

# ABRF-sPRG 2018-2019: Development and Characterization of a Stable-Isotope Labeled Phosphopeptide Standard

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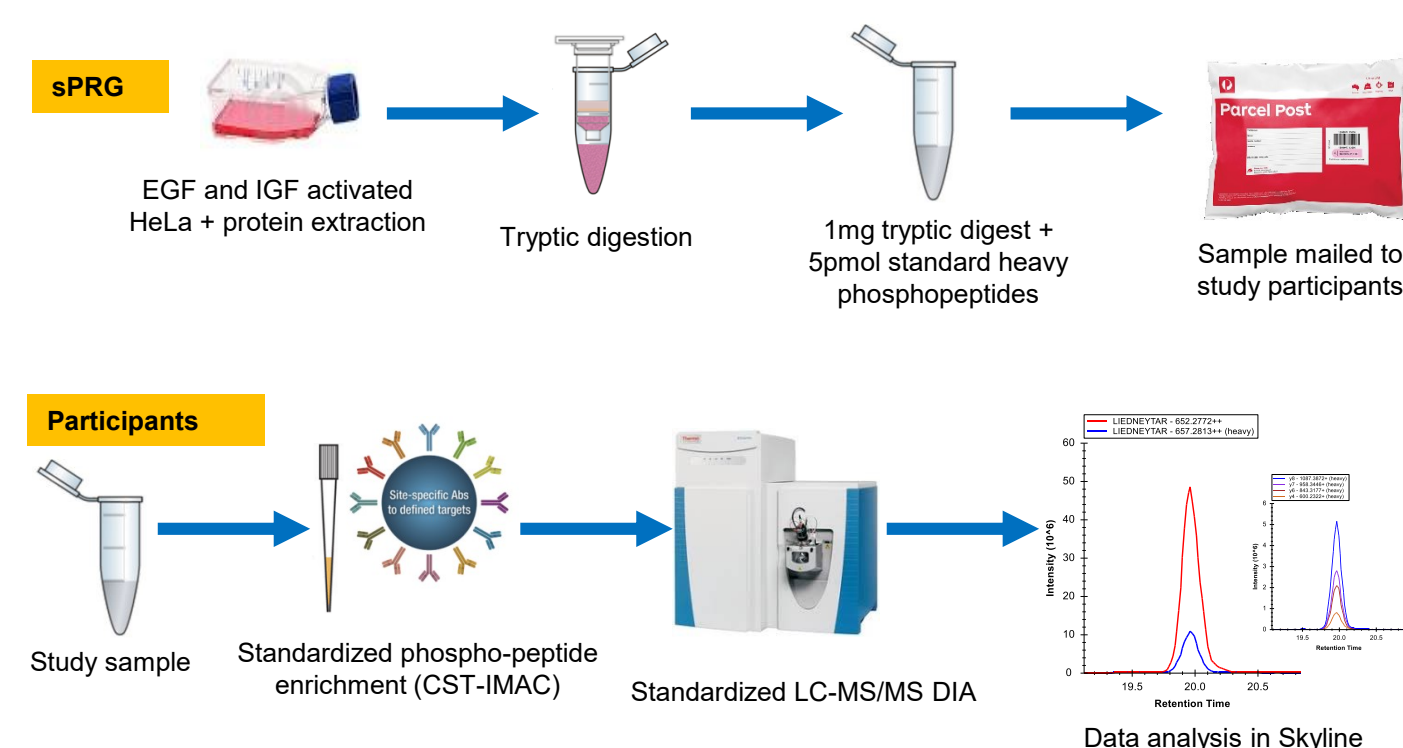
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## Introduction

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 acquisition method parameters.
- This pool will enable researchers to test the effectiveness of their enrichment workflows, act as an internal enrichment and chromatography calibrant, and as a pre-built biological assay for a wide variety of signaling pathways.

## Methods/Study Design



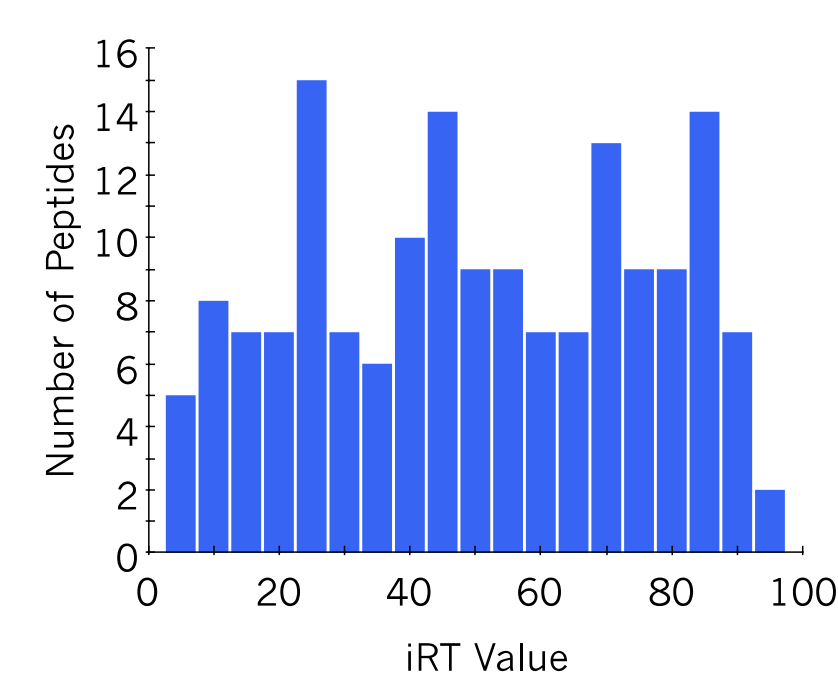
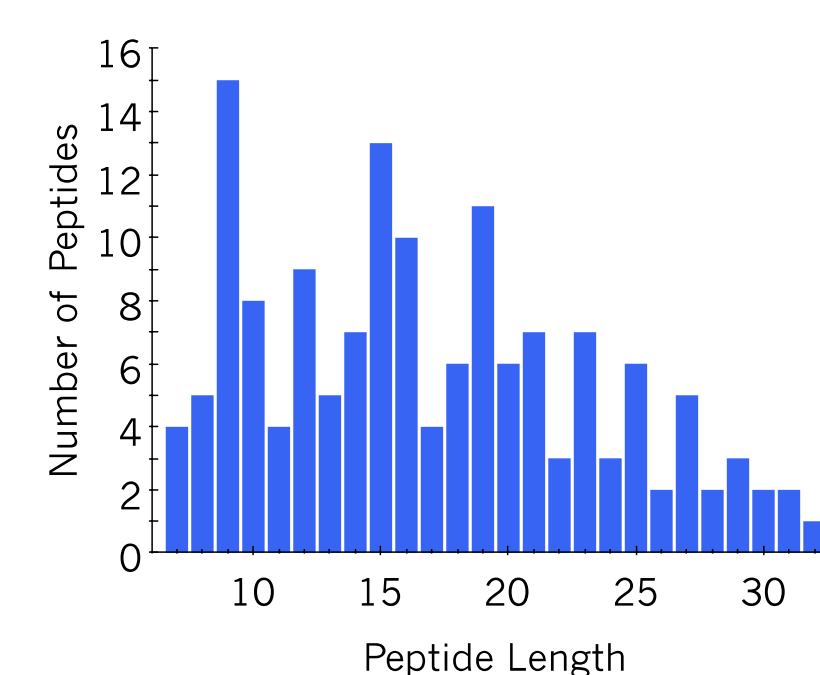
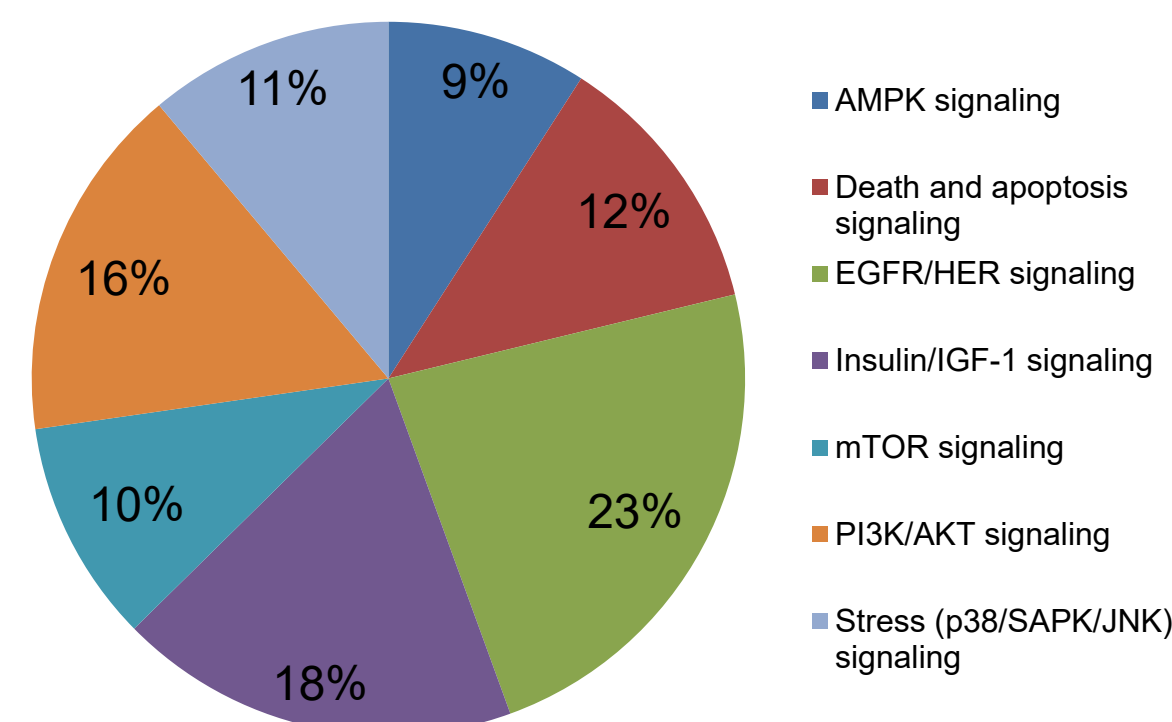
The sPRG prepared protein lysates from activated HeLa cells and digested with trypsin on S-Trap columns (Protifi). Study participants were sent 10pmol of pure heavy isotope phospho-peptide standard (Thermo) and 1mg of tryptic HeLa lysate spiked with 5pmol of heavy standard (Thermo). Participants were provided standardized and optimized methods to enrich phospho-peptides (IMAC, CST) and acquire LC-MS/MS data using DIA (8X gas phase fractions). Raw data was analyzed by the sPRG using Skyline.

## Generating a Synthetic Phosphopeptide Standard Year 1: 2016-2017

**Site breakdown:**  
96 Serine  
26 Threonine  
36 Tyrosine

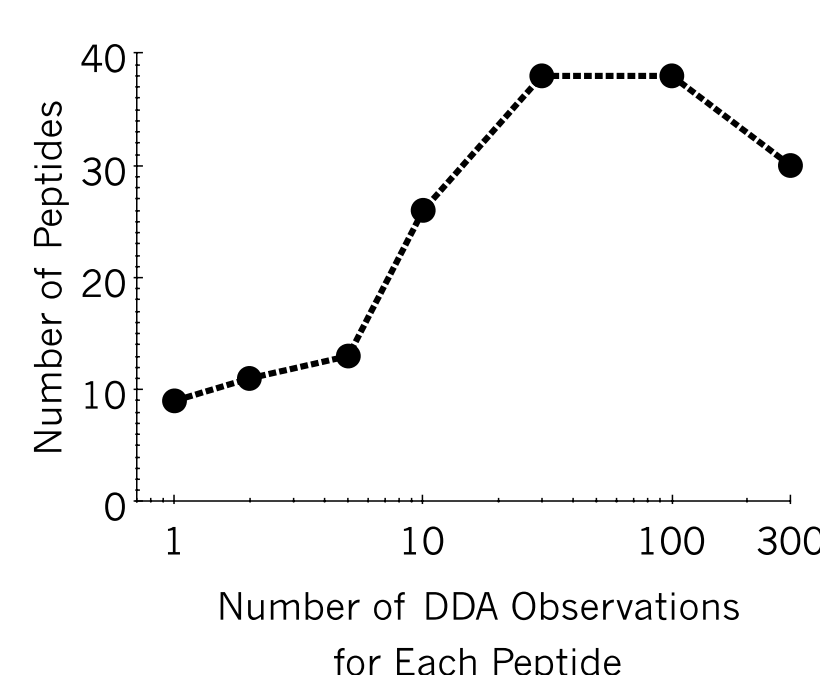
**Occupancy breakdown:**  
143 single  
6 double  
1 triple

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



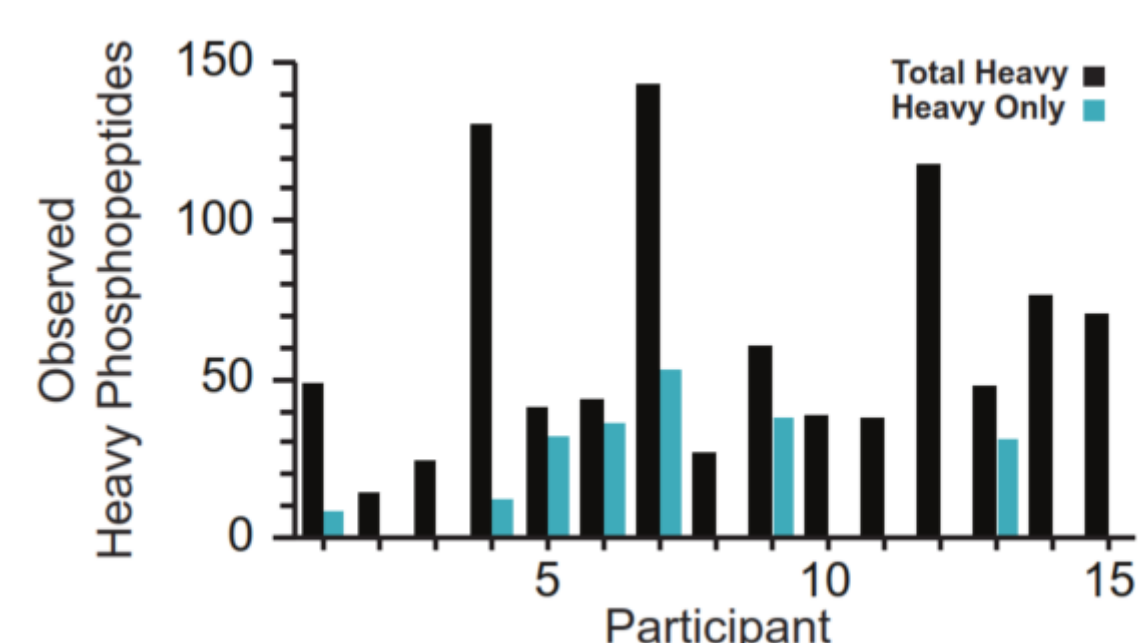
Phospho-peptides span wide dynamic range in previous DDA experiments (Phosphopedia database)

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

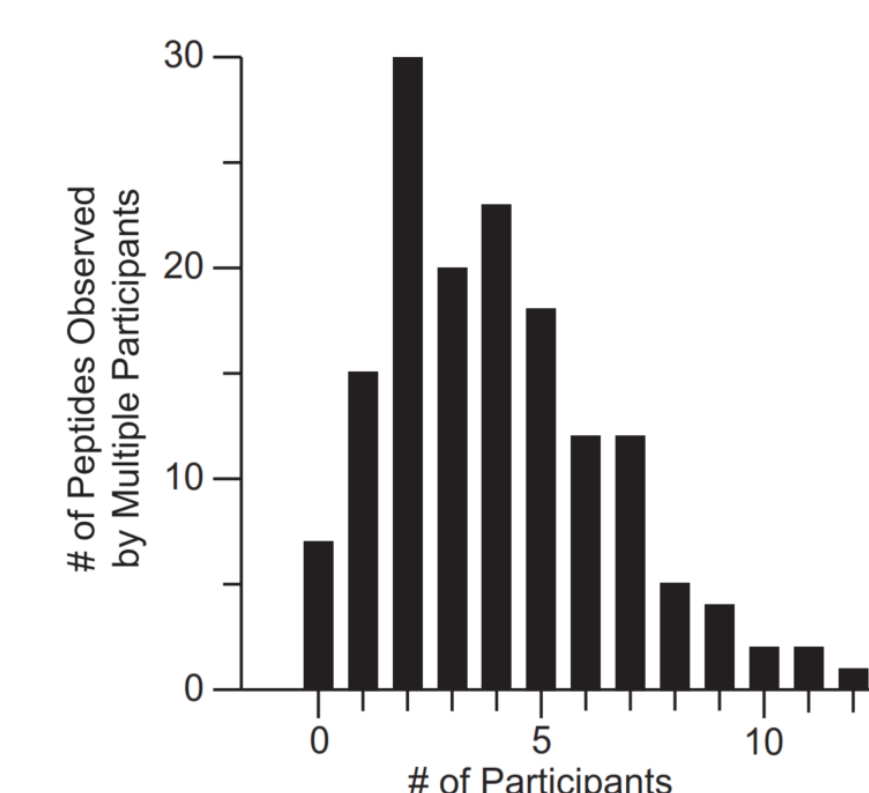
Initial characterization of the pooled heavy isotope phosphopeptide standard using PRM gave clean fragmentation for 86% of peptides (2pmol injected on Thermo Fusion, QE, or QE-HF)

## 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

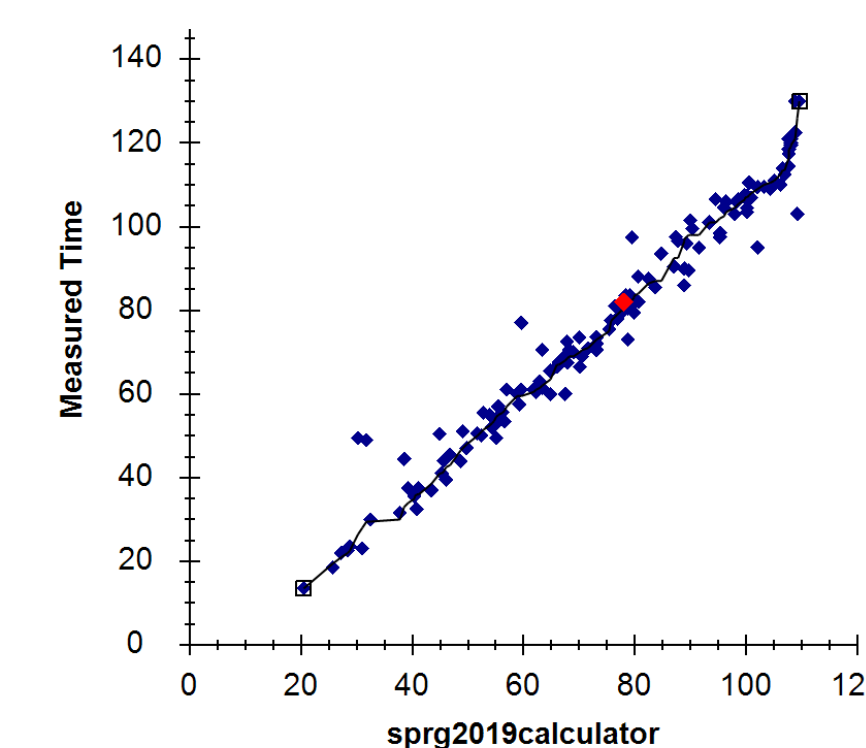
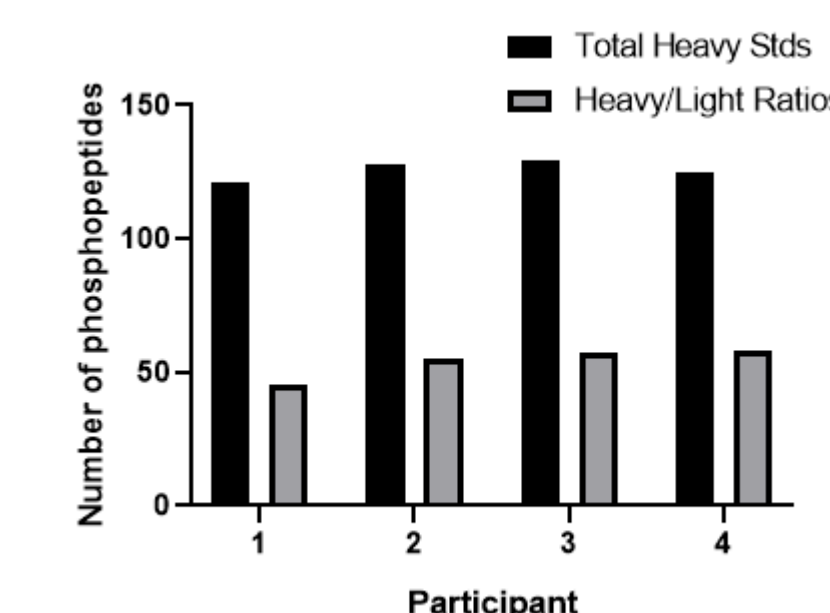


Poor cross-lab reproducibility; most peptides were observed at least once but with only a few being detected by multiple participants

## Year 3: 2018-2019

Standardized sample prep and acquisition methods  
N=7 participating labs

Phospho enrichment = CST IMAC  
LC-MS/MS acquisition = 8X 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

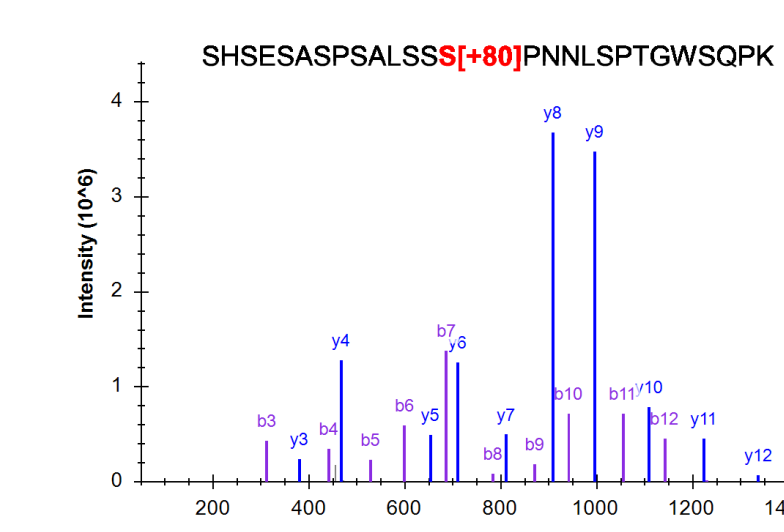
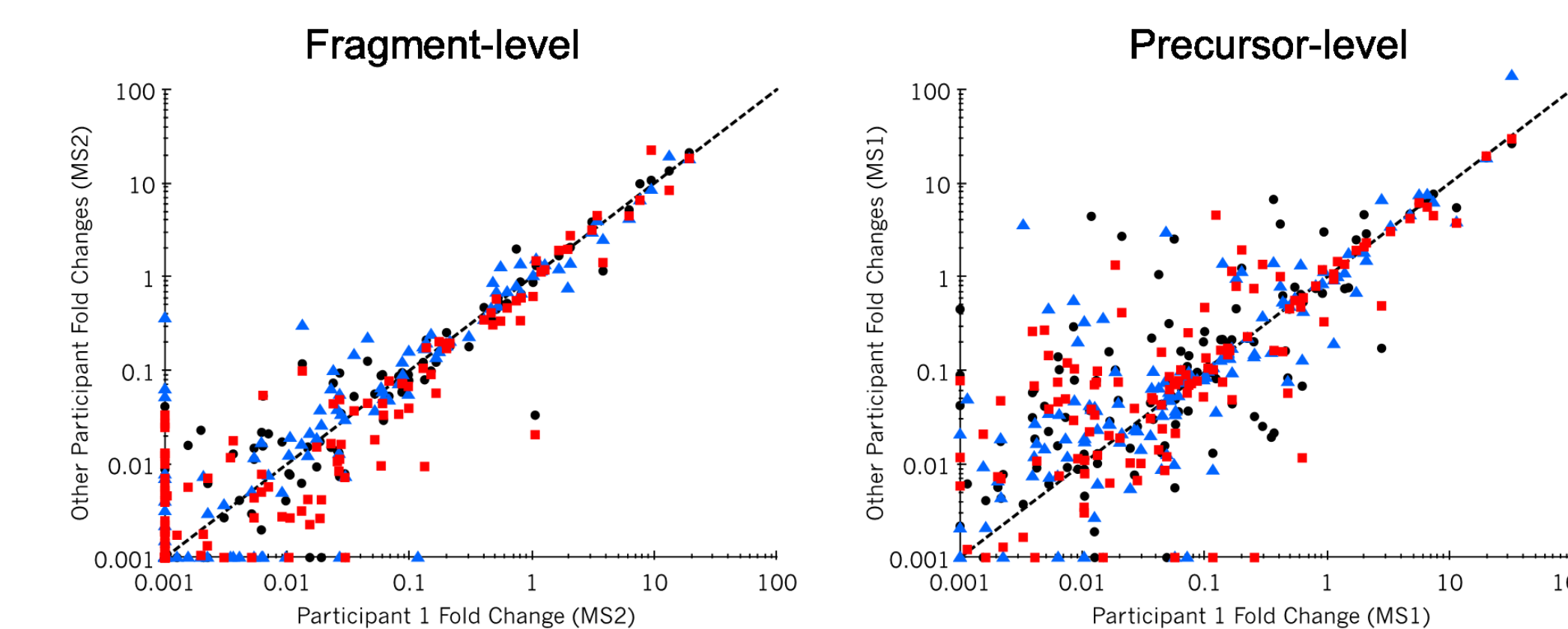


Excellent reproducibility across labs with standardized enrichment and acquisition methods; on average 126 peptides were detected and 54 quantified (81% and 35% respectively of the total possible)

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



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

