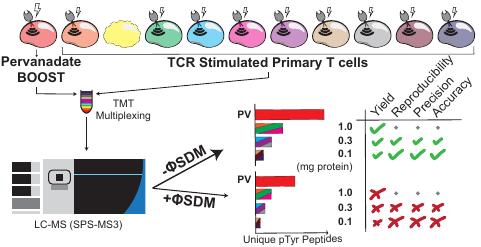
Mouse primary T cell phosphoyrosine proteomics enabled by BOOST

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The Broad Spectrum Optimization of Selective Triggering (BOOST) approach was recently developed to increase the quantitative depth of the tyrosine phosphoproteome by mass spectrometry-based proteomics. While BOOST has been demonstrated in the Jurkat T cell line, it has not been demonstrated in scarce mice primary T cells. Here, we show the first phosphotyrosine proteomics performed in mice primary T cells using BOOST. Using BOOST, we identified and precisely quantificatied more than unique pTyr peptides using only mg of protein from stimulated primary T cells from mice. We further reveal the importance of the phase-constrained spectrum deconvolution method (SDM) parameter on Orbitrap instruments that, when disabled, enhanced quantitation depth, accuracy, and precision in low-abundance samples. Using samples with contrived ratios, we find that disabling SDM allows for up to a two fold increase in the number of statistically significant intensity ratios while enabling SDM degrades quantitation, especially in low-abundance samples.

For TOC only (Note this will be a bit smaller in reality)



Keywords:

* **Mice**
* **T cell**
* **BOOST**
* **TMT**
* **SH2 superbinder**
* **Phosphotyrosine proteomics**
* **Phase-constrained spectrum deconvolution method**

# Introduction

Kinase signaling cascades regulate key cellular processes including growth, differentiation, and trancsriptional regulation. In T cells, binding of antigen-loaded peptide major histocompatibility complex on antigen-presenting cells to the T cell receptor (TCR) initiates early tyrosine kinase-mediated signaling, leading to serine/threonine kinase activation that regulate transcriptional activation(Gaud, Lesourne, and Love 2018). Signal initiation from begins by recruitment of the Src family tyrosine kinases Lck and Fyn, which phosphorylate tyrosine residues in immunotyrosine activation motifs (ITAMs) on the intracellular domain of TCR complex subunits TCR, CD3, CD3, and CD3(Palacios and Weiss 2004; Gaud, Lesourne, and Love 2018). Next, the tyrosine kinase Zap70 binds to phosphorylated ITAMs, is itself phosphorylated by TCR proximal Lck, and directed toward substrates associated with the critical scaffolding protein linker for activation of T cells (LAT) by Lck(Lo et al. 2018). Zap70 and another Lck-activated tyrosine protein kinase named Itk phosphorylate and activate many LAT-associated proteins, culminating in serine/threonine kinase activation upstream of cytokine expression and actin cytoskeletal regulation. Despite the importance of tyrosine phosphorylation in the early stages of TCR signaling, tyrosine phosphorylation is scarce, accounting for less than of all phosphorylation events(Hunter and Sefton 1980; Hunter 2009).

Due to the scarce nature of tyrosine phosphorylation, large-scale phosphotyrosine (pTyr) proteomic studies of TCR signaling in mice primary T cells are often impaired by low yield. Recently, Locard-Paulet et al. (2020) performed a phosphoproteomic study with million antibody-stimulated CD4 T cells from mice per replicate using the pTyr- pTyr enrichment kit. Using this approach, Locard-Paulet et al. (2020) identified a total of unique pTyr sites from unique pTyr peptides, which is comparable to phosphoproteomic studies of TCR and chimeric antigen receptor signaling in primary human T cells(Joshi et al. 2017; Salter et al. 2021; Ramello et al. 2019). Phosphotyrosine-specific enrichment methods provide improved pTyr sequencing compared to general phosphopeptide enrichment strategies like immobilized metal affinity chromatography (IMAC) or titanium dioxide (TiO), which are more common in phosphoproteomic studies in primary T cells from mice(Navarro et al. 2011; Prado et al. 2021). One phosphoproteomic study of TCR signaling in mice using million cells per replicate and IMAC phosphopeptide enrichment identified only unique pTyr peptides (Iwai et al. 2010), whereas other studies studies report - (about to ) of their total yield as unique pTyr peptides using IMAC or TiO(Álvarez-Salamero et al. 2020; Navarro et al. 2011). As demonstrated in Iwai et al. (2010), decreasing the number of cells and therefore the amount of protein input can severely limit pTyr quantitation depth in primary T cells from mice.

To increase the accuracy, precision, and reporducibility of pTyr quantitation in low protein input samples, both experimental and computational approaches are being developed. For example, recent improvements in pTyr enrichment reagents, namely the superbinder SH2 enrichment method(Kaneko et al. 2012; Bian et al. 2016; Dong et al. 2017; Tong et al. 2017; Yao et al. 2018, 2019), have improved quantitation depth of the pTyr proteome(Chua et al. 2020; Chua and Salomon 2021; Griffith et al. 2021). Additionally, the use of isobaric labeling reagents like tandem mass tags (TMT) have allowed for accurate quantitation in multiplexed samples with a higher probability of identifying unique peptides compared to label-free quantitation(Thompson et al. 2003, 2019; Wiese et al. 2007; Werner et al. 2012; McAlister et al. 2012; O’Connell et al. 2018). To improve the spectral quality and speed of acquiring TMT samples Fourier transfrom-mass spectrometers (FT-MS), instrument settings like the phase-constrained spectrum deconvolution method (SDM) are available. By applying SDM, FT spectra are deconvolved into frequency distributions, allowing for efficient extraction of the harmonic components of oscillating ions and ultimately achieving higher mass accuracy and resolution in shorter times(Grinfeld et al. 2017).

Recently, we combined the multiplexing capability of TMT, the selectivity of superbinder SH2, and the ability to inhibit tyrosine phosphatases *in vivo* to develop the broad-spectrum optimization of selective triggering (BOOST) method to increase pTyr quantitation depth in proteomics experiment(Chua et al. 2020). During the development of BOOST we used Jurkat T cells, a model system for studying TCR signaling(Abraham and Weiss 2004), however we have not demonstrated the BOOST method in the more biologically relevant primary T cells from mice. Here, we demonstrate the first pTyr proteomics study in primary T cells from mice using the BOOST method. By using predetermined protein input amounts, we show that BOOST increases the sequencing depth of low abundance samples, yielding more than unique pTyr peptides. We also show that acquiring samples using SDM degrades quantitation in low-abundance samples. By using samples with contrived ratios, samples acquired with SDM disabled have higher replicate reproducibility, are more accurate, and are more precise than equivalent samples acquired with SDM enabled.

# Materials and Methods

## Stimulation of mice primary T cells

CD8 thymocytes from B6 mice were harvested and blasted in cell culture using IL-2. Cells were rested in BSA T cell media for hours at cells/ml prior to stimulation. To initiate T cell stimulation, g/mL -CD3 antibody and g/mL streptavidin were added to the cells resuspended at cells/ml for minutes at C. After minutes of stimulation, cells were lysed with 1% (w/v) sodium dodecyl sulfate (SDS) in mM Tris-HCl (pH ). Pervanadate treatment was performed by incubating cells with M PV (prepared by mixing equal volume of mM sodium orthovanadate and mM hydrogen peroxide) for minutes at C.

## Sample processing

Lysate was applied through QIAshredder Mini Spin Column by centrifugation at g at C for minutes. Protein concentration was determined using Pierce BCA Protein Assay (Thermo Fisher Scientific, 23225), after which it was reduced by mM dithiothreitol at room temperature for minutes. Lysate was subsequently processed and digested using the filter-aided sample preparation (FASP) method(Wiśniewski et al. 2009) as described previously. Digested peptides were collected and acidified by trifluoroacetic acid and desalted using Sep-Pak C18 Cartridge (Waters WAT020515) as described previously(Ahsan and Salomon 2017). Desalted peptides were labeled using a Tandem Mass Tag as described previously(Chua et al. 2020). TMT-labeled peptides were mixed and purified for phosphotyrosine peptides as described previously(Chua et al. 2020).

## Liquid chromatography tandem mass spectrometry

For offline basic (pH ) fractionation, peptides were separated on a mm mm Acquity BEH C18 column (Waters) using an UltiMate UHPLC system (ThermoFisher Scientific) with a -minute gradient from to Buffer Bbasic into fractions, which are subsequently consolidated into super-fractions (Buffer A = mM ammonium hydroxide in (v/v) HPLC-grade water, (v/v) HPLC-grade acetonitrile; Buffer B = mM ammonium hydroxide in HPLC-grade acetonitrile). Each super-fraction was further separated on an in-line mm m reversed phase analytical column packed in-house with XSelect CSH C18 m resin (Waters) using an UltiMate RSLCnano system (ThermoFisher Scientific), at a flow rate of nL/min. Peptides were eluted using a -minute gradient from to Buffer B, followed by a -minute gradient to Buffer B (Buffer A = (v/v) formic acid in (v/v) HPLC-grade water, (v/v) HPLC-grade acetonitrile; Buffer B = (v/v) formic acid in (v/v) HPLC-grade acetonitrile). Data was acquired in DDA mode on a Orbitrap Eclipse Tribrid mass spectrometer (ThermoFisher Scientific) with a positive spray voltage of kV using multinotch TMT-MS3 settings(McAlister et al. 2014). Cycle time was set at 2.5 seconds. At the MS1 level scans, precursor ions (charge states ) acquired on the Orbitrap detector with the scan range of m/z, resolution, maximum injection time of ms, automatic gain control (AGC) target of , and a dynamic exclusion time of seconds. MS1 precursor ions were isolated on the quadrupole using an isolation window of m/z for MS2 scans. MS2 scans were acquired in centroid mode on the ion trap detector on a scan range of m/z via higher-energy dissociation (HCD, energy) activation with an AGC target of , maximum injection time of ms. Using synchronous precursor selection (SPS)(McAlister et al. 2014), notches were further isolated on the quadrupole using an MS2 isolation window of m/z for MS3 scans, which are acquired on the Orbitrap detector on a scan range of m/z in a mass resolution of via HCD activation ( energy) with a AGC target of and maximum injection time of ms in centroid mode.

## Database Search Parameters and Acceptance Criteria for Identifications

Raw files were processed in MaxQuant(Cox and Mann 2008) version using the integrated peptide search engine Andromeda(Cox et al. 2011). MS/MS spectra were searched against a mouse UniProt database (Mus musculus, last modified 12/01/2019) comprised of forward protein sequences. False discovery rate (FDR) for peptide spectrum matches (PSM) was set at using a reverse decoy database approach. Carbamidomethylation (cysteine) was set as fixed modification, whereas oxidation (methionine), acetylation (protein N-termini) and phosphorylation (serine, threonine, tyrosine) were set as variable modifications. Trypsin enzyme specificity was used with up to missed cleavages. Main search peptide tolerance was set as ppm, while FTMS and ITMS MS/MS match tolerances were set as ppm and Da, respectively. MS3 reporter ion mass tolerance was set at mDa, using isotopic correction factors provided by the manufacturer (Lot UK291564, Lot UH283151).

## Data Analysis & Code Availability

All analysis and data visualization were preformed on Ubuntu LTS in the Windows Subsystem for Linux version using Python with the packages “Matplotlib" (Version ), “SciPy" (Version ), “pandas" (Version ), “NumPy" (Version ), “Biopython" (version ), and “matplotlib-venn" (version ) and is available in Supporting Folder . Analysis of unique PSMs was performed using the MaxQuant output file “evidence.txt" (Supporting Folder ). Unique PSMs were defined by a non-redundant amino acid sequence (including posttranslational modifications), the charge state of the peptide, and the least number of missing values across all TMT channels. In the cases where redundancy was still present, we kept the peptide with the highest median reporter intensity. For assigning flanking sequences to each peptide and generating Supporting Figure , the MaxQuant output file “Phospho (STY)Sites.txt" (Supplementary Folder ) was used. For determining previously annotated pTyr sites we used the PhosphoSitePlus$^{\text{\textregistered}}$ (<www.phosphosite.org>)(Hornbeck et al. 2015) posttranslational modification database file “Phosphorylation\_site\_dataset.txt" (Supporting Folder ). For general pathway annotation, we downloaded the files [“wikipathways-20220110-gmt-Homo\_sapiens.gmt"](https://wikipathways-data.wmcloud.org/20220110/gmt/wikipathways-20220110-gmt-Homo_sapiens.gmt) and [“wikipathways-20220110-gmt-Mus\_musculus.gmt"](https://wikipathways-data.wmcloud.org/20220110/gmt/wikipathways-20220110-gmt-Mus_musculus.gmt) (Supporting Folder ) from the community managed biological pathway database [WikiPathways](https://www.wikipathways.org/index.php/WikiPathways)(Martens et al. 2021). Before analysis, all peptides flagged as “potential contaminants" or “reverse hits" were removed, and reporter ions from the PV-treated sample (TMT126) and the Blank channel (TMT127N) were excluded from further analysis unless otherwise stated. Statistical significance between the mean corrected reporter intensities for mg, mg, and mg protein input samples was determined using unpaired Student’s T-tests to calculate -values before correcting for multiple hypotheses (generating -values) using the method of Benjamini & Hochberg(Benjamini and Hochberg 1995). For all comparisons, statistical significance was only attained for peptides where reporter intensity values were present for all three replicates. In line with the previous literature, we did not impute or interpolate missing values at any point during data analysis(Chua et al. 2020; Chua and Salomon 2021). To evaluate replicate reproducibility, we performed least squares linear regression(Grus 2019) on pairwise comparisons between replicates for each protein input amount in a given experiment, removing peptides for which one or both replicates contained missing values. For all volcano plots, we plotted as a function of Ratio of Mean pTyr Intensities for comparisons between mg and mg, mg and mg, and mg and mg of protein input for each TMT mix. For cases where volcano plots were constructed for each portion of a Venn diagram, separate volcano plots for each overlapping portion were constructed using the reporter intensity data from each experiment. For BOOST Factor plots, only pTyr peptides with at least one reporter ion value were used, and we used the following equation to calculate BOOST Factor for each pTyr peptide:

The results of all analysis are provided in Supporting Tables -, including references to the identification number(s) for each peptide in the original MaxQuant output file(s).

# Results

## Experimental Design & Rationale

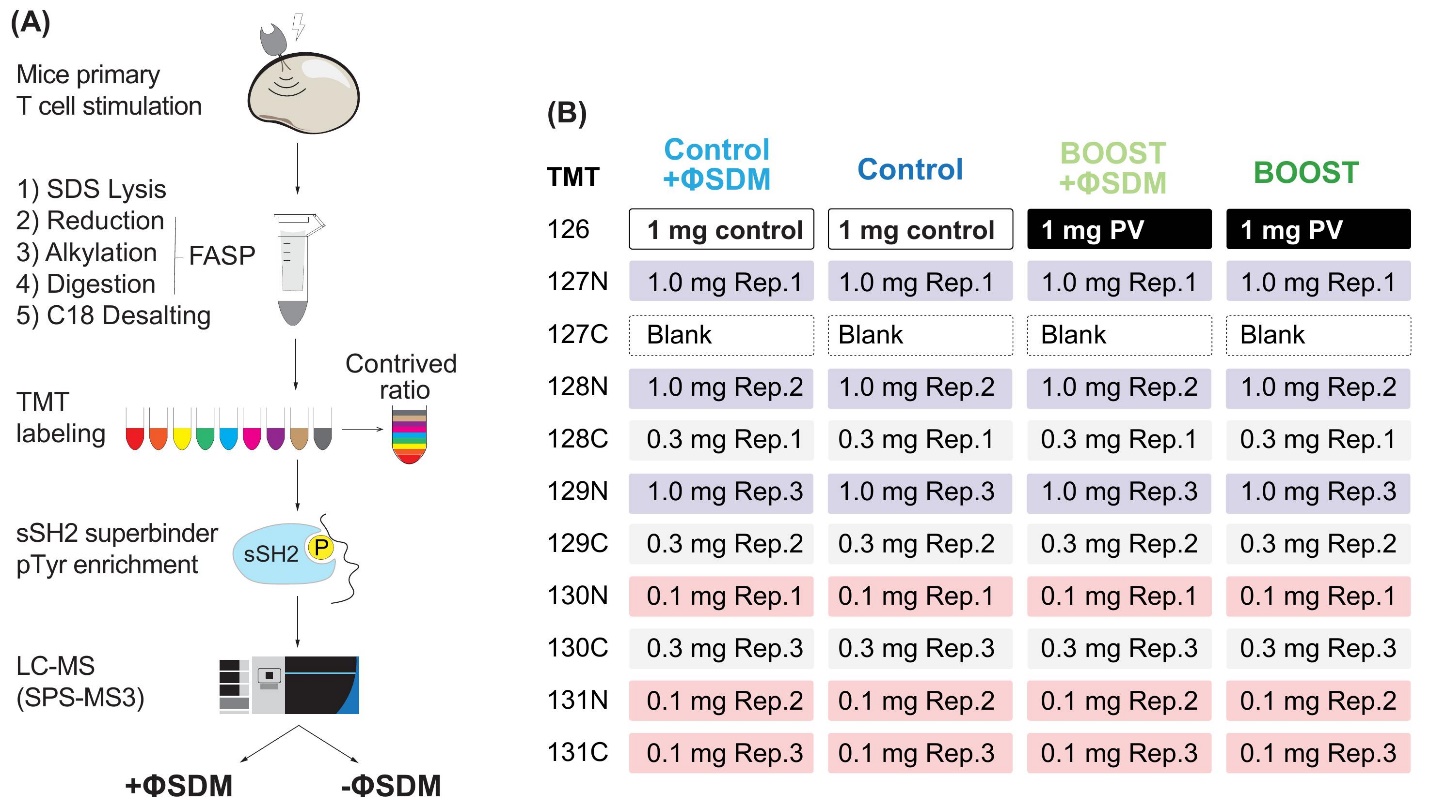


Figure 1: Schematic of (A) experimental workflow and (B) TMT mix and channel design.

In this study, we sought to determine the feasibility of the recently developed BOOST method for pTyr proteomics in primary T cells from mice. In short, BOOST is a method used to increase the precursor ion triggering and fragmentation of pTyr-containing peptides using a pervanadate (PV) treated sample in multiplexed TMT experiments, thus increasing quantitation depth of low-abundance posttranslational modifications(Chua et al. 2020). Our design is similar to that of our previous BOOST studies(Chua et al. 2020; Chua and Salomon 2021), using one mg PV treated sample (or mg protein control) and predetermined protein samples from stimulated primary mouse T cells to define the accuracy and precision of the BOOST method in primary cells (Figure [1](#experimental_design)A,B). In PV BOOST experiments, there is the potential for reporter ion interference between nearby channels(Stopfer, Conage-Pough, and White 2021). In our previous study we found evidence of reporter ion interference from channel 126 (+PV) to 127C, however we found no evidence of leakage from 126 to 127N(Chua and Salomon 2021). Therefore, we included a “Blank" channel (127C) to catch potential reporter ion interference from the mg PV-treated sample (126; Figure [1](#experimental_design)B). To enrich for pTyr-containing peptides, we used the superbinder SH2 method(Yao et al. 2018, 2019; Chua et al. 2020; Griffith et al. 2021) prior to acquisition and analysis by LC-MS and MaxQuant, respectively. To understand how the SDM affects pTyr quantitation in BOOST experiments, our BOOST and control TMT mixes were acquired with (SDM) and without the SDM on an Orbitrap Eclipse Tribrid mass spectrometer (Figure 1A). From all experiments (BOOST and control, with and without the SDM), the majority of identified phosphorylation sites were localized to tyrosines () with of pTyr sites being assigned with probability (Supporting Figure ).

## Disabling SDM Increases pTyr Quantitation Depth

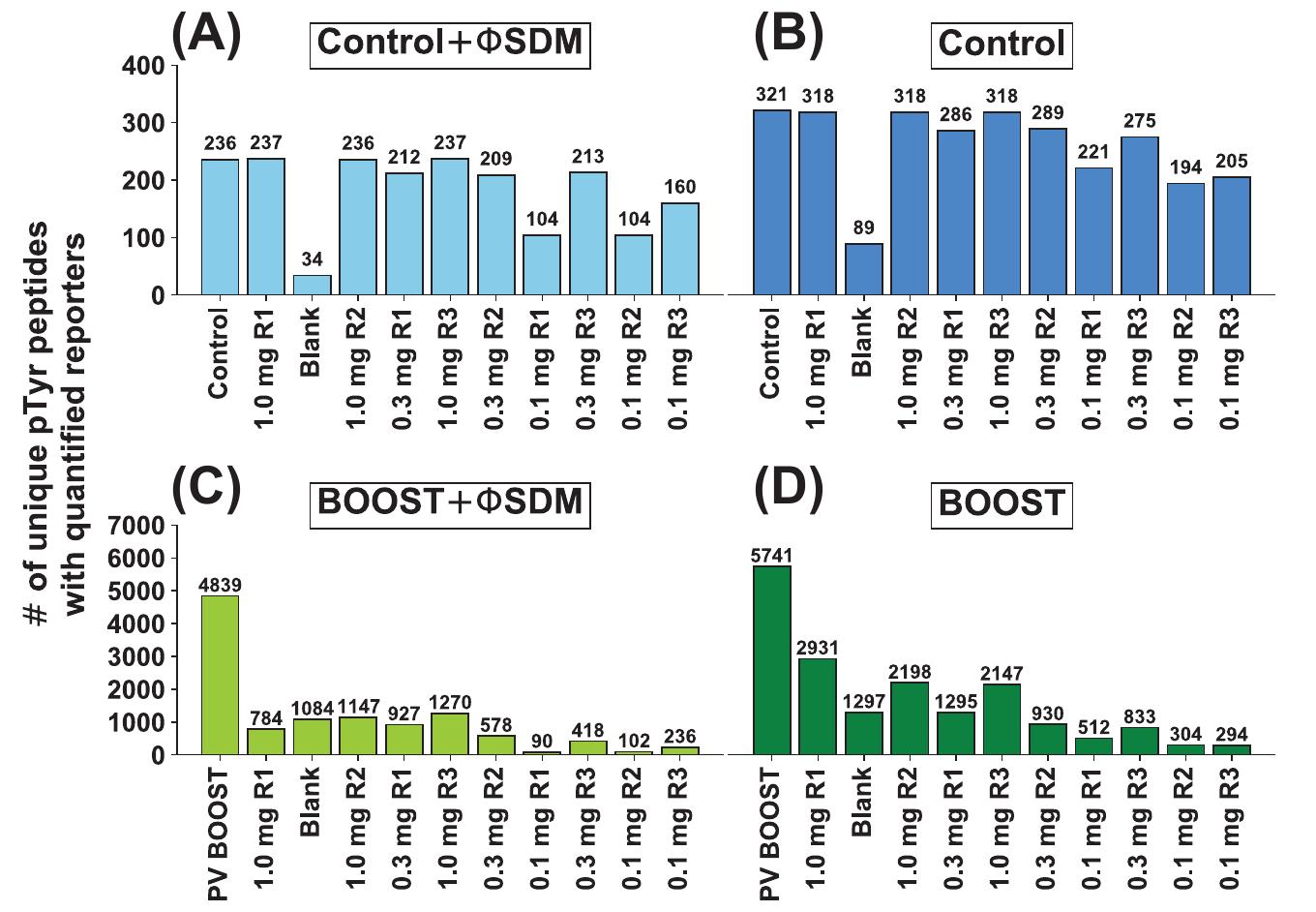


Figure 2: Quantitation depth is improved in BOOST and mg Control experiments when the SDM is disabled. The number of unique pTyr peptides identified in each TMT channel for the (A) mg Control experiment with the SDM enabled, (B) mg Control experiment with the SDM disabled, (C) BOOST experiment with SDM enabled, (D) BOOST experiment with the SDM disabled. The exact number of unique pTyr peptides is indicated above each bar.

To our surprise, disabling the SDM increased the number of pTyr peptides with quantifiable reporters in both the BOOST and control TMT mixes. In the mg PV-treated samples, we observed unique pTyr peptides with the SDM disabled and only with the SDM enabled. On average, mg protein input samples using BOOST yielded quantifiable pTyr peptides with the SDM disabled compared to when the SDM is enabled, a fold increase. We observed improvement when disabling the SDM in both and mg protein input samples in BOOST, with an average of and quantifiable pTyr peptides compared to and with the SDM enabled, respectively (Figure [2](#depth_nans)C,D). The increased quantitation depth also came with more complete data. The average percentage of missing data for mg, mg, and mg samples using BOOST dropped from , , and to , , and when the SDM was disabled, respectively (Supporting Figure C,D). While the control samples did benefit from disabling the SDM, the magnitude of improvement was smaller (-fold for mg, -fold for mg, and -fold for mg; Figure [2](#depth_nans)A,B) and the percentage of missing values between replicates were similar (Supporting Figure [2](#depth_nans)A,B). Importantly, after removing missing values, the median intensities for each replicate TMT channel were consistent and aligned well with their predetermined starting amounts (Supporting Figure ). The consistency of the reporter intensity values for the mg protein samples suggests there was minimal reporter ion interference from the mg PV-treated sample (126) into the mg R1 sample (127N) and reaffirms our experimental design (Figure [1](#experimental_design))

## Disabling SDM Increases the Reproducibility, Accuracy, Precision of pTyr Quantitation in Low Abundance Samples

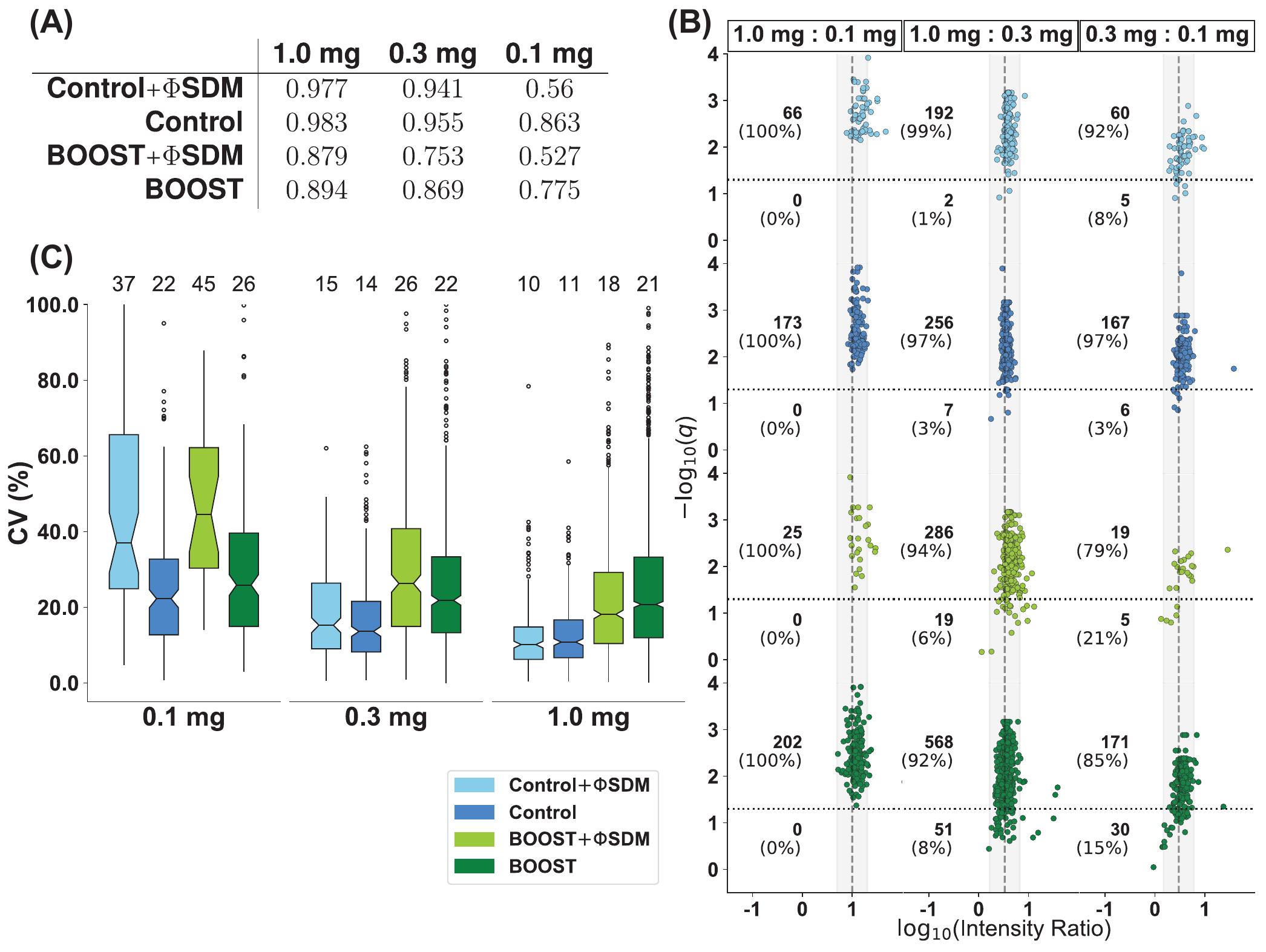


Figure 3: With the SDM disabled, replicate intensity values are more reproducible, more significant ratios of pTyr peptides are observed, and the coefficient of variation for each condition are consistent. (A) Table showing the average coefficient of determination () from least squares linear regression preformed on replicates. (B) Volcano plot showing contrived ratios for each TMT mix as indicated. The numbers and proportions [in percentages] of ratios above and below a -value of [horizontal black, dotted lines] are indicated. The grey, dashed line indicates the theoretically expected ratio, and the grey shaded area represents -fold above and below theoretically expected ratios. (C) Box-and-whisker plots showing the percentage coefficient of variation of the triplicate intensities for each protein input as indicated. The median coefficient of variation percentages are show above each boxplot. Color labels apply to (B) and (C).

Interestingly, we observed a substantial increase in replicate reproducibility after disabling the SDM in both BOOST and control conditions, especially in low abundance samples. We assessed replicate reproducibility by performing simple least squares regression in a pairwise manner on replicates for mg, mg, and mg protein inputs for BOOST and control experiments acquired with and without the SDM (Figure [3](#accuracy_and_precision)A, Supporting Figures -). When the SDM was disabled, we observed higher average values for the coefficient of determination (), a measure of the linear relationship of between data, in all conditions. This effect was clearest in the low abundance samples, where the average for BOOST experiments with mg of protein increased from to by disabling the SDM (Figure [3](#accuracy_and_precision)A, Supporting Figures , ). We observed similar results in the control samples, where disabling the SDM increased the from to (Figure [3](#accuracy_and_precision)A, Supporting Figures , ). Our data suggest that disabling the SDM increases the linear relationship between replicates and, therefore, replicate reproducibility in both BOOST and control experiments.

In addition to increasing reproducibility, disabling the SDM also increased the accuracy and precision of pTyr quantitaiton. We assessed accuracy by observing clustering around the theoretically expected peptide intensity ratios in volcano plots (Figure [3](#accuracy_and_precision)B). In both the control and BOOST experiments with the SDM disabled, we observed tight clustering of values around theoretical truth, especially in the mg to mg comparison. In contrast, enabling the SDM decreased both clustering around the theoretical truth and the number of peptides with a statistically significant difference in mean reporter intensity. Disabling the SDM lead to a -fold increase in statistically significant ratios between the mg and mg protein input conditions for control experiments, and a -fold increase for BOOST experiments (Figure [3](#accuracy_and_precision)B). The increased number of statstically significant ratios with the SDM disabled was coupled with an increase in quantitative precision in low abundance samples. For mg protein input samples, disabling the SDM decreased the median coefficient of variation (CV) from to in control experiments and to in BOOST experiments, while the CV for mg and mg samples remained similar between control and BOOST experiments (Figure [3](#accuracy_and_precision)C). Together, our data suggest that disabling the SDM for multiplexed TMT experiments with low protein input amounts substantially increases the quality of pTyr quantitation.

## The Magnitude of pTyr Quantitation is Improved when SDM is Disabled

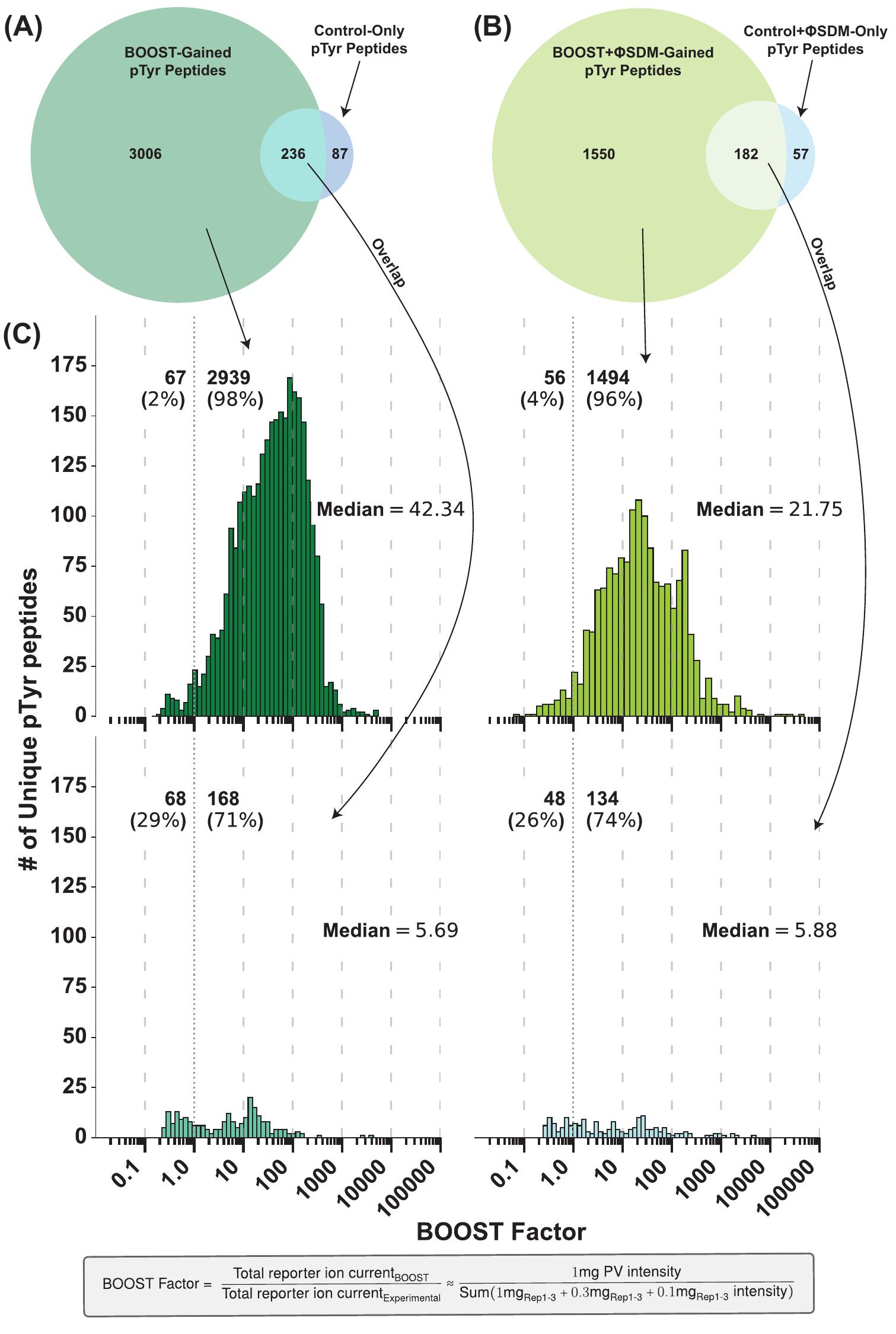


Figure 4: The number of peptides and the magnitude of BOOST factor are increased when the SDM is disabled. (A) Venn diagram showing the number of unique pTyr peptides observed when the SDM is disabled in the BOOST, the mg Control, and in both the BOOST and mg Control TMT mixes. (B) Venn diagram showing the number of unique pTyr peptides observed when the SDM is disabled in the BOOST, the mg Control, and in both the BOOST and mg Control TMT mixes.

Because the goal of BOOST is to improve quantitation of low abundance peptides, we chose to examine the magnitude of improvement with the SDM disabled. We first determined the populations of peptides unique to BOOST (“BOOST-Gained"), unique to the control (“Control-Only"), or found in both experiments (“Overlap") when the SDM was disabled or enabled (Figure [4](#boost_factor)A,B, Supporting Tables -). While the percentage of BOOST-Gained peptides was high without () or with () the SDM, we identified -fold more BOOST-Gained peptides with the SDM disabled than with the SDM enabled (Figure [4](#boost_factor)A,B). The accuracy of reporter intensity measurements was almost identical in overlapping peptides identified in the BOOST and control experiments with the SDM disabled, with a large increase in the number of significant BOOST-Gained peptides in all contrived ratios (Supporting Figure ). In contrast, the accuracy and yield of significant overlapping peptides were severely lowered in the BOOST experiment compared to the control with the SDM enabled (Supporting Figure ). When comparing the unique pTyr peptides observed in the BOOST experiments with or without the SDM, we found that () of the unique pTyr peptides were observed exclusively with the SDM disabled, whereas only () of pTyr peptides were observed exclusively with the SDM (Supporting Figure A). For the control samples, the majority of the unique pTyr peptides were observed both with and without the SDM () although disabling the SDM led to a modest increase in the percentage of unique pTyr peptides acquired ( versus ; Supporting Figure B). Our data suggest that the SDM degrades the accuracy of measruements in control-overlapping pTyr peptides and limits the potential to identify unique pTyr peptides.

In our paper describing the BOOST method, we determined the magnitude of quantitative improvement in BOOST experiments using “BOOST factors", defined as the ratio of the reporter intensity from the PV-treated sample to the sum of reporter intensities from experimental channels for a specific peptide (Figure [4](#boost_factor)C, bottom)(Chua et al. 2020). A peptide with a BOOST factor exceeding occurs when the reporter ion current of the PV-treated sample is greater than the reporter ion current of the experimental channels, indicating the peptide is generally scarce in the experimental samples(Chua et al. 2020). Overall, the majority of BOOST-Gained peptides had BOOST factors greater than regardless of whether the SDM was enabled or disabled. However, disabling SDM shifted the median BOOST factor value from to (Figure [4](#boost_factor)C). The overlapping peptides had relatively similar BOOST factor distributions with (median ) or without (median ) the SDM, suggesting that BOOST-Gained peptides were lower in abundance with or without the SDM and that disabling the SDM increased acquisition of low abundance peptides. To account for BOOST-Gained pTyr peptides with missing values, we filtered the data to contain pTyr peptides where intensity ratios and statistical significance could be attained and plotted their cumulative distributions (Supporting Figure ). For low abundance ratios ( mg to mg, and mg to mg, ) acquired without the SDM, of the significantly changing pTyr peptides had a BOOST factor less than , which shifted to about in the higher abundance ratio ( mg to mg). For ratios acquired with the SDM enabled, three pTyr peptides with BOOST factors less than had statistically significant ratios for mg to mg and seven pTyr peptides with BOOST factors less than had statistically significant ratios for mg to mg (Supporting Figure ). When we included ratios where at least one replicate value was identified for each protein input sample, the distribution of BOOST factors were almost identical for low abundance samples with of pTyr peptides having BOOST factors less than (Supporting Figure ). These data suggest that disabling the SDM increases the quantity and quality of low abundance pTyr peptides observed using the BOOST method in primary T cells from mice.

## BOOST Reveals pTyr Sites Critical for T cell Receptor Signaling in Primary T cells

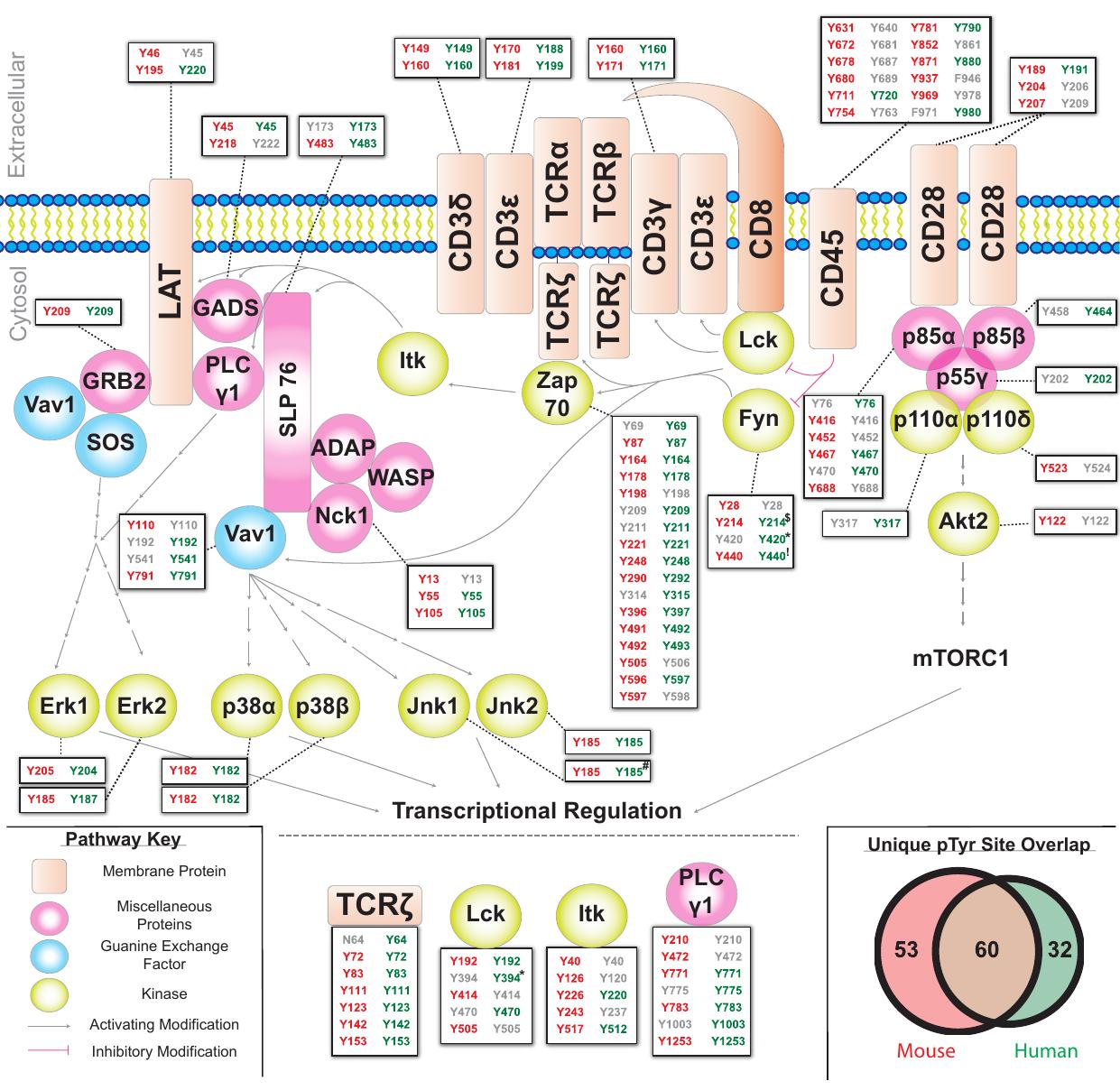


Figure 5: BOOST pTyr proteomcis reveals a comparable number of unique TCR-related pTyr sites in primary T cells from mice and Jurkat T cells. Unique pTyr sites identified in our BOOST experiment with the SDM disabled are colored in red. Unique pTyr sites identified by Chua et al. (2020) (2020) in the Jurkat T cell model system for studying TCR signaling are colored in green. Inset shows the overlap between unique pTyr sites in our BOOST experiment and the BOOST experiment performed by Chua et al. (2020). Special characters next to site numbers indicate the presence of PSMs that arise from multiple proteins: \* = Lck/Fyn/Yes1/Src, $ = Fyn/Yes1, ! = Fyn/Yes1/Src, # = Jnk1/Jnk3.

To show the efficacy of BOOST pTyr proteomics in primary T cells we used -CD3 antibodies to stimulate TCR signaling (Figure [1](#experimental_design)). Therefore, we expected to observe many unique pTyr sites on TCR signaling proteins. In accordance with our expectations, we observed a total of unique pTyr sites on proteins in the Kyoto Encyclopedia of Genes and Genomes (KEGG) T cell receptor signaling pathway (Figure [5](#biologically_interesting), Supporting Figure ). To determine the profiling range of unique pTyr site sequencing in primary T cells using BOOST, we compared our BOOST experiment with the SDM disabled to our previously published BOOST experiment in Jurkat T cells(Chua et al. 2020). Our Jurkat BOOST experiment was acquired using an Orbitrap Fusion Lumos Tribrid mass spectrometer without the SDM and had a similar BOOST-gained yield to our BOOST experiment(Yu et al. 2020; Chua et al. 2020), and is therefore suitable for comparison with our BOOST data. We observed pTyr sites in both mouse and Jurkat BOOST experiments (Figure [5](#biologically_interesting) inset), with the majority of these sites on ITAMs (TCR, CD3//; ), the tyrosine kinase Zap70 (), and the canonical activation sites on mitogen activated protein kinases (Erk1/2, p38/, Jnk; ). We found unique pTyr sites exclusive to BOOST in primary T cells from mice, compared to unique pTyr sites exclusive to BOOST in Jurkat T cells (Figure [5](#biologically_interesting) inset). Notably, using the BOOST method in primary T cells from mice uncovered pTyr sites on CD45, a phosphatase critical for the activation of Lck(Sieh, Bolen, and Weiss 1993), and pTyr sites on Tec, a non receptor tyrosine kinase with overlapping function with Itk in TCR signaling(Yang et al. 1999; Aoki et al. 2004). Of the unique pTyr sites observed on Tec, the activation site (Y519) was quantified in all three replicates in the , , and mg samples of the BOOST experiment, whereas it was quantified in some but not all replicates of all conditions of the mg Control experiment. Similar results were found for SHP-1, Itk, Zap70, and Zap70 from our BOOST experiment in primary T cells from mice (Supporting Table ). These results suggest that using BOOST in primary T cells from mice increases pTyr profiling depth similarly to what we observed previously using Jurkat T cells(Chua et al. 2020; Chua and Salomon 2021).

# Discussion

To improve our understanding of the critical tyrosine phosphorylation events involved in TCR signaling and other cellular processes, accurate methods to perform deep profiling of the pTyr proteome are required. Although the accuracy of LC-MS/MS techniques are desirable for pTyr proteomics, the low abundance of tyrosine phosphorylation events in the proteome hinder the frequently used global phosphoenrichment methods like TiO or IMAC(Navarro et al. 2011; Prado et al. 2021; Thingholm et al. 2006; Thingholm and Larsen 2016b, 2016a). Although recent developments in pTyr-specific enrichment techniques like p-Tyr-1000 and the superbinder SH2 method have improved pTyr proteomics(Kaneko et al. 2012; Bian et al. 2016; Dong et al. 2017; Tong et al. 2017; Yao et al. 2018, 2019; Chua et al. 2020), the issue of low pTyr abundance is apparent when using samples that are difficult or expensive to collect, such as primary cells from humans or mice(Salter et al. 2021; Ramello et al. 2019; Locard-Paulet et al. 2020). Increasing quantitative yield in low abundance samples has been acheived in multiplexed TMT experiments using a carrier proteome sample(Petelski et al. 2021; Stopfer, Conage-Pough, and White 2021; Cheung et al. 2021), and we developed the BOOST method using a pervanadate treated sample to increase quantitative yield of pTyr peptides(Chua et al. 2020; Chua and Salomon 2021).

Here, we demonstrate the first use of the BOOST method in primary T cells from mice, defining the accuracy, precision, and profiling depth of the mouse T cell pTyr proteome in low abundance samples. Our multiplexed TMT experiments were designed to minimize reporter ion interference from the pervanadate channel by including a “Blank" (127C) channel where maximal reporter ion interference has been observed previously(Chua and Salomon 2021; Stopfer, Conage-Pough, and White 2021). Using BOOST, we were able to quantify more than , , and unqiue pTyr peptides in mg, mg, and mg protein samples, respectively (Figure [2](#depth_nans)D), while maintaining accuracy and precision (Figure 3, Supporting Figure ). Using BOOST allowed for BOOST-gained pTyr peptides to be quantified with pTyr peptides that were low abundance in the samples (Figure [4](#boost_factor)C). This included unique pTyr sites on proteins involved in the T cell receptor signaling pathway, with of these sites being uniquely identified in mice when compared to our the pTyr sites we originally identified in Jurkat T cells (Figure [5](#biologically_interesting), Supporting Figure )(Chua et al. 2020). Of the TCR signaling proteins indentified using BOOST, many of the unique pTyr sites were found in all replicates of the and/or mg samples in the BOOST experiment and not in the mg Control experiment. Together, our data suggest that including a pervanadate BOOST channel increases quantitative depth of low abundance peptides in higher abundance samples and overall quantitation in low abundance samples without large comprimises to accuracy or precision.

We also examined the influence of the acquisition parameter SDM, a computational method that increases acquisition rate of FT-MS by efficient and noise tolerant deconvolution of FT spectra(Grinfeld et al. 2017), on our BOOST and mg Control samples. Although previous research has shown that using the SDM on long gradients or scarce samples may reduce the efficiency of the algorithm due to low ion currents(Grinfeld et al. 2017; Yu et al. 2020; Kelstrup et al. 2018), the influence of the SDM on TMT mixes with carrier proteome channels has yet to be evaluated. Our data are in agreement with previous literature suggesting that enabling the SDM degrades low abundance samples. We observed a decrease of about to unique pTyr peptides across our mg Control samples with the SDM enabled, with the largest loss in the mg R1 sample ( to unique pTyr peptides; Figure 2A,B). Surprisingly, enabling the SDM degraded the quality of data from BOOST experiments. We observed a large reduction of unique pTyr peptide yield in experimental channels, with the largest difference being mg R1 dropping from unique pTyr peptides to unique pTyr peptides with the SDM enabled (Figure [2](#depth_nans)C,D). We also observed a reduction in accuracy (Figure [3](#accuracy_and_precision)B), precision (Figure [3](#accuracy_and_precision)C), and replicate reproducibility (Supporting Figures , ) with the SDM enabled. Our study indicates that disabling the SDM, or “Turbo-TMT" on the method editor on Orbitrap instruments, subtstantially improves the quantitation depth of low abundance posttranslational modification samples, especially when a BOOST channel is present.

With increased interest in using proteomics to study rare or specific posttranslational modifications(Millan-Ariño et al. 2020; Yao et al. 2019; Fulzele and Bennett 2018) and the proteomes of single-cells(Petelski et al. 2021; Cheung et al. 2021; Vistain and Tay 2021), reliable methods to increase multiplexing capabilities(Arul and Robinson 2018), posttranslational modification selection(Pieroni et al. 2020), and quantitation(Chua et al. 2020; Chua and Salomon 2021; Pino et al. 2020) are desired. These experimental techniques will come with a wave of computational methods to further improve quantitation(Grinfeld et al. 2017; Sinitcyn et al. 2021; Bilbao et al. 2018), which will require stringent testing for both experimental-computational method compatibility and to understand the range of biological processes that these methods can work with. In this study, we displayed both of these features for the BOOST method by showing that BOOST and the SDM were incompatible and that BOOST can increase the yield of pTyr peptides in primary T cells from mice, which are notoriously difficult to perform large scale pTyr proteomics experiments on(Locard-Paulet et al. 2020). By using this study as a benchmark for the BOOST method in primary T cells from mice, future research into the pTyr proteome of primary T cells from mice is possible using far less sample than is conventionally used in shotgun proteomics.

# Conclusion

Our study defines the accuracy, precision, and profiling depth of multiplexed TMT experiments using a pervandate BOOST channel to increase quantitative yield of pTyr peptides in stimulated primary T cells from mice. We found that including the BOOST channel increases the quantitative yield of unique pTyr peptides without jeoprodizing accuracy, precision, or replicate reproducibility in low abundance samples. The majority of the unique pTyr peptides observed in the BOOST channel and at least one experimental channel were scarce in the samples, suggesting that BOOST increases identification of rare pTyr peptides. Surprisingly, we found that enabling the SDM degrades the quality of data in BOOST experiments, almost halving the unique pTyr peptide yield and reducing accuracy, precision, and replicate reproducibility in low abundance samples. Using BOOST, we were able to quantify many unique pTyr sites on proteins involved in TCR signaling in experimental channels. Together, our study shows that multiplexed TMT experiments using a pervanadate BOOST channel increase quatitative yield of meaningful unique pTyr peptides in primary T cells from mice and that the SDM should not be used during BOOST experiments.

# Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/>. Supporting Information includes:

* All tables generated after MaxQuant analysis of .raw files (“summary.txt", “evidence.txt", “peptides.txt", “modificationSpecificPeptides.txt", “Oxidation (M)Sites.txt", “Phospho (STY)Sites.txt", “proteinGroups.txt", “allPeptides.txt", “msScans.txt", “mzRange.txt", “msmsScans.txt", and “msms.txt") (.ZIP)
* All Python3 code used to perform data analysis and representation, including statistical analyses, replicate reproducibility assessments, BOOST factor analysis, and comparisons between TMT experiments (.ZIP)
* All output from statistical analyses performed and selected MaxQuant output required for statistical analysis (.XLSX)

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# Data Availability

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository(Vizcaı́no et al. 2012) with the dataset identifier PXD025853 (Username: reviewer\_pxd025853.ac.uk , Password: RDtiS7iG).

# References

Abraham, Robert T, and Arthur Weiss. 2004. “Jurkat T Cells and Development of the T-Cell Receptor Signalling Paradigm.” *Nature Reviews Immunology* 4 (4): 301–8.

Ahsan, Nagib, and Arthur R Salomon. 2017. “Quantitative Phosphoproteomic Analysis of T-Cell Receptor Signaling.” In *The Immune Synapse*, 369–82. Springer.

Álvarez-Salamero, Candelas, Raquel Castillo-González, Gloria Pastor-Fernández, Isabel R Mariblanca, Jesús Pino, Danay Cibrian, and Marı́a N Navarro. 2020. “IL-23 Signaling Regulation of Pro-Inflammatory T-Cell Migration Uncovered by Phosphoproteomics.” *PLoS Biology* 18 (3): e3000646.

Aoki, Naohito, Shuichi Ueno, Hiroyuki Mano, Sho Yamasaki, Masayuki Shiota, Hitoshi Miyazaki, Yumiko Yamaguchi-Aoki, Tsukasa Matsuda, and Axel Ullrich. 2004. “Mutual Regulation of Protein-Tyrosine Phosphatase 20 and Protein-Tyrosine Kinase Tec Activities by Tyrosine Phosphorylation and Dephosphorylation.” *Journal of Biological Chemistry* 279 (11): 10765–75.

Arul, Albert B, and Renã AS Robinson. 2018. “Sample Multiplexing Strategies in Quantitative Proteomics.” *Analytical Chemistry* 91 (1): 178–89.

Benjamini, Yoav, and Yosef Hochberg. 1995. “Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing.” *Journal of the Royal Statistical Society: Series B (Methodological)* 57 (1): 289–300.

Bian, Yangyang, Lei Li, Mingming Dong, Xuguang Liu, Tomonori Kaneko, Kai Cheng, Huadong Liu, et al. 2016. “Ultra-Deep Tyrosine Phosphoproteomics Enabled by a Phosphotyrosine Superbinder.” *Nature Chemical Biology* 12 (11): 959–66.

Bilbao, Aivett, Bryson C Gibbons, Gordon W Slysz, Kevin L Crowell, Matthew E Monroe, Yehia M Ibrahim, Richard D Smith, Samuel H Payne, and Erin S Baker. 2018. “An Algorithm to Correct Saturated Mass Spectrometry Ion Abundances for Enhanced Quantitation and Mass Accuracy in Omic Studies.” *International Journal of Mass Spectrometry* 427: 91–99.

Cheung, Tommy K, Chien-Yun Lee, Florian P Bayer, Atticus McCoy, Bernhard Kuster, and Christopher M Rose. 2021. “Defining the Carrier Proteome Limit for Single-Cell Proteomics.” *Nature Methods* 18 (1): 76–83.

Chua, Xien Yu, Theresa Mensah, Timothy Aballo, Samuel G Mackintosh, Ricky D Edmondson, and Arthur R Salomon. 2020. “Tandem Mass Tag Approach Utilizing Pervanadate Boost Channels Delivers Deeper Quantitative Characterization of the Tyrosine Phosphoproteome.” *Molecular & Cellular Proteomics* 19 (4): 730–43.

Chua, Xien Yu, and Arthur Salomon. 2021. “Ovalbumin Antigen-Specific Activation of Human T Cell Receptor Closely Resembles Soluble Antibody Stimulation as Revealed by Boost Phosphotyrosine Proteomics.” *Journal of Proteome Research*, 10.1021/acs.jproteome.1c00239.

Cox, Jürgen, and Matthias Mann. 2008. “MaxQuant Enables High Peptide Identification Rates, Individualized Ppb-Range Mass Accuracies and Proteome-Wide Protein Quantification.” *Nature Biotechnology* 26 (12): 1367–72.

Cox, Jurgen, Nadin Neuhauser, Annette Michalski, Richard A Scheltema, Jesper V Olsen, and Matthias Mann. 2011. “Andromeda: A Peptide Search Engine Integrated into the Maxquant Environment.” *Journal of Proteome Research* 10 (4): 1794–1805.

Dong, Mingming, Yangyang Bian, Yan Wang, Jing Dong, Yating Yao, Zhenzhen Deng, Hongqiang Qin, Hanfa Zou, and Mingliang Ye. 2017. “Sensitive, Robust, and Cost-Effective Approach for Tyrosine Phosphoproteome Analysis.” *Analytical Chemistry* 89 (17): 9307–14.

Fulzele, Amit, and Eric J Bennett. 2018. “Ubiquitin diGLY Proteomics as an Approach to Identify and Quantify the Ubiquitin-Modified Proteome.” In *The Ubiquitin Proteasome System*, 363–84. Springer.

Gaud, Guillaume, Renaud Lesourne, and Paul E Love. 2018. “Regulatory Mechanisms in T Cell Receptor Signalling.” *Nature Reviews Immunology* 18 (8): 485–97.

Griffith, Alijah A, Kenneth P Callahan, Nathan Gordo King, Qian Xiao, Xiaolei Su, and Arthur R Salomon. 2021. “SILAC Phosphoproteomics Reveals Unique Signaling Circuits in Car-T Cells and the Inhibition of B Cell-Activating Phosphorylation in Target Cells.” *Journal of Proteome Research*, 10.1021/acs.jproteome.1c00735.

Grinfeld, Dmitry, Konstantin Aizikov, Arne Kreutzmann, Eugen Damoc, and Alexander Makarov. 2017. “Phase-Constrained Spectrum Deconvolution for Fourier Transform Mass Spectrometry.” *Analytical Chemistry* 89 (2): 1202–11.

Grus, Joel. 2019. *Data Science from Scratch: Sirst Principles with Python*. O’Reilly Media.

Hornbeck, Peter V, Bin Zhang, Beth Murray, Jon M Kornhauser, Vaughan Latham, and Elzbieta Skrzypek. 2015. “PhosphoSitePlus, 2014: Mutations, Ptms and Recalibrations.” *Nucleic Acids Research* 43 (D1): D512–D520.

Hunter, Tony. 2009. “Tyrosine Phosphorylation: Thirty Years and Counting.” *Current Opinion in Cell Biology* 21 (2): 140–46.

Hunter, Tony, and Bartholomew M Sefton. 1980. “Transforming Gene Product of Rous Sarcoma Virus Phosphorylates Tyrosine.” *Proceedings of the National Academy of Sciences* 77 (3): 1311–5.

Iwai, Leo K., Christophe Benoist, Diane Mathis, and Forest M White. 2010. “Quantitative Phosphoproteomic Analysis of T Cell Receptor Signaling in Diabetes Prone and Resistant Mice.” *Journal of Proteome Research* 9 (6): 3135–45. <https://doi.org/10.1021/pr100035b>.

Joshi, Rubin N, Nadine A Binai, Francesco Marabita, Zhenhua Sui, Amnon Altman, Albert JR Heck, Jesper Tegnér, and Angelika Schmidt. 2017. “Phosphoproteomics Reveals Regulatory T Cell-Mediated Def6 Dephosphorylation That Affects Cytokine Expression in Human Conventional T Cells.” *Frontiers in Immunology* 8: 1163.

Kaneko, Tomonori, Haiming Huang, Xuan Cao, Xing Li, Chengjun Li, Courtney Voss, Sachdev S Sidhu, and Shawn SC Li. 2012. “Superbinder Sh2 Domains Act as Antagonists of Cell Signaling.” *Science Signaling* 5 (243): ra68–ra68.

Kelstrup, Christian D, Konstantin Aizikov, Tanveer S Batth, Arne Kreutzman, Dmitry Grinfeld, Oliver Lange, Daniel Mourad, Alexander A Makarov, and Jesper V Olsen. 2018. “Limits for Resolving Isobaric Tandem Mass Tag Reporter Ions Using Phase-Constrained Spectrum Deconvolution.” *Journal of Proteome Research* 17 (11): 4008–16.

Lo, Wan-Lin, Neel H Shah, Nagib Ahsan, Veronika Horkova, Ondrej Stepanek, Arthur R Salomon, John Kuriyan, and Arthur Weiss. 2018. “Lck Promotes Zap70-Dependent Lat Phosphorylation by Bridging Zap70 to Lat.” *Nature Immunology* 19 (7): 733–41.

Locard-Paulet, Marie, Guillaume Voisinne, Carine Froment, Marisa Goncalves Menoita, Youcef Ounoughene, Laura Girard, Claude Gregoire, et al. 2020. “LymphoAtlas: A Dynamic and Integrated Phosphoproteomic Resource of Tcr Signaling in Primary T Cells Reveals Itsn 2 as a Regulator of Effector Functions.” *Molecular Systems Biology* 16 (7): e9524.

Martens, Marvin, Ammar Ammar, Anders Riutta, Andra Waagmeester, Denise N Slenter, Kristina Hanspers, Ryan A. Miller, et al. 2021. “WikiPathways: Connecting Communities.” *Nucleic Acids Research* 49 (D1): D613–D621.

McAlister, Graeme C, Edward L Huttlin, Wilhelm Haas, Lily Ting, Mark P Jedrychowski, John C Rogers, Karsten Kuhn, et al. 2012. “Increasing the Multiplexing Capacity of Tmts Using Reporter Ion Isotopologues with Isobaric Masses.” *Analytical Chemistry* 84 (17): 7469–78.

McAlister, Graeme C, David P Nusinow, Mark P Jedrychowski, Martin Wuühr, Edward L Huttlin, Brian K Erickson, Ramin Rad, Wilhelm Haas, and Steven P Gygi. 2014. “MultiNotch Ms3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression Across Cancer Cell Line Proteomes.” *Analytical Chemistry* 86 (14): 7150–8.

Millan-Ariño, Lluı́s, Zuo-Fei Yuan, Marlies E Oomen, Simone Brandenburg, Alexey Chernobrovkin, Jérôme Salignon, Lioba Körner, Roman A Zubarev, Benjamin A Garcia, and Christian G Riedel. 2020. “Histone Purification Combined with High-Resolution Mass Spectrometry to Examine Histone Post-Translational Modifications and Histone Variants in Caenorhabditis Elegans.” *Current Protocols in Protein Science* 102 (1): e114.

Navarro, Maria N, Jurgen Goebel, Carmen Feijoo-Carnero, Nick Morrice, and Doreen A Cantrell. 2011. “Phosphoproteomic Analysis Reveals an Intrinsic Pathway for the Regulation of Histone Deacetylase 7 That Controls the Function of Cytotoxic T Lymphocytes.” *Nature Immunology* 12 (4): 352–61.

O’Connell, Jeremy D, Joao A Paulo, Jonathon J O’Brien, and Steven P Gygi. 2018. “Proteome-Wide Evaluation of Two Common Protein Quantification Methods.” *Journal of Proteome Research* 17 (5): 1934–42.

Palacios, Emil H, and Arthur Weiss. 2004. “Function of the Src-Family Kinases, Lck and Fyn, in T-Cell Development and Activation.” *Oncogene* 23 (48): 7990–8000.

Petelski, Aleksandra A, Edward Emmott, Andrew Leduc, R Gray Huffman, Harrison Specht, David H Perlman, and Nikolai Slavov. 2021. “Multiplexed Single-Cell Proteomics Using Scope2.” *Nature Protocols* 16 (12): 5398–5425.

Pieroni, Luisa, Federica Iavarone, Alessandra Olianas, Viviana Greco, Claudia Desiderio, Claudia Martelli, Barbara Manconi, et al. 2020. “Enrichments of Post-Translational Modifications in Proteomic Studies.” *Journal of Separation Science* 43 (1): 313–36.

Pino, Lindsay K, Seth C Just, Michael J MacCoss, and Brian C Searle. 2020. “Acquiring and Analyzing Data Independent Acquisition Proteomics Experiments Without Spectrum Libraries.” *Molecular & Cellular Proteomics* 19 (7): 1088–1103.

Prado, Douglas S, Richard T Cattley, Corey W Shipman, Cassandra Happe, Mijoon Lee, William C Boggess, Matthew L MacDonald, and William F Hawse. 2021. “Synergistic and Additive Interactions Between Receptor Signaling Networks Drive the Regulatory T Cell Versus T Helper 17 Cell Fate Choice.” *Journal of Biological Chemistry* 297 (6).

Ramello, Maria C., Ismahène Benzaïd, Brent M. Kuenzi, Maritza Lienlaf-Moreno, Wendy M. Kandell, Daniel N. Santiago, Mibel Pabón-Saldaña, et al. 2019. “An Immunoproteomic Approach to Characterize the Car Interactome and Signalosome.” *Science Signaling* 12 (568): eaap9777. <https://doi.org/10.1126/scisignal.aap9777>.

Salter, Alexander I., Anusha Rajan, Jacob J. Kennedy, Richard G. Ivey, Sarah A. Shelby, Isabel Leung, Megan L. Templeton, et al. 2021. “Comparative Analysis of Tcr and Car Signaling Informs Car Designs with Superior Antigen Sensitivity and in Vivo Function.” *Science Signaling* 14 (697): eabe2606. <https://doi.org/10.1126/scisignal.abe2606>.

Sieh, Monica, JB Bolen, and A Weiss. 1993. “CD45 Specifically Modulates Binding of Lck to a Phosphopeptide Encompassing the Negative Regulatory Tyrosine of Lck.” *The EMBO Journal* 12 (1): 315–21.

Sinitcyn, Pavel, Hamid Hamzeiy, Favio Salinas Soto, Daniel Itzhak, Frank McCarthy, Christoph Wichmann, Martin Steger, et al. 2021. “MaxDIA Enables Library-Based and Library-Free Data-Independent Acquisition Proteomics.” *Nature Biotechnology* 39 (12): 1563–73.

Stopfer, Lauren E, Jason E Conage-Pough, and Forest M White. 2021. “Quantitative Consequences of Protein Carriers in Immunopeptidomics and Tyrosine Phosphorylation Ms2 Analyses.” *Molecular & Cellular Proteomics* 20.

Thingholm, Tine E, Thomas JD Jørgensen, Ole N Jensen, and Martin R Larsen. 2006. “Highly Selective Enrichment of Phosphorylated Peptides Using Titanium Dioxide.” *Nature Protocols* 1 (4): 1929–35.

Thingholm, Tine E, and Martin R Larsen. 2016a. “Phosphopeptide Enrichment by Immobilized Metal Affinity Chromatography.” In *Phospho-Proteomics*, 123–33. Springer.

———. 2016b. “The Use of Titanium Dioxide for Selective Enrichment of Phosphorylated Peptides.” In *Phospho-Proteomics*, 135–46. Springer.

Thompson, Andrew, Jürgen Schäfer, Karsten Kuhn, Stefan Kienle, Josef Schwarz, Günter Schmidt, Thomas Neumann, and Christian Hamon. 2003. “Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by Ms/Ms.” *Analytical Chemistry* 75 (8): 1895–1904.

Thompson, Andrew, Nikolai Wölmer, Sasa Koncarevic, Stefan Selzer, Gitte Böhm, Harald Legner, Peter Schmid, et al. 2019. “TMTpro: Design, Synthesis, and Initial Evaluation of a Proline-Based Isobaric 16-Plex Tandem Mass Tag Reagent Set.” *Analytical Chemistry* 91 (24): 15941–50.

Tong, Jiefei, Biyin Cao, Gregory D Martyn, Jonathan R Krieger, Paul Taylor, Bradley Yates, Sachdev S Sidhu, Shawn SC Li, Xinliang Mao, and Michael F Moran. 2017. “Protein-Phosphotyrosine Proteome Profiling by Superbinder-Sh2 Domain Affinity Purification Mass Spectrometry, sSH2-Ap-Ms.” *Proteomics* 17 (6): 1600360.

Vistain, Luke F, and Savaş Tay. 2021. “Single-Cell Proteomics.” *Trends in Biochemical Sciences* 46 (8): 661–72.

Vizcaı́no, Juan Antonio, Richard G Côté, Attila Csordas, José A Dianes, Antonio Fabregat, Joseph M Foster, Johannes Griss, et al. 2012. “The Proteomics Identifications (Pride) Database and Associated Tools: Status in 2013.” *Nucleic Acids Research* 41 (D1): D1063–D1069.

Werner, Thilo, Isabelle Becher, Gavain Sweetman, Carola Doce, Mikhail M Savitski, and Marcus Bantscheff. 2012. “High-Resolution Enabled Tmt 8-Plexing.” *Analytical Chemistry* 84 (16): 7188–94.

Wiese, Sebastian, Kai A Reidegeld, Helmut E Meyer, and Bettina Warscheid. 2007. “Protein Labeling by iTRAQ: A New Tool for Quantitative Mass Spectrometry in Proteome Research.” *Proteomics* 7 (3): 340–50.

Wiśniewski, Jacek R, Alexandre Zougman, Nagarjuna Nagaraj, and Matthias Mann. 2009. “Universal Sample Preparation Method for Proteome Analysis.” *Nature Methods* 6 (5): 359–62.

Yang, Wen-Chin, Marguerite Ghiotto, Bernadette Barbarat, and Daniel Olive. 1999. “The Role of Tec Protein-Tyrosine Kinase in T Cell Signaling.” *Journal of Biological Chemistry* 274 (2): 607–17.

Yao, Yating, Yangyang Bian, Mingming Dong, Yan Wang, Jiawen Lv, Lianfang Chen, Hongwei Wang, Jiawei Mao, Jing Dong, and Mingliang Ye. 2018. “SH2 Superbinder Modified Monolithic Capillary Column for the Sensitive Analysis of Protein Tyrosine Phosphorylation.” *Journal of Proteome Research* 17 (1): 243–51.

Yao, Yating, Yan Wang, Shujuan Wang, Xiaoyan Liu, Zhen Liu, Yanan Li, Zheng Fang, Jiawei Mao, Yong Zheng, and Mingliang Ye. 2019. “One-Step Sh2 Superbinder-Based Approach for Sensitive Analysis of Tyrosine Phosphoproteome.” *Journal of Proteome Research* 18 (4): 1870–9.

Yu, Qing, Joao A Paulo, Jose Naverrete-Perea, Graeme C McAlister, Jesse D Canterbury, Derek J Bailey, Aaron M Robitaille, et al. 2020. “Benchmarking the Orbitrap Tribrid Eclipse for Next Generation Multiplexed Proteomics.” *Analytical Chemistry* 92 (9): 6478–85.