ABRF-sPRG 2016-2017: Development and Characterization of a Stable-Isotope Labeled Phosphopeptide Standard

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Introduction

The mission of the ABRF proteomics Standards Research Group (sPRG) is to design and develop standards and resources for mass-spectrometry-based proteomics experiments. 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 study designed to target various issues encountered in phosphopeptide experiments. We have constructed a pool of heavy-labeled 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, mTOR signaling, PI3K/AKT signaling, and stress (p38/SAPK/JNK) 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 phosphoproteomic 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.

Methods

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

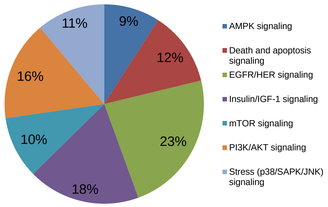
In the HeLa experiment, 200ng of digested HeLa lysate was added to 2pmol of phosphopeptide standard. For TiO2 experiment, 10pmol of phosphopeptide standard was added to 1mg of tryptic peptides from a 231 cell lysate and phosphopeptide were isolated with TiO2 kit (Pierce). 1/20 of eluted peptides were analyzed. Samples were analyzed by DDA on a Thermo Fusion.

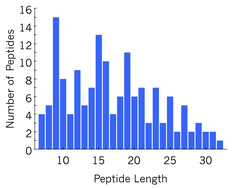
Generating a Synthetic Phosphopeptide Standard

Site breakdown:
96 Serine
26 Threonine
36 Tyrosine

Occupancy breakdown:
143 single
6 double
1 triple

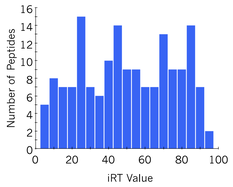
In an effort to build a biologically interesting standard, we selected 150 phosphopeptides from 89 proteins covering a variety of known signaling pathways. In addition to being previously observable by DDA, we've picked peptides that contain sites targeted by commercial antibodies.

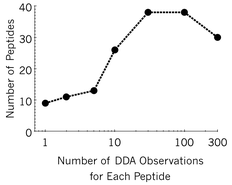




One difficulty in choosing phospho-peptides for synthesis is that the most easily observed forms often contain missed cleavages and can be extremely long. We have made an effort to balance observability with synthesis success.

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

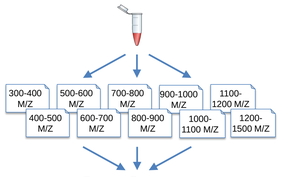




The peptides span a wide dynamic range in DDA experiments. While the majority of peptides have been observed at least 10 times in the online Phosphopedia database, some peptides have only been observed once.

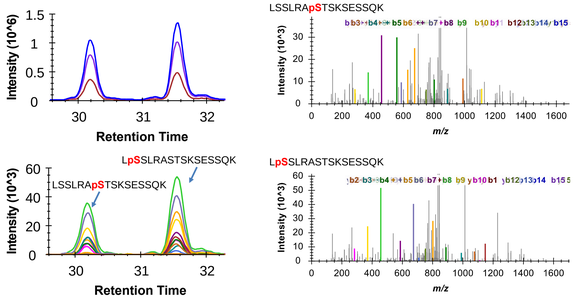
Phosphopeptide Characterization by DIA/PRM

We performed extensive characterization of the standard using both DIA and PRM experiments. For DIA, we acquired 10 gas phase fractions with a Thermo QE-HF orbitrap mass spectrometer, each spanning 100 M/Z. This deep fractionation, coupled with overlapping acquisition windows, allowed us to collect 3 M/Z precursor isolation windows with cycle times below 2 seconds, effectively producing PRM-quality analysis for peptides with precursors from 300 to 1200 M/Z, and 6 M/Z isolation from 1200 to 1500 M/Z.



Peptide Type	Count	Percent
good signal	121	85.8%
low signal	6	4.3%
smear	9	6.4%
no signal	5	3.5%

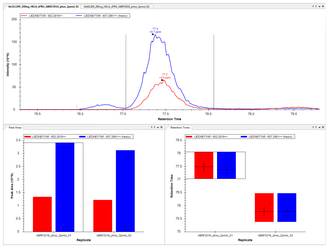
Following these experiments we ran confirmatory PRM experiments to validate our detections. All told, we characterized 86% of the peptides with clean fragmentation signals and only failed to observe 5 peptides (3.5%). We believe these peptides are either too hydrophilic to survive on C18 trap columns, or were not synthesized in sufficiently observable quantities.



We ordered some of the peptides (such as LSSLRASTSKSESSQK from RPS6, above) as multiple positional isomers. In this particular example, RPS6 can be phosphorylated at either S235 and S240, and each isomer elutes at a different retention time and produces a unique fragmentation pattern. While many of the multiple isomeric peptides could be localized to different retention time points with our data, some could not and were not included in our detection table above.

Phosphopeptide Detection in Spiked Lysates

We also performed experiments to analyze the phosphopeptide standard spiked into both HeLa lysate and MDA-MB-231 cell lysate followed by enrichment with a TiO2 column. With a DDA experiment, we are able to detect over 66% of the peptides in the HeLa background.



The standard can be used to unambiguously detect endogenous phosphopeptides. For example, in the above figure the phosphopeptide LIEDNEY[80]TAR of Lyk is shown to co-elute with the isotope-labeled standard.

In our first TiO2 enrichment experiment, we were able to detect over 50% of the peptides. Further experiments will be needed to see if all phosphopeptides will be able to withstand the rigors of TiO2 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 TiO2 or IMAC columns. Participants can use either their laboratories standardized protocol or a suggested protocol provided by sPRG. Data will then be analyzed by the sPRG and presented at ABRF2018.

Your participation in this study would be greatly appreciated.

Acknowledgements

The ABRF Proteomics Standards Research Group (sPRG) would like to thank Thermo Fisher Scientific for providing greatly discounted synthetic peptides.