Introduction

The mission of the ABRF proteomics Standards Research Group (sPRG) is to design and develop standards and resources for mass-spectrometry-based phospho-proteomics research. Recent advances in methodology have made phosphopeptide analysis a tractable problem for core facilities. Here we report on the development of a two-year sPRG experiment to target various issues encountered in phosphopeptide experiments. We have constructed a pool of deconvoluted phosphopeptides that will enable core facilities to rapidly develop assays. Our pool contains over 150 phosphopeptides that have previously been observed in mass spectrometry data sets. The specific peptides have been chosen to cover as many known biologically interesting phosphosites as possible, from seven different signaling pathways: AMPK signaling, death and apoptosis signaling, EGFR/HER signaling, insulin/IGF-1 signaling, mTORC1 signaling, and stress (G38/SAPK/JUN) signaling. We feel this pool will enable researchers to test the effectiveness of their enrichment workflows and to provide a benchmark for a cross-lab study. Currently, the standard is being tested in the sPRG members’ laboratories to establish its properties. Later this year we will invite ABRF members and non-members to participate in the second half of our study, using this controlled standard in a HeLa S3 background to evaluate their phosphopeptide data acquisition and analysis workflows. We hope this standard is helpful in a number of ways, including enabling phosphopeptide sample workflow development, as an internal enrichment and chromatography calibrant, and as a pre-built biological assay for a wide variety of signaling pathways.

Generating a Synthetic Phosphopeptide Standard

In an effort to build a biologically standard, we selected 150 phosphopeptides from 89 proteins covering a variety of signaling pathways. In an effort to prove previously observable by DDA, we chose a variety of phosphopeptides that contain sites targeted by commercial antibodies.

One difficulty in choosing phospho-peptides for synthesis is that the most easily observed sites are often missed cleavages and can be extremely long. We have made an effort to balance observability with synthetic success.

In addition to producing a biologically useful standard, we wanted the peptides to be relatively balanced across chromatographic time to allow them to function as an internal retention standard as well.

Methods

For peptide characterization, 2pmol of phosphopeptide standard was injected per experiment. Samples were analyzed by either DDA/DA/PRM on Thermo Fusion, QI, or QI-HF.

In the HeLa experiment, 20ng of digested HeLa lysate was added to 2pmol of phosphopeptide standard. For TO2 experiment, 10pmol of phosphopeptide standard was added to 1mg of tyrosine peptides from a 231 cell lysate from the sPRG standard kit (Pierce). 1/20 of eluted peptides were analyzed. Samples were analyzed by DDA on a Thermo Fusion.

The peptides span a wide dynamic range in DDA experiments. While the majority of peptides have been observed at least once in the online Phosphopedia database, some peptides have only been observed once.

Phosphopeptide Detection in Spiked Lysates

We also performed extension experiments to analyze the phosphopeptide standard spiked into both HeLa and MDA-MB-231 cell lysates followed by enrichment with a TO2 column. With a DDA experiment, we observed detect over 66% of the peptides in the sPRG pool and 33% of the phosphopeptides.

The standard can be used to unambiguously detect endogenous phosphopeptides. For example, in the above figure the phosphopeptide LILEDEY90(TAR) of Lyk is shown to co-elute with the sPRG phosphopeptide standard.

In our first TO2 enrichment experiment, we were able to detect over 50% of the peptides. Further experiments will be needed to see if this level of enrichment will be able to withstand the rigors of TO2 enrichment protocols.

Plans for a Future Study

The sPRG is planning to send out these phosphopeptide standards in fall of 2017 to willing participants. We will ask to analyze the pure phosphopeptide standards as well as standards mixed with HeLa lysates. Further studies will involve the isolation of the phosphopeptides mixed with lysates by enrichment either through TO2 or IMAC columns. Participants can use either their laboratories standardized protocol or a suggested protocol provided by sPRG. Data will be analyzed using the sPRG and presented at ABRF 2018.

Your participation in this study would be greatly appreciated.

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