digestion method in which IP eluates were reconstituted with 6M Urea, 50mM TEAB (pH 8.5) followed by reduction, alkylation and trypsin (Thermo Fisher Scientific PN#90057) digestion overnight at 37oC. The digested samples were acidified with TFA.
MS sample preparation: A set of 32 high-purity Pierce™ stable isotopically labeled (SIL) peptides corresponding to 13 proteins from AKT/mTOR pathway was spiked at 20 fmol in 500 ng of HCT116, i) after mIP, or ii) with no enrichment.
For the preparation of the dilution series solutions in "low complexity" matrix, the set of 32 SIL peptides was spiked in various calibrated amounts (7 points from 50 amol to 200 fmol, and one matrix blank) in 1 pmol of a 6-protein mix digest (Thermo Fisher Scientific PN#88342) supplemented with 20 fmol of synthetic unlabeled forms of the peptides. For the preparation of the dilution series solutions in "high" complexity matrix, the set of 32 SIL peptides was spiked in various calibrated amounts (7 points from 50 amol to 200 fmol, and one matrix blank) in 500 ng of a HeLa digest (Thermo Fisher Scientific PN#88329) supplemented with 20 fmol of synthetic unlabeled forms of the peptides.
All samples were supplemented with 30 fmol of a mixture of PRTC peptides (Thermo Fisher Scientific, PN#88321).

LC-MS/MS Analysis

For single-signaling pathway monitoring experiments, chromatographic separations were performed on an Evosep One system (Evosep, Odense, Denmark) equipped with Evosep C_{18} EvoTips and C18 analytical columns (3 µm, 0.1 x 80 mm operated at 1 or 1.2 µL/min, or 3 µm, 0.15 x 50 mm operated at 1.5 µL/min). For the multi-pathway monitoring experiment, chromatographic separations were performed on an UltiMate 3000 RSLC system in capillary flow mode equipped with C_{18} trap cartridges (5 µm, 0.3 x 5 mm operated at 100 µL/min) and analytical column (2 µm,, 0.15 x 150 mm operated at 3 µL/min). The various gradient lengths used are detailed in relevant figures. Evosep One and Ultimate 3000 RSLC systems were coupled to Q Exactive HF-X MS and Q Exactive HF quadrupole-Orbitrap MS instruments, respectively.
Mass spectrometers were operated with several PRM-based acquisition schemes including dRT-PRM[2], IS-PRM[1] (using the instrument application programming interface, iAPI). Under its main implementation ("sequential"), the IS-PRM technique alternated between i) a "watch mode", in which internal standards (IS) were continuously measured in their (dynamically corrected) elution time monitoring windows at fast scanning rates, and ii) a "quantitative mode" (triggered by the real-time detection of the IS by means of spectral matching), which measured the corresponding pairs of IS and endogenous peptides serially over their elution profile, using optimized acquisition parameters (Figures 1). For all PRM, dRT-PRM, and IS-PRM (Quant. mode) experiments on Q Exactive HF-X instrument, PRM scans employed an Orbitrap resolution of 60,000 (at m/z 200) and maximum fill times of 116 ms. The watch mode of IS-PRM employed an Orbitrap resolution of 7,500 (at m/z 200) and maximum fill times of 10 ms.

Figure 1. Peptide monitoring strategies in PRM and advanced PRM methods.



Considerations for High-Throughput LC-PRM Analysis

Figure 2. Determination of the chromatographic properties of Evosep system operated at throughputs of 60- 100-, and 200-samples analyzed/day, based on LC-MS analysis of 15 PRTC peptides

PRTC Peptides		PRTC Peptides	
1	SSAAPPPPPR	8	ELGQSGVDTYLQTK
2	GISNEGQNASIK	9	GLILVGGYGTR
3	HVLTSIGEK	10	GILFVGSGVSGGEEGAR
4	DIPVPKPK	11	SFANQPLEVVYSK
5	IGDYAGIK	12	LTILEELR
6	TASEFDSAIAQDK	13	NGFILDGFPR
7	SAAGAFGPELSR	14	ELASGLSFPVGFK
		15	LSSEAPALFQFDLK

Throughput	Duty cycle (min)	Gradient length (min)	Elution window (min)	Avg peak width (s)
60 samples/day 8 cm x 100 µm ID	24	21	12.39	11
100 samples/day 8 cm x 100 µm ID	14.4	12	5.73	7
200 samples/day 5 cm x 150 µm ID	7.2	5	2.6	5

The chromatographic properties of the Evosep system were determined from LC-MS analyses of 15 PRTC peptides, covering the typical elution range of tryptic peptides (Figure 2). Three gradient lengths (from 5 to 21 min) were evaluated on two different column formats, enabling throughputs of 60-, 100-, and 200-samples analyzed per day, owing to minimized overhead in duty cycle (≤ 3 min). Based on the total elution window and peptide chromatographic peak widths associated with each set-up, the number of peptides that can be included in conventional PRM, dRT-PRM and IS-PRM experiments were predicted (Table 1). Predictions were performed for both ideal situation under which the elution times of the peptides are evenly distributed over the LC separation, and more common situation under which peptide elution times are compressed into chromatographic sub-ranges. As compared with conventional PRM, the higher acquisition efficiency of dRT-PRM, and especially IS-PRM, enabled significant increase in experiment scale at constant analytical throughput.

Table 1. Estimation of the scale achievable by PRM, dRT-PRM and IS-PRM analyses.

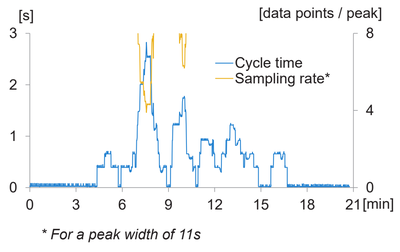
Throughput	Method MS	MS parameters[a] (Res.-Fill Time)	Monitoring window[b]	Nb targets (pairs HL peptides) – Theoretical[c,d]	Nb targets (pairs HL peptides) - Typical (/2-3)[d]
60 samples/day	PRM	60k - 116ms	0.9	98	32-48
60 samples/day	dRT-PRM	60k - 116ms	0.45	188	62-93
60 samples/day	IS-PRM	60k - 116ms[e]	0.45	391	130-195
100 samples/day	PRM	60k - 116ms	0.66	37	12-18
100 samples/day	dRT-PRM	60k - 116ms	0.33	70	23-34
100 samples/day	IS-PRM	60k - 116ms[e]	0.33	149	49-74
200 samples/day	PRM	60k - 116ms	0.5	15	5-7
200 samples/day	dRT-PRM	60k - 116ms	0.25	28	9-13
200 samples/day	IS-PRM	60k - 116ms[e]	0.25	62	20-30

[a] For Q Exactive HF-X MS; b Estimated as 5-6 and 2-3 x peak width in conventional PRM and dRT-PRM/IS-PRM analyses; [c] For an even distribution of peptide elution times over the LC separation; [d] For a sampling rate of 6 data points/peak; [e] In QUANT mode

AKT/mTOR Pathway Monitoring by Fast LC-PRM

Fast LC-PRM methods were applied to the monitoring of a single signaling pathway. A total of 13 proteins were defined as main components of AKT/mTOR pathway (Figure 3, right panel). Two to four peptides were selected as surrogate of each protein. The Evosep LC method, allowing 60 samples/day analytical throughput, was selected as suited to PRM analysis of the 32 pairs of SIL and endogenous peptides corresponding to the target proteins (Table 1 and Figure 3, left panel).

Figure 3. Establishment of conventional PRM assays for AKT/mTOR surrogate peptides.



** For a peak width of 11s*

Proteins	Pep. Nb	Proteins	Pep. Nb
AKT1	2	INSR	3
AKT1S1	2	IRS1	2
AKT2	2	MTOR	4
AKT3	2	PTEN	2
GSK3A	2	RPS6KB1	2
GSK3B	3	TSC2	3
IGF1R	3		

[#] n.a., LOD > 0.78 fmol, <5 points to determine linearity range, hydrophobic peptides;
[##] Carry-over in blank matrix

PRM assays were developed by measuring the dilution series of the 32 SIL peptides in "low" and "high" complexity matrices (6-protein mix and HeLa digests, respectively) supplemented with constant amount of corresponding synthetic unlabeled peptides (Figure 4, left panel). Peptide assays characteristics (*i.e.*, LOD, LOQ, and linearity range) were determined, as illustrated with the LOQs in low complexity matrix presented in Figure 4 (right panel).

Figure 4. Characterization of conventional PRM assays for AKT/mTOR surrogate peptides.

Dilution points	Amount SIL peptides (fmol)
CC1	200.00
CC2	50.00
CC3	12.50
CC4	3.13
CC5	0.78
CC6	0.20
CC7	0.05
« Blank »	0.00

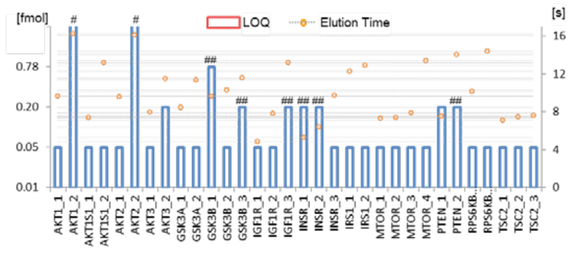
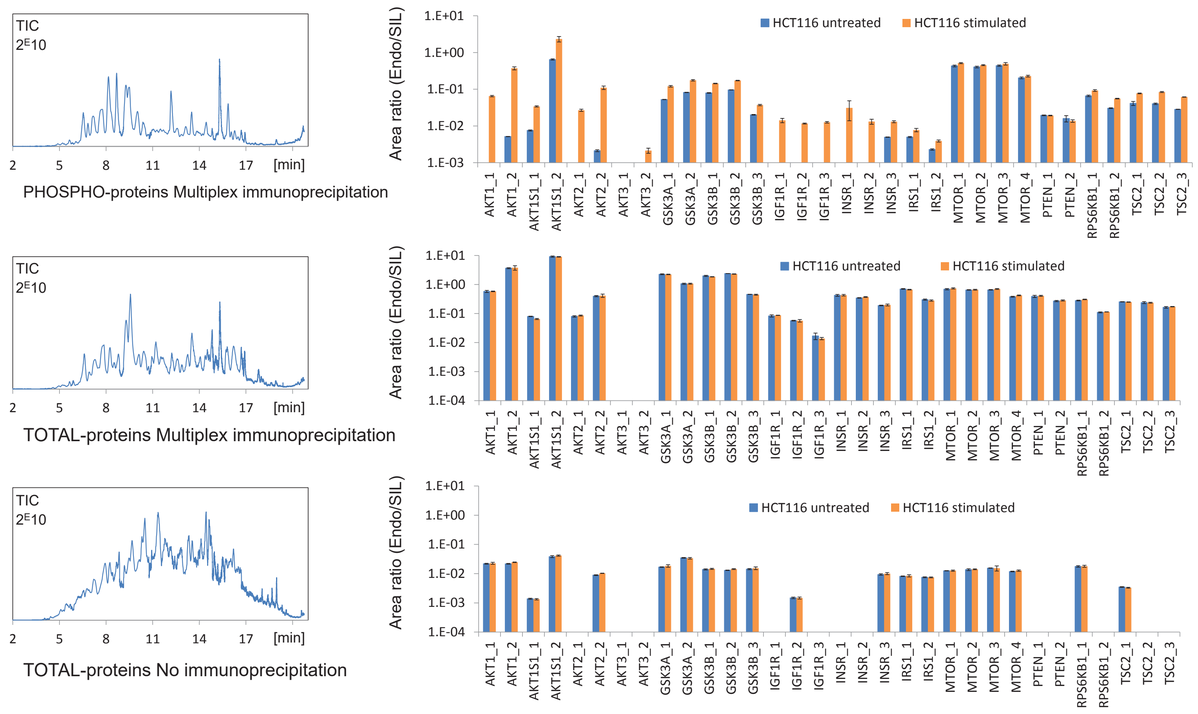


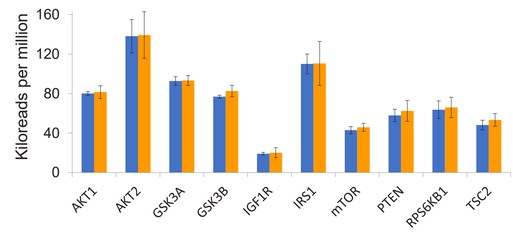
Figure 5. PRM analyses of the surrogate peptides of AKT/mTOR pathway "Phospho-" and "Total-" proteins



The PRM assays developed for the quantification of AKT/mTOR surrogate peptides were applied to untreated and hIGF-1 stimulated HCT116 digest prepared i) by multiplex immunoprecipitattion targeting phosphoproteins of the pathway (Figure 5, upper panel), ii) by multiplex immunoprecipitation targeting "total"proteins of the pathway (Figure 5, middle panel), and iii) without enrichment (Figure 5, lower panel). The overall protein digest amount injected on the LC column for conventional PRM analyses was 0.5 µg for non-enriched samples and samples prepared by multiplex immunoprecipitation, as illustrated with the total ion chromatograms displayed in left panels of Figure 5. Peptide surrogates were quantified based on the measurements of pairs of SIL and endogenous in triplicated LC-PRM analyses. While hIFG-1 stimulation did not induce changes in total proteins abundances, it modified the activation status of most of them, as illustrated by the significant increase in the peptide abundance of phosphoproteins (especially IGF1R, INSR, and AKT proteins). The multiplexed immunoprecipitation steps allowed differentiated quantification of phospho- and total-proteins but also quantification of additional peptides, benefiting from the decrease in sample complexity and the enrichment of targets

Figure 6. RNA expression of AKT/mTOR pathway components.

For each IGF treatment condition, total RNA was isolated from three HCT116 pellets containing one million cells each using the Purelink RNA Mini Kit from Invitrogen. The total RNA was DNase treated with Turbo DNase from Invitrogen and 10ng of the DNased treated total RNA was converted to cDNA with SuperScript IV VILO Mastermix from Invitrogen. Ampliseq-on-Chef libraries were made with a custom designed AKT pathway RNA panel and the Ion Ampliseq Kit for Chef DL8. Libraries were 540 templated on the Ion Chef and sequenced on the Ion 540 chip with the Ion GeneStudio Prime. Within a dataset, total reads for each pathway gene were normalized for sequencing coverage by converting to reads per million (rpm) and then, across the datasets, the rpm counts were further normalized with a conversion factor based on the average total housekeeping gene rpm.

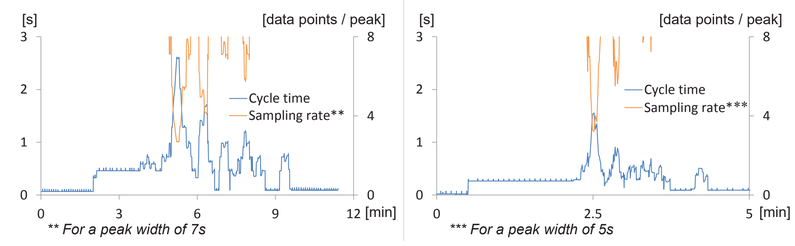


Throughput Capabilities of Advanced PRM Methods

AKT/mTOR pathway monitoring experiments were repeated using advanced PRM methods. The higher efficiency of dRT-PRM and IS-PRM acquisition schemes enabled the selection of faster Evosep LC methods (Table 1), allowing 100- and 200-samples/day analytical throughput, respectively, while keeping same PRM scans settings (quant. mode for IS-PRM). The analyses exhibited sampling rates similar to those of conventional PRM analyses using "60 samples/day" LC method (Figure 7).

Figure 7. Analyses of the 32 pairs of SIL and endo. peptides surrogate of the 13 AKT/mTOR proteins.

A) dRT-PRM - "100 samples/day" LC method. **B) IS-PRM - "200 samples/day" LC method.**



*** For a peak width of 7s*
**** For a peak width of 5s*

The compressed gradients used with advanced PRM methods did not affect the sensitivity of measurements in low complexity matrix (*e.g.*, multiplexed immunoprecipitated samples) and only moderately compromised it in a more complex matrix (Figures 8 and 9).

Figure 8. Limits of detection of peptides in "low" and "high" complexity matrices.

A) PRM - "60 samples/day" LC method. **B) dRT-PRM - "100 samples/day" LC method.**

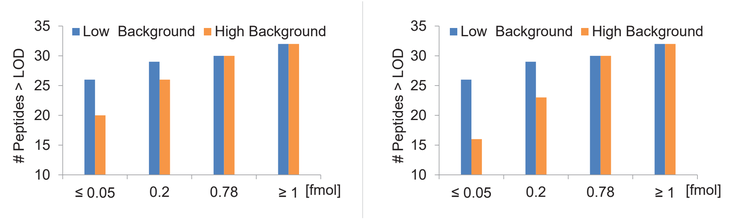
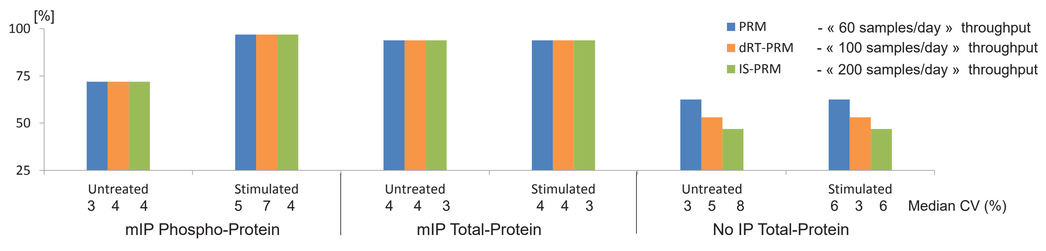


Figure 9. Success rate and reproducibility of quantification of the 32 endogenous peptides surrogate of the 13 AKT/mTOR proteins by conventional PRM, dRT-PRM, and IS-PRM analyses across mIP / no IP experiments.



CONCLUSIONS

- The developed fast LC-PRM set-ups exhibited the ability to quantify with high sensitivity several dozens of endogenous peptides in one hundred samples within one day under high efficiency acquisition modes.
- Advanced PRM methods combined with multiplexed immunoprecipitation increased the throughput of pathway monitoring without compromising data quality.

REFERENCES

1. Gallien S, Kim SY, and Domon B; Mol. Cell. Proteomics, 2015
2. Thoeing S, Gallien S, Arrey T, Xuan Y, Lange O, Strupat K ,and Kellmann M, PO-64997, Thermo Fisher Scientific, 2017

TRADEMARKS/LICENSING