Phospho-signaling network quantified in single mass spec run

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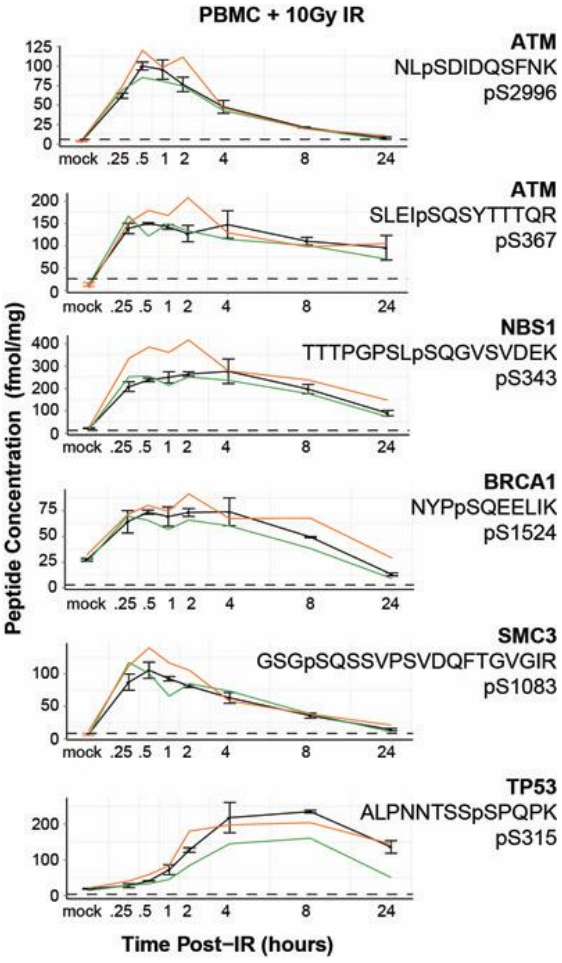

Kinetic profile of phosphorylation events involved in DNA damage repair. The multiplexed IMAC-MRM assay was used to quantify phospho-signaling in peripheral blood mononuclear cells treated with 10 Gy ionizing radiation. The kinetic response curves for representative analytes (ATMpS2996, ATMpS367, NBS1pS343, BRCA1pS1524, SMC3pS1083, and TP53pS315) are displayed. Each line represents distinct blood samples from a single patient collected weeks apart after repeated radiation therapy. Dotted line is the lower limit of quantification.

Image provided by Dr. Amanda Paulovich (Clinical Research Division)

Many cellular processes require rapid responses to changes in their environment often far faster than transcriptional alterations alone could manage. Posttranslational modification is a key mechanism that fills the need for rapid protein regulation. There are over 500 kinases documented in the human genome and they are involved in nearly every cellular process; thus protein phosphorylation has been one of the most heavily studied modifications. However, identifying and quantifying protein phosphorylation has always been a difficult task. First, phospho-proteins are often in low abundance and below detection limits without enrichment. Second, detection limits rely on high-affinity phospho-specific antibody reagents, which are difficult to produce and often non-specific. In the last two decades protein mass spectrometry has overcome the need for antibodies to

identify phospho-peptides. Yet, even these new powerful techniques require enrichment of phospho-peptides to identify less common species. Moreover, reliably quantifying phospho-peptides has been a challenge as results have not been universally reproducible. To address these needs, scientists in the Paulovich Lab (Clinical Research Division) have developed a reproducible assay that quantifies numerous phosphorylation changes in a large protein network. In a study published in *Molecular and Cellular Proteomics* these researchers generated a standard operating protocol with limited variability for enriching phospho-peptides and identified a pool of reference standards to quantify phosphorylation changes involved in DNA damage repair.

Researchers made use of immobilized metal affinity chromatography (IMAC) to enrich cellular protein lysates for phospho-peptides. As a proof-of-concept the lysates were derived from cells exposed to DNA damaging agents in order to identify phosphorylation events involved in the repair process. After trypsin digestion, lysates are simplified and enriched for negatively charged phospho-peptides using iron cations coordinated to a solid matrix. Reducing the sample pH causes negatively charged amino acids to become protonated, making the electrostatic interaction between Fe^{3+} ions and phosphate modifications highly specific. Using a single step IMAC purification followed by traditional protein mass spectrometry, a median of 4307 phospho-peptides was detected, representing an enrichment of 87% over crude lysate.

Phospho-peptides known to regulate DNA damage repair were synthesized using heavy isotopes to generate a reference pool that can be spiked into samples, and recognized by their altered mass. The peptide concentration in the reference pool is known so precise, relative quantification of phosphorylation events is possible. Finally, to increase the reliability and ease of analysis researchers used multiple reaction monitoring (MRM) rather than traditional MS-MS. In MS-MS peptides are fragmented and every possible fragment is detected, whereas in MRM only specific fragments are monitored. MRM further increases sensitivity and rate of detection.

With this assay in hand, scientists optimized the single step IMAC enrichment and quantification of the DNA damage peptides to be highly reproducible. To ensure reproducibility, results from the assay were verified using IMAC beads from different manufacturer lots and when IMAC beads were recharged with fresh Fe^{3+} ions. Dr. Paulovich who is also a member of the Early Detection Initiative spoke to possible applications of this NextGen protein assay system, saying, "The current assay panel is being used to look for underlying causes of inter-patient variation in sensitivity to radiation therapy, but the IMAC-MRM approach can be applied to quantify the activation state of many cell signaling pathways, and thus can be applied to a plethora of biological questions."

Historically, the standard for analyzing phosphorylation events has been immunoblotting with phospho-specific antibodies, which are often non-specific. Moreover, many phospho-specific antibodies are not commercially available. The IMAC-MRM assay bypasses both of these challenges, and does so extremely quickly. "The IMAC-MRM assay described in this current study focuses on the cellular DNA damage response, essentially replacing 107 Western blots with a single, 80 minute mass spectrometry run, producing easy-to-interpret data of superior quantitative quality and specificity." said Dr. Paulovich. This approach represents a new 'system level' method for the detection and quantification of complex signaling networks.

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Kennedy JJ, Yan P, Zhao L, Ivey RG, Voytovich UJ, Moore HD, Lin C, Pogosova-Agadjanyan EL, Stirewalt DL, Reding KW, Whiteaker JR, Paulovich AG. (2015). Immobilized metal affinity chromatography coupled to multiple reaction monitoring enables reproducible quantification of phospho-signaling. *Mol Cell Proteomics*. doi: 10.1074/mcp.O115.054940. [Epub ahead of print.]