**Introduction**

The mission of the ABRF Proteomics Standards Research Group (sPRG) is to identify and implement technical standards that reflect the ABRF’s commitment to accuracy, clarity, and consistency in the field of proteomics. There is broad interest in quantifying protein phosphorylation alterations in cellular signaling pathways under different conditions. The transient nature and low abundance of many phosphorylation sites makes this analysis challenging. Here we report on the follow up of a multi-year sPRG study designed to target various issues encountered in phosphopeptide experiments.

- We have constructed a pool of over 150 heavy-labeled phosphopeptides from seven different signaling pathways that will enable core facilities to rapidly develop phosphopeptide assays.
- We previously benchmarked this standard in a cross lab study where we mixed the standard into an activated HeLa tryptic digest and distributed to over 60 ABRF member and nonmember laboratories around the world. We asked participants to enrich phosphopeptides and report ratios of the heavy phosphopeptides to the endogenous levels.
- In the current “invite-only” study, we continue validation of the standard within various RG group/ABRF members’ laboratories using an optimized phosphopeptide enrichment protocol and instrument method parameters.
- This pool will enable researchers to test the effectiveness of their enrichment workflows, act as an internal enrichment and chromatography calibrator, and as a pre-built biological assay for a wide variety of signaling pathways.

**Methods/Study Design**

The sPRG prepared protein lysates from activated HeLa cells and digested with trypsin on ST-Trap columns (Protify). Study participants were sent 10gpm of pure heavy isotope phospho-peptide standard (Thermo) and a mix of tryptic HeLa lysate spiked with the heavy standard (Thermo). Participants were provided standardized and optimized methods to enrich phospho-peptides (IMAC, CST) and acquire LC-MS/MS data using DIA (BX gas phase fractionations). Raw data was analyzed by the sPRG using Skyline.

**Generating a Synthetic Phosphopeptide Standard**

**Year 1: 2016-2017**

- **Site breakdown:**
  - Serine: 36
  - Threonine: 26
  - Tyrosine: 36
- **Occupancy breakdown:**
  - 143 single
  - 6 double
  - 1 triple

150 heavy isotope phosphopeptides from 89 proteins associated with known signaling pathways and commercially available antibodies.

By necessity of synthesis and detection, many contain missed cleavages and are longer length.

Balanced across chromatographic retention time for use as internal RT standard.

**Phosphopeptide Detection in Spiked Lysates**

**Year 2: 2017-2018**

Open sample prep and acquisition methods

N=15 participating labs

Phospho-peptide standard as observed by study participants (n=15) had a wide range of detection efficiencies with some reporting only the heavy spike-in and not the endogenous.

Poor cross-lab reproducibility; most peptides were observed at least once but with only a few being detected by multiple participants.

**Year 3: 2018-2019**

Standardized sample prep and acquisition methods

N=7 participating labs

Phospho-enrichment + CST IMAC LC-MS/MS acquisition = BX gas phase fractionated DIA injections each with 4 m/z fully overlapping windows spanning 100 m/z across a 400-1200 total m/z range.

Excellent reproducibility across labs with standardized enrichment and acquisition methods; on average 126 peptides were detected and 54 quantified (81% and 35% respectively of the total possible).

A Skyline RT calculator built off the heavy standard reliably predicted peptide RTS across labs.

**Cross-lab Heavy/Light Phosphopeptide Quantification**

Cross-lab ratio analysis is more consistent with DIA and fragment level quantification.

**Figure:**

- **Interpretation of complex phosphopeptide signatures and positional isomers is aided by heavy standards and DIA acquisition**