Tandem Mass Tag Approach Utilizing Pervanadate BOOST Channels Delivers Deeper Quantitative Characterization of the Tyrosine Phosphoproteome

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In Brief
There are clear advantages to experimental approaches that reduce the amount of necessary cellular material for wide-scale quantitative profiling of the tyrosine phosphoproteome, particularly in biological systems where material is limited. The incorporation of boost channels in a multiplexed tandem mass tag experiment containing cells treated with the broad-spectrum tyrosine phosphatase inhibitor pervanadate enabled up to 6.3-fold deeper quantification of the tyrosine phosphoproteome while maintaining accuracy and precision.

Graphical Abstract

- BOOST
  - non-pTyr
  - pTyr
  m/z

+ BOOST
  - non-pTyr
  - pTyr
  m/z

selective triggering of precursor fragmentation

Highlights
- Detection of low-abundance phosphotyrosine-containing peptides is challenging.
- Multiplexed TMT allows inclusion of modification(pTyr)-saturated boost channels.
- Boost channels facilitate selection of pTyr precursor ions for fragmentation.
- Quantitation depth is increased while maintaining accuracy and precision.

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Tandem Mass Tag Approach Utilizing Pervanadate BOOST Channels Delivers Deeper Quantitative Characterization of the Tyrosine Phosphoproteome*

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Dynamic tyrosine phosphorylation is fundamental to a myriad of cellular processes. However, the inherently low abundance of tyrosine phosphorylation in the proteome and the inefficient enrichment of phosphotyrosine (pTyr)-containing peptides has led to poor pTyr peptide identification and quantitation, critically hindering researchers' ability to elucidate signaling pathways regulated by tyrosine phosphorylation in systems where cellular material is limited. The most popular approaches to wide-scale characterization of the tyrosine phosphoproteome use pTyr enrichment with pan-specific, anti-pTyr antibodies from a large amount of starting material. Methods that decrease the amount of starting material and increase the characterization depth of the tyrosine phosphoproteome while maintaining quantitative accuracy and precision would enable the discovery of tyrosine phosphorylation networks in rarer cell populations. To achieve these goals, the BOOST (Broad-spectrum Optimization Of Selective Triggering) method leveraging the multiplexing capability of tandem mass tags (TMT) and the use of pervanadate (PV) boost channels (cells treated with the broad-spectrum tyrosine phosphatase inhibitor PV) selectively increased the relative abundance of pTyr-containing peptides. After PV boost channels facilitated selective fragmentation of pTyr-containing peptides, TMT reporter ions delivered accurate quantitation of each peptide for the experimental samples while the quantitation from PV boost channels was ignored. This method yielded up to 6.3-fold boost in pTyr quantification depth of statistically significant data derived from controlled ratios, compared with TMT without PV boost channels or intensity-based label-free (LF) quantitation while maintaining quantitative accuracy and precision, allowing quantitation of over 2300 unique pTyr peptides from only 1 mg of T cell receptor-stimulated Jurkat T cells. The BOOST strategy can potentially be applied in analyses of other post-translational modifications where treatments that broadly elevate the levels of those modifications across the proteome are available. Molecular & Cellular Proteomics 19: 730–743, 2020. DOI: 10.1074/mcp.TIR119.001865.

Tyrosine phosphorylation is a major biochemical currency in cellular signaling that regulates vital cellular processes. Dysregulation of these processes underlies many disease states such as immune disorders and oncogenesis. As such, comprehensive mapping of the tyrosine phosphoproteome is essential to understanding the key regulatory mechanisms of these diseases, and for designing improved therapeutic strategies. Mass spectrometry (MS) has become a robust platform for wide-scale quantitative profiling of the phosphorylated tyrosine (pTyr) proteome, but because tyrosine phosphorylation makes up only 0.05% of the total phosphoproteome in a typical vertebrate cell (1), MS-based detection of pTyr-containing peptides remains a challenge. pTyr peptide enrichment has improved pTyr proteomics, yet the conventional approach of using pan-specific anti-pTyr antibodies such as P-Tyr-1000 suffers from limitations such as prohibitive cost and low efficiency (2). Nevertheless, more than 10,000 out of the estimated 300,000 phosphorylatable tyrosine phosphorylation sites (3) have been identified using the MS-based approach (4). However, these results were achieved using large amounts of starting material (~5 mg) in combination with the broad-spectrum tyrosine phosphatase inhibitor PV to inflate the total pTyr peptide abundance to non-physiological levels (3, 5). For more physiologically relevant samples without broad-spectrum tyrosine phosphatase inhibition, several groups reported using up to 16–35 mg of protein to attain a small fraction of the pTyr sites identified by PV treatment (4, 6). Therefore, a method that requires less starting material while delivering a deeper characterization of the tyrosine phosphoproteome with quantitative
accuracy and precision is necessary for the discovery of tyrosine phosphorylation networks in cell types where material is limited.

The multiplexing technology of TMT in concert with selective MS/MS triggering of pTyr peptides facilitated by PV can be leveraged to address this issue. TMT enables simultaneous MS analysis and comparative quantitation of multiple samples (7, 8). Each of the TMT channels consists of samples labeled by variants of an amine-reactive isobaric tag, each containing a unique mass reporter. Because all samples are pooled and analyzed concurrently, a peptide labeled by different tag variants appears as one composite peak at the MS1 scan level. Subsequently, fragmentation and reporter ion intensity readout yield relative quantitation between the various experimental channels. Including cells treated with PV as a subset of the TMT channels greatly increases the relative abundance of multiplexed signal from pTyr-containing peptides in all other experimental channels. Here we investigated PV-enhanced selective MS/MS fragmentation of pTyr precursor ions and evaluated the accuracy and precision of relative quantitative information from pTyr peptides in the experimental channels where PV treatment is absent. We also systematically quantified the magnitude of improvement of TMT reporter ion intensities with the addition of PV-treated samples in a multiplexed TMT experiment to probe the basis of the observed improvements in pTyr quantitation depth.

EXPERIMENTAL PROCEDURES

**Cell Culture**—Jurkat T cells (clone E6–1) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 containing 2.05 mM l-glutamine supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM l-Glutamine (HyClone, Logan, UT) and 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), in a humidified incubator with 5% CO2 at 37 °C. Cells used in this work were tested mycoplasma-free using the Universal Mycoplasma Detection Kit from ATCC according to manufacturer’s instructions.

**T-cell Receptor Stimulation and Pervanadate Treatment**—For T-cell receptor stimulation, Jurkat T cells were rinsed and resuspended in plain RPMI at 1 x 10^6 cells/ml, then rested for 20 min at 37 °C. Cellular stimulation was carried out with 2.5 μg/ml of OKT3 and OKT4 antibodies (eBioscience, San Diego, CA) each for 30 s followed by antibody crosslinking with 22 μg/ml of goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) for an additional 5 min at 37 °C. T cell receptor stimulation was halted by resting the cells on ice and then cells were lysed in lysis buffer [4% SDS, 200 mM Tris-HCl pH 7.6, 2 μM MS-SAFE Protease and Phosphatase Inhibitor (Sigma-Aldrich)] and rested on ice immediately afterward. For PV treatment, 50 mM PV stock solution was prepared by mixing equal volume of 100 mM sodium orthovanadate and 100 mM hydrogen peroxide. Jurkat T cells were rinsed and resuspended in plain RPMI at 1 x 10^6 cells/ml, then rested at 37 °C for 15 min. The cells were then incubated with 500 μM PV for 20 min at 37 °C. PV treatment was halted by resting the cells on ice and then cells were lysed with the lysis buffer.

**TMT Labeling, Contrived Ratio Mixture**—Lysate was clarified and then reduced, alkylated, and digested using the filter-aided sample preparation method (9). Protein concentration of lysate was quantified using Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA). Digested peptides were desalted using Sep-Pak C18 Cartridge (Waters, Milford, MA, WAT020515) as described (10). Desalted peptides were labeled using a Tandem Mass Tag 11-plex isobaric label reagent set (Thermo Fisher Scientific #A37725) following the manufacturer’s instructions. Briefly, each of the 0.8 mg of label (dissolved in 41 μl anhydrous acetonitrile) was used to label 1 mg of protein (reconstituted in 100 μl of 100 mM triethylammonium bicarbonate) at room temperature for 1 h, subsequently quenched with 8 μl of 5% hydroxyamine for 15 min at room temperature. Protein derived from receptor-stimulated cells were labeled by mass reporters 131N and 131C, designated "PV boost channels." TMT labeling efficiency analysis was then performed to ensure near-complete labeling of peptides. Median intensity information for each labeling reaction was used to normalize labeled peptide abundance before mixing in contrived ratios as follows: 3 replicates each of 0.1 mg, 0.3 mg, and 1.0 mg of protein were mixed to achieve the contrived ratios of 1:0.1 mg, 1:0.3 mg, and 0:3:0.1 mg during quantitation. For the "TMT+PV" sample, 1.0 mg each of labeled PV sample in the 131N and 131C boost channels was added to the contrived ratio mixture. For comparative analysis, an identical mixture without the boost channels was set up as the "TMT-PV" sample. Each labeled peptide mixture was diluted with water to reduce the acetonitrile concentration and acidified with 0.1% formic acid, prior to desalting using Sep-Pak C18 Cartridge (Waters WAT020515) and subsequently solvent evaporation using speedvac vacuum concentrator before proceeding with pTyr peptide enrichment.

**SH2 Superbinder Expression and Purification**—The Src SH2 domain was isolated, mutated, and subcloned into the petM-11 expression vector to produce the SH2 super binder, as previously described (4). Briefly, the petM-11 SH2 super binder vector was transformed into BL21 E. coli (DE3), and protein expression was induced with 0.1 mM IPTG overnight at 18 °C with shaking at 225 rpm. The cells were lysed in lysis buffer (2% Triton X-100, 1 mg/ml Lysozyme, 25 U/ml Benzonase, 1× Roche Complete protease inhibitor mixture, 20 mM Sodium Phosphate, 500 mM Sodium Chloride, 0.5 mM Magnesium Chloride, and 20 mM Imidazole, pH7.2) and homogenized through a French pressure cell press. The lysate was cleared by centrifugation at 30,000 g for 45 min at 4 °C, and the supernatant was run through a 0.22-μm filter and applied to a Ni-NTA column (GE Healthcare, Chicago, IL) to bind the SH2 super binder protein. Following binding, the Ni-NTA column was washed extensively with lysis buffer. The protein was eluted from the column with 400 mM imidazole and then concentrated using an Amicon Ultra 3 kDa protein spin concentrator (Millipore Sigma, Burlington, MA). The concentrated protein solution was purified further through a S-100 HR gel filtration column (GE Healthcare). Protein concentration was determined via BCA assay, and the purified SH2 super binder domain was stored at −20 °C in 40% (v/v) glycerol with 0.02% (w/v) sodium azide, 100 mM Sodium Bicarbonate, and 500 mM Sodium Chloride. The purified SH2 super binder domain was immobilized on CNBr-activated Sepharose (GE Healthcare) following the manufacturer’s guidelines and was stored at 4 °C at a concentration of 2.4 mg protein per ml slurry, 50 mg Sepharose beads per ml slurry in IAP buffer (50 mM MOPS-NaOH pH7.2, 10 mM sodium phosphate, 50 mM sodium chloride) supplemented with 0.02% (w/v) sodium azide.

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1 The abbreviations used are: pTyr, phosphorylated tyrosine; BOOST, Broad-spectrum Optimization Of Selective Triggering; MS, mass spectrometry; TMT, tandem mass tags; PSM, peptide spectrum match; FDR, false discovery rate; PV, pervanadate; sSH2, Src Homology 2 superbinder; HPLC, high pressure liquid chromatography.
Phosphotyrosine Peptide Enrichment—4.2 mg (TMT-PV) or 6.2 mg (TMT+PV) of desalted, TMT-labeled and mixed peptides were solubilized in 1.4 ml IAP buffer and then incubated with ~1 mg SH2 superbinder beads overnight at 4 °C on a rotator. The beads went through three 1 ml ice-cold IAP buffer washes and one 1 ml ice-cold unbuffered milli-Q water wash. Following the washes, pTyr peptides were eluted from the beads two times, each using 100 μl 0.15% (v/v) TFA for 10 min on a 1150 rpm mixer. The eluted peptides were desalted using 100 μl C18 tips (Thermo Scientific Pierce, Waltham, MA) following the manufacturer’s guidelines and then dried on a speed-vac. To allow for the most reasonable and direct comparison between LF and TMT without significantly altering the sample-to-bead ratio, the following setup was performed. For LF experiment, the input material was maintained at 4.2 mg of receptor-stimulated protein, identical to the TMT-PV experiment. The 100 μl eluate was fractionated by volume into one, three and ten part equivalents of 36 fractions on an offline reverse-phase 10 cm (1 mm ID) Acquity peptides were pre-fractionated under basic conditions (pH 10) into unbuffered milli-Q water wash. Following the washes, pTyr peptides through three 1 ml ice-cold IAP buffer washes and one 1 ml ice-cold superbinder beads overnight at 4 °C on a rotator. The beads went dehydrated using 100 μl C18 tips (Thermo Scientific Pierce, Waltham, MA) following the manufacturer’s guidelines and then dried on a speed-vac. For TMT samples, additional modifications included TMT-11plex labels on N termini and lysine residues, where reporter ion MS3 with reporter mass tolerance of 0.005 Da was defined. All downstream analyses and graphical plotting were performed in R or Microsoft Excel based on MaxQuant output files.

Experimental Design and Statistical Rationale—The rationale of the experimental design is described in detail in the results section. Equal amounts of peptide were used in each labeling reaction so that the amount of peptide and label remained proportional in separate labeling steps. Three technical replicates each of 0.1 mg, 0.3 mg and 1.0 mg labeled peptides using the protein concentration derived from BCA Protein Assay were combined to create the contrived ratios of 10:1, 10:3, and 3:1. Technical, not biological, replicates were chosen to minimize biological sample variation that would otherwise hinder our ability to determine the quantitative accuracy of this experiment from predetermined contrived ratios, which is one of the core elements in the data analysis. LF experiments with a similar setup were included as a control alongside TMT experiments for comparison. Similar amounts of peptide input were used for pTyr peptide enrichment in all comparisons to maintain approximately equal sample-to-bead ratio. To observe the effect of PV boost channel to a fixed amount of peptide, the experimental sample peptide amounts were kept constant between the TMT-PV and TMT+PV samples (4.2 mg), and then 2 mg PV peptide was added only to the TMT+PV sample. This resulted in a total of 4.2 mg peptide for the TMT-PV sample and 6.2 mg (4.2 mg experimental + 2 mg PV) for the TMT+PV sample. Thus, the only difference between these two conditions is the addition of 2 mg PV peptide to the TMT+PV sample. Both TMT and LF experiments are limited to approximately equal instrument time. Each TMT experiment had a total of twelve fractionated LC/MS runs, whereas LF experiment had a total of eleven fractionated LC/MS runs. LF LC/MS runs had a similar or greater total number of MS and MS/MS scans compared with TMT experiments, making both LF and TMT experiments highly comparable. To determine statistical significance, Welch’s two sample t test was used to calculate p values from ratios containing all three intensity values present for both numerator and denominator (no missing values). To correct for multiple-hypothesis testing, the Benjamini-Hochberg (FDR) procedure was used to adjust p values to obtain q values (15). Q values less than 0.05 were considered statistically significant. Whenever appropriate, values were corrected for heteroscedasticity to allow for parametric testing by log transformation. Coefficient of determination of a linear regression model was calculated to examine the reproducibility and correlation of peptide intensities between replicates in a pairwise fashion.

RESULTS

Design of PV BOOST—To examine whether the inclusion of PV treated samples in a subset of multiplexed TMT experi-
1. Lysis
2. Reduction
3. Alkylation
4. Tryptic Digestion
5. Desalting

FASP

pTyr peptide enrichment
“superbinder-SH2”

Receptor stimulation
Pervanadate treatment
Jurkat T cells

TMT-PV

Contrived ratios mix
pTyr peptide enrichment
“superbinder-SH2”

Eluate fractionation by volume
1X x 3
“0.1 mg”
3X x 3
“0.3 mg”
10X x 3
“1.0 mg”
10X x 2
“1.0 mg”

LC-MS

MaxQuant

Molecular & Cellular Proteomics 19.4
733
ment would selectively trigger more pTyr precursor ion fragmentation thereby increasing the overall number of quantified TMT reporters in the TMT experimental channels, “TMT+PV” and “TMT-PV” sample mixtures, as well as a LF control were prepared. To maximize TMT channel utilization, 2 out of 11 TMT channels were designated as PV boost channels while the remaining 9 experimental channels contained predetermined peptide input. 3 replicates each of 0.1 mg, 0.3 mg, and 1.0 mg protein were used to construct contrived ratios of 1:0.1 mg, 1:0.3 mg, and 0.3:0.1 mg (Fig. 1). These contrived ratios were then used for accuracy and precision analyses. Because LF and TMT have inherently different sample preparation and quantification methods, each experiment was limited to approximately the same instrument time to set up a valid comparison between the different methods. This resulted in approximately equal total number of MS scans (supplemental Fig. S1A).

TMT Labeling Efficiency—In order to enable direct comparison from the same starting material, TMT labeling was performed prior to pTyr peptide enrichment. To maximize the amount of pTyr peptides labeled in this experiment, the amount of peptide labeled per TMT kit was optimized. Greater than 99% labeling was achieved when up to 1 mg of protein input for each TMT channel was used per 0.8 mg of labeling reagent before pTyr peptide enrichment (supplemental Fig. S1B, S1C). Equally high labeling efficiencies were observed by other groups when similar label-to-peptide ratios were used (16).

Determination of pTyr Peptide Enrichment Strategy—To determine the most efficacious affinity reagent for pTyr peptide enrichment in this experiment, two enrichment methods were directly compared across 3 replicates each of PV-treated Jurkat T cells, using the recently described and more cost-effective Src SH2 domain Superbinder (sSH2) (4) and the more commonly employed anti-pTyr P-Tyr-1000 antibody. Overall, enrichment by sSH2 is preferred because it enables the detection of more unique pTyr sites/PSM, delivers more pTyr peptides with higher intensities, and similarly to P-Tyr-1000 antibody, lacks strong amino acid sequence preference (supplemental Fig. S2, S3). The selective enrichment of pTyr peptides by sSH2 was consistent with published reports (17) (supplemental Table S1). Because of the superior performance of sSH2, this affinity reagent was chosen as the enrichment strategy for subsequent experiments.

Peptide Intensities Reproducibly Mirror Protein Input—First, the overall pTyr peptide intensities of each experimental input protein condition were quantified, as the intensity magnitudes should correlate with the amount of input material. Specifically, the distributions of signal intensities of TMT reporter ions or LF MS1 precursors from each condition were examined. As expected, the order of overall magnitude of pTyr peptide intensities reproducibly increases from 0.1 mg, 0.3 mg, to 1.0 mg of protein input; with 1.0 mg of PV protein having the highest intensities among all conditions (Fig. 2A). Despite the presence of neighboring high-intensity PV boost channels, the distributions of peptide intensities were almost identical across replicates within the same condition, suggesting that cross-channel interference is unlikely. Moreover, peptide intensities between replicates in a pairwise fashion were highly reproducible, as the linear regression model coefficients of determination are greater than 0.97 across all conditions (supplemental Fig. S4).

PV Boost Channel Significantly Increased pTyr Quantification Depth—Next, we examined the “pTyr quantification depth”, which we define as the number of unique pTyr-containing peptides with a nonzero TMT reporter ion or LF MS1 precursor ion intensity. The pTyr quantification depth dramatically increased in the presence of PV boost channel when the same amount of input was analyzed in the experimental channels. For example, 1395–2319 pTyr peptide reporter ions were quantified in the receptor-stimulated 1 mg TMT experimental channels with the inclusion of PV boost channels, compared with only 147–166 pTyr peptide reporter ions without PV boost channels, an increase of 12.8-fold on average (Fig. 2B, supplemental Table S2). Although the observed improvement varied across the experimental channels, with a modest increase of 2.3-fold on average with 0.1 mg of protein input, PV boost channels enhanced the overall pTyr quantification depth in every condition investigated. Although a comparably high pTyr quantification depth was obtained from 1 mg of PV protein in both LF and TMT experiments, we observed on average a 12.3-fold reduction in pTyr quantification depth in the LF experiment from 1 mg protein input compared with TMT+PV. PV boost channels also elevated the pTyr quantification depth 14.2-fold on average of peptides containing unique pTyr sites with 1 mg of protein input (supplemental Fig. S5, supplemental Table S2). Overall, PV boost channels always resulted in a gain in pTyr quantification depth when an
identical amount of input material was analyzed across all conditions investigated.

Analysis of pTyr Peptide Intensity Ratio Accuracy and Precision—We next investigated whether the substantial gain in pTyr quantification depth comes at the expense of accuracy or precision. Because of the presence of missing values (discussed in the next section), peptide intensity ratios were generated from a subset of the data lacking missing values where statistical significance could be calculated. Volcano plot analysis revealed that the inclusion of PV boost channels yielded the highest number of quantified pTyr peptide intensity ratios with statistical fold-change while maintaining the same level of accuracy observed in the TMT-PV and LF control experiments (Fig. 2C). For example, in the expected abundance ratio of 1:0.3 mg, the number of intensity ratios with significant fold change was increased from 48 in LF and 80...
TMT PV BOOST Channel Expands pTyr Quantitation Depth

Table I
Tabulation of quantified pTyr intensity ratios derived from contrived protein input in LF, TMT-PV and TMT+PV experiments. The numbers of quantified ratios are calculated by requiring a minimum of one or three values on both the numerator and denominator; the latter is further analyzed to determine statistically significant fold change of the intensity ratios with a maximum q value of 0.05. Fold increase in quantified pTyr peptide intensity ratios observed in the presence of PV boost channels relative to TMT-PV and LF experiments are also shown.

| Number of quantified pTyr peptide intensity on numerator and denominator | Contrived ratio of protein input | Quantified pTyr peptide intensity ratios | Fold increase in quantified pTyr peptide intensity ratios |
|---|---|---|---|
| | LF | TMT-PV | TMT+PV | TMT+PV |
| Minimum 1 (all possible) | 0.3:0.1 mg | 84 | 101 | 237 | 2.8 | 2.4 |
| | 1:0.1 mg | 81 | 103 | 279 | 3.4 | 2.7 |
| | 1:0.3 mg | 93 | 142 | 780 | 8.4 | 5.5 |
| Minimum 3 (no missing values) | 0.3:0.1 mg | 42 | 49 | 87 | 2.1 | 1.8 |
| | 1:0.1 mg | 41 | 49 | 87 | 2.1 | 1.8 |
| | 1:0.3 mg | 53 | 83 | 267 | 5.0 | 3.2 |
| Minimum 3 + q<0.05 (statistically significant) | 0.3:0.1 mg | 26 | 11 | 69 | 2.7 | 6.3 |
| | 1:0.1 mg | 39 | 48 | 85 | 2.2 | 1.8 |
| | 1:0.3 mg | 48 | 80 | 239 | 5.0 | 3.0 |

Table II
The median peptide signal intensity ratios and q values for each condition was calculated and compared to contrived ratios of 1:0.1 mg, 1:0.3 mg, and 0.3:0.1 mg.

| Contrived ratio of protein input | Condition | Median intensity ratio | Median q value |
|---|---|---|---|
| | LF | 11.61 | 1.3 × 10^{-2} |
| | TMT-PV | 9.46 | 5.0 × 10^{-3} |
| 1:0.1 mg (10.00) | TMT+PV | 10.19 | 3.0 × 10^{-3} |
| | LF | 4.38 | 1.3 × 10^{-2} |
| | TMT-PV | 3.96 | 4.8 × 10^{-3} |
| | TMT+PV | 3.65 | 8.3 × 10^{-3} |
| 1:0.3 mg (3.33) | LF | 2.69 | 1.9 × 10^{-2} |
| | TMT-PV | 2.30 | 7.6 × 10^{-2} |
| | TMT+PV | 2.66 | 2.2 × 10^{-2} |
| 0.3:0.1 mg (3.00) | LF | 11.61 | 1.3 × 10^{-2} |
| | TMT-PV | 9.46 | 5.0 × 10^{-3} |
| | TMT+PV | 10.19 | 3.0 × 10^{-3} |
| | LF | 4.38 | 1.3 × 10^{-2} |
| | TMT-PV | 3.96 | 4.8 × 10^{-3} |
| | TMT+PV | 3.65 | 8.3 × 10^{-3} |

Table III
Coefficient of variation percent (CV%) for TMT experiments. Median CV% for all paired quantified peptide intensities are indicated. The corresponding standard deviation is included in parenthesis. p values are generated using Welch’s two sample t-test to indicate statistical difference between the two populations of CV%

| Protein input (mg) | Median CV% (±S.D.) TMT+PV | Median CV% (±S.D.) TMT-PV | p value |
|---|---|---|---|
| 1.0 | 15.0 (±19.7) | 12.3 (±11.2) | 1.0 × 10^{-2} |
| 0.3 | 23.6 (±21.4) | 27.0 (±19.2) | 2.6 × 10^{-1} |
| 0.1 | 27.5 (±22.3) | 16.5 (±18.6) | 1.8 × 10^{-1} |

In TMT-PV to 239 in TMT+PV, representing a 5-fold and 3-fold increase, respectively (Table I). We also observed a 6.3-fold boost in the number of intensity ratios with significant fold change in the low abundant 0.3:0.1 mg condition in the presence of PV boost channels (Table I), underscoring the potential of this strategy when the detection limit is challenged. Importantly, PV boost channels did not compromise quantitative accuracy in the experimental channels as most of the measured ratios were in good agreement with the expected values (Fig. 2C). For example, in the expected ratio of 1:0.3 mg (3.33), TMT-PV had a median intensity ratio of 3.65 compared with TMT-PV with 3.96 and label-free with 4.38 (Fig. 2D, Table II).

To further probe the peptides detected in both TMT experiments, the list of identified pTyr peptides in the experimental channels was subdivided into populations that were uniquely observed in TMT+PV (“boost-gained”), uniquely observed in TMT-PV (“absent”) and those that were observed in both (“overlap”) (supplemental Fig. S6, supplemental Table S3–S5). Venn diagram analysis of these subpopulations reveals that there was 91-fold more boost-gained peptides than those that were absent when PV boost channels were included (2925 versus 32) (supplemental Fig. S6). To individually probe their quantitative accuracy, the subsets of these populations in which an intensity ratio and its statistical fold-change could be determined were carefully inspected in separate volcano plot analyses. Notably, the accuracy of boost-gained peptides was similar to those that were detected in the overlap population in the TMT+PV sample, both containing ~80–98% of reporter intensity ratios with statistically significant fold-change. Overall, this strategy can deliver considerable gains in pTyr quantitation depth without impairing quantitative accuracy.

Next, the precision of TMT reporter quantification was examined through three-replicate analyses. Although the coefficient of variation percentages (CV%) were not significantly different for the 0.1 mg and 0.3 mg of protein inputs, for 1.0 mg of input, TMT-PV had a slightly smaller median CV% (12.3%) compared with TMT+PV (15.0%) with statistical significance (Table III). Nonetheless, this difference in the precision of reporter ion quantification for one of the three input amounts still allowed the detection of more significantly changed intensity ratios in TMT+PV when comparing 1.0:3 mg or 1:0.1 mg (Fig. 2C). LF experiments had a comparable CV% of 16.8–21.7% (Table IV). Moreover, compared with the
**Quantitative Accuracy was Preserved with Minimal Missing Values** — Although we had established that each TMT channel labeling reagent can be used to label 1 mg of input material with >99% labeling efficiency (supplemental Fig. S1B, S1C), this experimental setup was designed to intentionally include substantially lower protein abundances of 0.1 mg and 0.3 mg to not only allow the construction of contrived ratios, but also to challenge the detection limit of this method. Because of the low abundance of pTyr peptides, 0.1 mg samples yielded the highest number of missing values (supplemental Fig. S7A). Conversely, no more than 7% of the identified pTyr peptides in the PV samples of both LF and TMT experiments lack quantified intensity values (supplemental Fig. S7A). As a result, each pTyr peptide contained varying number of missing values in which an intensity is not assigned (supplemental Fig. S7B). Although analysis in the absence of missing values is preferred, ratios of peptide intensity can be constructed even in the presence of missing values if there is a minimum of one value each in the numerator and denominator respectively. To facilitate such analysis in the presence of unevenly distributed numbers of missing values, the number and accuracy of intensity ratios were systematically examined as a function of missing values (zero to a maximum of four) out of six possible intensities used for ratio construction in this experimental setup (Fig. 3A). In general, TMT+PV delivered more quantifiable intensity ratios across zero to four missing values compared with TMT-PV or LF experiments within the same condition (Fig. 3B). Although the accuracy of intensity ratios started to deteriorate beyond three or more missing values, greater than 80% of the intensity ratios were captured by two or fewer missing values (Fig. 3C–3D). Most importantly, these additional ratios were similarly as accurate in the presence or absence of boost channels (Fig. 3D). Overall, PV boost channels improved quantification depth while also maintaining good accuracy in the absence or presence of a small number of missing values.

**Analysis of Dynamic Range and Boost Factor** — Because the abundance of pTyr peptides in the boost channels and experimental channels can vary by several orders of magnitude, it is imperative to investigate how boost channels affect dynamic range and quantitative accuracy. Dynamic range is defined as the difference between the highest and lowest channel intensity, including PV boost channels. The dynamic range of the TMT+PV sample is consistently higher than that of the TMT-PV sample because of the presence of PV boost channels in the former (supplemental Fig. S8A–S8F). However, dynamic range was not a good predictor of accuracy due the lack of a strong correlation (Pearson correlation coefficient range of −0.17 to 0.16) (supplemental Fig. S8A–S8F).

Using an alternative approach, we systematically probed the magnitude of improvement delivered by PV boost channels through calculation of “boost factors”. Because of the combinatorial nature of TMT quantification, the ratio of summed TMT reporter ion intensities of the PV-treated sample over that of the receptor-stimulated sample can be used as a proxy for the ratio between the total reporter ion current of the PV boost channels and the total reporter ion current of the experimental channels. We define this ratio as the “boost factor” (Fig. 5). Overall, boost-gained peptides had a median boost factor of ~12-fold higher (58 versus 4.9) than the overlapping peptides identified in both the TMT+PV and TMT-PV samples (supplemental Fig. S9A). The difference could be explained by the 8-fold lower median experimental reporter ion intensities in the boost-gained peptides compared with the overlapping peptides (supplemental Fig. S9B). In other words, boost-gained peptides in the experimental channels were correlated to physiologically low-abundance pTyr peptides. The bulk of the reporter ion current for these peptides was from the PV boost channels and not from the experimental channels.

**Coefficient of variation percent (CV%) for LF experiments.** Median CV% for all quantified peptide intensities are indicated. The corresponding standard deviation is included in parenthesis.

| Protein input (mg) | Median CV% (±S.D.) LF |
|-------------------|----------------------|
| 1.0 mg            | 17.3 (±16.8)         |
| 0.3 mg            | 16.8 (±15.3)         |
| 0.1 mg            | 21.7 (±17.5)         |

**PV Boost Channels Elevated MS1 pTyr Precursor Ion and Reporter Ion Intensities** — We hypothesize that the gain in quantitation depth in the TMT+PV sample is attributed to an increase in abundance of pTyr MS1 precursor ions and consequently, an increase in total TMT reporter ion abundance. To test this hypothesis, the pTyr peptides that were quantified in both TMT-PV and TMT+PV samples were compared directly. As anticipated, the average MS1 precursor ion intensities were significantly higher in the TMT+PV than in the TMT-PV sample (Fig. 4A). Comparisons of the average ratio of the summed TMT reporter ion intensities revealed a similar trend when reporter intensities originating from PV boost channels are included (Fig. 4B), while not significantly changing the reporter intensities of the experimental channels between the 2 samples when PV boost channels are ignored (Fig. 4C). Overall, PV boost channels elevated the signal abundance of pTyr precursor ions and reporter ion intensities and that ultimately resulted in deeper quantitation depth in the experimental channels without significant inflation of the experimental channel reporter intensities.
The correlation between boost factor and the detection of significantly changing ratios was examined (Fig. 5). The 2925 boost-gained pTyr peptides had boost factors spanning six logarithmic orders of magnitude, with 98.3% of the peptides having a boost factor above 1 (supplemental Fig. S9). A boost factor above 1 indicates that the total reporter ion current in the boost channels exceeds that of the experimental channels in total. Because many of these boost-gained peptides contained varying numbers of missing values, we focused on the subset of data in which a ratio fold-change and statistical significance could be determined. A cumulative count plot of the intensity ratios with significant fold-change (q<0.05) as a function of the number of missing values is shown in Fig. 3.

The number of quantified pTyr peptide intensity ratios is further increased without compromising accuracy in the presence of a minimal number of missing values. A, Schema for peptide intensity ratio construction in the context of missing values. A maximum of 4 missing values is allowed when a maximum of 6 possible intensities are used to construct the ratios. B, Bar charts showing the number of quantified pTyr peptide intensity ratios as a function of the number of missing values in calculating the ratio, from zero to four. C, Line graphs showing the cumulative percentage of quantified pTyr peptide intensity ratios as a function of the number of missing values. 100% indicates the sum of all quantified intensity ratios for the same condition depicted in (B). D, Ratio accuracy boxplot as a function of the number of missing values. Horizontal dashed lines indicate the expected ratios of 1:0.1 mg, 1:0.3 mg and 0.3:0.1 mg, respectively.
function of boost factors was generated. The plot revealed that in this experiment, in order to obtain at least 90% of all the boost-gained significantly changed ratios, a boost factor of at least 5 was required (Fig. 5 boxed line graph, supplementary Table S6). A boost factor of at least 58 was enough for the detection of all significant ratios across all conditions. We also observed a comparably similar trend when identical analyses using all ratios with no missing values and all possible ratios were performed (supplemental Fig. S9, supplemental Table S6). Overall, despite the wide dynamic range of boost channels, most of the boost-gained peptides were obtained within one or two orders of magnitude of boost factors, depending on the stringency for missing values and statistical significance.

**DISCUSSION**

Any meaningful interpretation of pTyr proteomic data relies on the accurate identification and quantification of pTyr peptides. In data dependent acquisition, the isolation and the fragmentation of pTyr peptides are highly disfavored because of their relative low abundance (18). The work presented here integrates TMT, PV treatment and sSH2 to improve the subpar quantitation depth of the tyrosine phosphoproteome. We leveraged the multiplexing capability of TMT and included PV boost channel as a subset of the 11 TMT channels to increase the likelihood of otherwise low-abundance pTyr peptide being triggered for MS/MS fragmentation (Fig. 1). PV boost channels are only used for facilitating the selective fragmentation of pTyr precursor ions whereas quantitative information of the experimental channels can be extracted from their corresponding reporter ion intensities. Coupled to the efficacious and cost-effective sSH2 as an alternative to antibody-based approach to enrich for pTyr peptides, BOOST delivers a deep quantitative characterization of the tyrosine phosphoproteome where cellular material is limited.

Previous attempts to improve pTyr proteome quantitation depth have been largely focused on the enrichment of pTyr peptides (4, 19, 20, 21), with some degree of success. Although total phosphopeptides can be enriched using titanium dioxide, this method predominantly allows for the detection of phosphoserine- and phosphothreonine-containing peptides, with one study reporting less than 150 pTyr sites from 10 mg of protein (<1% pTyr of total phosphopeptide) (22). Conventional wide-scale pTyr proteomics relies on the availability of commercial pan-specific anti-pTyr antibodies, such as 4G10, P-Tyr-1000, P-Tyr-100, and PY99 for pTyr peptide enrichment. Recently, sSH2, which is more cost-effective than the other enrichment methods, was shown to have >13 times greater binding capacity compared with 4G10 for wide-scale pTyr proteomic experiments (4). The most recently developed triple-monoclonal antibody mixture, P-Tyr-1000, is perceived as the benchmark affinity reagent for wide-scale pTyr pro-

![Fig. 4. PV boost channels elevated the intensities of pTyr-containing (A) MS1 precursor ions, (B) total TMT reporter ions including PV boost channels and (C) total TMT reporter ions excluding PV boost channels. Log10 MS1 precursor ion intensities or summed TMT reporter ion intensities of the peptides that were identified and quantified in both TMT-PV and TMT+PV conditions were plotted in a pairwise fashion. Dashed line is a y = x linear function indicating equal intensity in both conditions along the line. The average ratio of TMT+PV intensities versus TMT-PV is indicated under the corresponding plot. p values are generated using one-sample t test to indicate statistical difference between the average ratios and a ratio of 1, the theoretical ratio if intensity of TMT+PV and TMT-PV are identical to each other.](image_url)
Supplemental Fig. S6

**Experimental channels are approximately equivalent.**

This plot is identical to the corresponding volcano plots above. Boost factor is 1 when the total reporter ion current between the boost and the experimental channels (Fig. S2–S3).

**Histogram depicts the distribution of the boost factors of all boost-gained peptides.** A subset of data in which a ratio fold-change and statistical significance can be calculated were used to generate a cumulative count plot of the significantly changed ratios ($q<0.05$) as a function of boost factors. The number of intensity ratios analyzed in this plot is identical to the corresponding volcano plots above. Boost factor is 1 when the total reporter ion current between the boost and experimental channels are approximately equivalent.

**Count of unique pY peptide**

The number of intensity ratios analyzed in this plot is identical to the corresponding volcano plots above. Boost factor is 1 when the total reporter ion current between the boost and experimental channels are approximately equivalent.

**Analysis of boost factor.** Venn diagram illustrates the number of identified pTyr peptides in the TMT experimental channels that were further categorized into subpopulations that were uniquely observed in TMT+PV (boost-gained), uniquely observed in TMT-PV (absent) and those that were observed in both conditions (overlap). Volcano plot analysis was performed for the boost-gained peptide intensities. Boost factor is defined as ratio of the summed TMT reporter ion intensities of the PV-treated samples (boost channels) over the summed TMT reporter ion intensities of the receptor-stimulated samples (experimental channels), which is a proxy for the ratio between the total reporter ion current of the boost channels and the total reporter ion current of the experimental channels. Histogram depicts the distribution of the boost factors of all boost-gained peptides. A subset of data in which a ratio fold-change and statistical significance can be calculated were used to generate a cumulative count plot of the significantly changed ratios ($q<0.05$) as a function of boost factors. The number of intensity ratios analyzed in this plot is identical to the corresponding volcano plots above. Boost factor is 1 when the total reporter ion current between the boost and experimental channels are approximately equivalent.

Concordant with the hypothesis that PV boost channels elevated pTyr MS1 precursor ion abundance, our data showed that for the same pTyr peptide identified in both TMT-PV and TMT+PV samples, pTyr-containing MS1 precursors and total reporter ion intensities were significantly higher when PV boost channels are included (Fig. 4). Although the magnitude of improvement varied depending on protein input, PV boost channels always generated deeper quantitation when the same condition was examined (Fig. 2B, supplemental Table S1). Most importantly, PV boost channels delivered comparable, if not better, quantitative accuracy, precision, and statistical significance when compared with TMT-PV or LF experiments across all conditions (Fig. 2C–2D, Tables I–IV). Although there is an upper limit to the total reporter ion current for MS3 quantitation, the addition of two PV boost channels of high intensity did not significantly alter the ratios in the experimental channels, suggesting that the upper saturation limit of quantitation was not exceeded in those channels (Fig. 2C, supplemental Fig. S6). Conversely, the number of missing data increased markedly as the amount of input protein was reduced to 0.1 mg in the experimental channels.
supplemental Table S1. This analysis revealed that we were successful at probing the detection limit of this approach as a smaller amount of protein was used here compared with >5 mg of protein typically utilized in wide-scale pTyr proteomic experiments. Overall, PV boost channels always increased pTyr quantitation depth when the same amount of protein input was analyzed.

The results of this experiment demonstrate that pTyr proteomics can be achieved with 1 mg of starting material or less, which is a more reasonable amount than the 5–20 mg typically used in a wide-scale pTyr proteome analysis (23, 24, 25, 26). Even if cellular material is not limiting, simply increasing the protein amount for pTyr enrichment has shown to be insufficient to substantially expand pTyr quantitation depth. One group reported less than 350 pTyr peptides from 5 mg of Jurkat protein using a similar sSH2 approach (17, 19), whereas another group identified only 558 pTyr sites from 35 mg of Jurkat protein (4). In contrast, the BOOST approach in this study quantified more than 2300 pTyr peptides from only 1 mg of Jurkat protein (Fig. 2B, supplemental Table S1).

Although analysis of the dynamic range of boost channels did not reveal a clear correlation with quantitative accuracy, characterization of boost factors provides an avenue to probe the magnitude of improvement delivered by PV boost channels as a result of elevated reporter ion current. Considering the combinatorial nature of TMT quantitation, we quantified boost factors by examining the total reporter ion current in PV boost channels in relation to that of the experimental channels (Fig. 5, supplemental Fig. S9A). It is noteworthy that boost-gained peptides were associated with relatively higher boost factors and were derived from substantially lower experimental reporter ion intensities (supplemental Fig. S9A–S9B). Analysis of the summed reporter ion intensities from the experimental channels suggests that the inclusion of PV boost channels greatly facilitated the selective fragmentation of pTyr precursor ions that otherwise would likely be below the detection limit of quantitation (supplemental Fig. S9B). Overall, despite the wide dynamic range of PV boost channels, most of the boost-gained peptides were obtained within one or two orders of magnitude of boost factors, depending on the stringency for missing values and statistical significance (supplemental Fig. S9C).

A valid consideration with the BOOST approach is the possibility that highly abundant pTyr peptides from PV boost channel might “leak” into neighboring channels to artificially inflate their quantified intensity because of isotopic impurities in the individual TMT labels. The reporter ion correction factors for each TMT label provided by the manufacturer were used as correction factors in MaxQuant to correct for this possibility in these experiments. Although the phenomenon of cross population reporter ion interference was recently reported in a multi-batch TMT experiment (27), we did not observe evidence of signal crossover between channels (Figs. 2A, supplemental Fig. S4). If PV leakage into the immediate neighboring channels were occurring, a deviation of peptide intensities in those channels would likely be observed. However, peptide intensities across replicates exhibited near-identical distribution and high reproducibility within the same condition, including those immediately neighboring the boost channels (Figs. 2A, supplemental Fig. S4). Nevertheless, to preemptively mitigate this potential risk in future experiments, we recommend designating the ultimate and, if necessary, penultimate TMT channels as the boost channels to minimize the effects of cross-label isotopic impurity affecting the +1 Da channel, which was shown to have a more pronounced effect than cross-label isotopic impurity affecting the –1 Da channel (27). We acknowledge that even though two boost channels were utilized in this specific experiment, maximization of the number of TMT experimental channels could necessitate the reduction to a single boost channel which may be accomplished by careful titration of the amount of material in the single boost channel as long as accuracy is not compromised.

The correlation between boost factors and the exact magnitude of improvement in pTyr quantitation depth may differ across different sample types. For example, this strategy, if applied to limited patient samples or rare primary cell sub-populations, may require the use of boost channels from a different source because of the scarcity of patient-derived samples or inability to stimulate patient samples with PV ex vivo. In situations where it is not possible to obtain additional cellular material for PV stimulation, proxy cell lines of the same species and cell type could potentially be used as the boost channels. For example, pTyr proteomics involving scarce patient-derived primary renal tissue might necessitate the use of HEK-293 cells for PV stimulation as the boost channel samples. For such studies, experimental parameters need to be further optimized with regards to the suitability of proxy cells, the optimal amount of cellular material in the experimental and boost channels and the arrangement of samples in TMT channels, as pTyr enrichment methods and the number of multiplexed channels continues to improve over time. Ultimately, the experimental parameters would likely depend on the researcher’s system, experimental goal, and availability of cellular material for the boost channel. In this study, we provided a template for such experimental design using the Jurkat model system and performed a systematic boost factor analysis to guide researchers in calibrating their experimental setup.

The use of PV boost channels in a multiplexed experiment is analogous to the carrier channel employed in a single-cell proteomics study where carrier cells facilitated the detection of single-cell channels (30), yet this strategy has not been applied to wide-scale analysis of post-translational modifications. We leveraged sSH2 as an alternative to the antibody-based approach to pTyr peptide enrichment, in combination with the multiplexing capability of TMT to include PV-treated samples as a subset of the 11 TMT channels, to deliver a deeper quantitative characterization of the tyrosine phospho-
proteome. This study may have a broader impact beyond pTyr proteomics because the BOOST strategy could potentially be extended to other post-translational modifications, where a broad-spectrum treatment for elevating the abundance of peptides harboring the modification of interest is available to be used in the boost channel in a multiplexed experiment. For example, sodium fluoride is a potent serine/threonine phosphatase inhibitor (31) that can be used to boost phosphoserine and phosphothreonine identification depth. Similarly, ubiquitin ligase activators (32) or proteasome inhibitors could be deployed to enhance the identification of ubiquitinated proteins. One could also use histone deacetylase inhibitors to study wide-scale histone acetylation. In short, the BOOST approach delivers a global enhancement that is widely applicable to the field of pTyr proteomics and may broadly improve analysis of other low-abundance post-translational modifications.

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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 with the data set identifier PXD016192.

TMT PV BOOST Channel Expands pTyr Quantitation Depth

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