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# Quantitative Label-Free Phosphoproteomics Strategy for Multifaceted Experimental Designs

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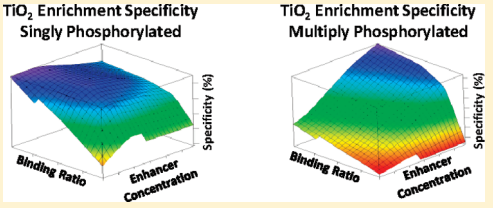
Supporting Information

**ABSTRACT:** Protein phosphorylation is a critical regulator of signaling in nearly all eukaryotic cellular pathways and dysregulated phosphorylation has been implicated in an array of diseases. The majority of MS-based quantitative phosphorylation studies are currently performed from transformed cell lines because of the ability to generate large amounts of starting material with incorporated isotopically labeled amino acids during cell culture. Here we describe a general label-free quantitative phosphoproteomic strategy capable of directly analyzing relatively small amounts of virtually any biological matrix, including human tissue and biological fluids. The strategy utilizes a $TiO_2$ enrichment protocol in which the selectivity and recovery of phosphopeptides were optimized by assessing a twenty-point condition matrix of binding modifier concentrations and peptide-to-resin capacity ratios. The quantitative reproducibility of the $TiO_2$ enrichment was determined to be 16% RSD through replicate enrichments of a wild-type *Danio rerio* (zebrafish) lysate. Measured phosphopeptide fold-changes from alpha-casein spiked into wild-type zebrafish lysate backgrounds were within 5% of the theoretical value. Application to a morpholino induced knock-down of G protein-coupled receptor kinase 5 (GRK5) in zebrafish embryos resulted in the quantitation of 719 phosphorylated peptides corresponding to 449 phosphorylated proteins from 200 $\mu$g of zebrafish embryo lysates.




The most commonly used LC-MS based quantitative phosphoproteomics experiments are currently based on the incorporation of stable isotope labels through stable-isotope labeling by amino acids in cell culture (SILAC), proteolytic digestion in the presence of $^{18}O$, or covalent peptide modifications with isobaric tags for relative and absolute quantification (iTRAQ) reagents (reviewed in refs 1−3). Although SILAC is very powerful for studies in transformed cell lines, analysis of primary cells, tissues or biological fluids using this method requires SILAC-labeled animals, which is expensive and has only be implemented in rodent, fly, and newt models.[4−6] In addition, a maximum of two or three unique experimental conditions may be directly compared using $^{18}O$ or SILAC labeling strategies, respectively, limiting the complexity of the experimental design which can be employed. The use of iTRAQ or TMT (Tandem Mass Tag) labeling addresses some of these limitations by covalently labeling peptides post-digestion from up to eight different experimental conditions, making the approach amenable to studying tissue or biological fluids including nonpooled biological replicates.[7] Those strategies, however, may introduce additional quantitative variation from the labeling procedure and may be cost prohibitive when working with samples (e.g., tissues) with relatively low levels of protein phosphorylation.[8] Importantly, quantitative information from iTRAQ/TMT experiments is only gained from precursor ions which were selected for MS/MS fragmentation and precludes the ability to perform follow-up experiments aimed at further interrogating qualitatively unidentified, but statistically significant, signals.[1]

As an alternative to stable isotope labeling approaches several label-free quantitation strategies, such as measuring area-under-the-curve (AUC) intensities of peptide precursor ions following accurate-mass and retention time alignment of raw LC-MS data, have been described in the literature.[9−11] In addition to experimental compatibility with tissue or biological fluids, this approach affords the capability of making quantitative measurements across a large number of biological samples without the requirement of pooling. Although label-free approaches allow for flexible experimental designs, they are often associated with rigorous analytical and informatic requirements, including: attention to reproducibility of sample preparation and chromatographic separations; automation of multiple LC-MS file alignment; and automation of precursor ion detection and quantitation.[1,2] An additional source of possible variation in any label-free quantitative phosphorylation study is phosphopeptide enrichment prior to LC-MS analysis, as each sample must be enriched independently. Collectively, these challenges have limited the number of global label-free quantitative phosphoproteomics descriptions in the literature.[12−18]

Here we describe a label-free quantitative phosphoproteomics workflow which addresses the above concerns and applied it to study the effects of a morpholino induced knock-down of G protein-coupled receptor kinase 5 (GRK5) in *Danio rerio*

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(zebrafish) embryos. To enhance the quantitative robustness of the label-free phosphoproteomic analysis, an optimized $TiO_2$ enrichment strategy was first developed using wild-type zebrafish embryo lysates enriched under a matrix of twenty unique conditions. The goal of this $TiO_2$ enrichment optimization was to reduce the percent of nonphosphorylated peptide interference, maintain high recovery of phosphopeptides and avoid bias of the enrichment for any particular subset of phosphorylated species.

## MATERIALS AND METHODS

**Chemicals and Materials.** MassPrep Enhancer and Rapigest SF acid-cleavable surfactant were purchased from Waters Corporation (Milford, MA). $TiO_2$ resin was purchased from Protea Biosciences (Morgantown, WV) as part of the $TiO_2$ SpinTip Sample Prep Kit (product number SP-154−24). Approximately 193 mg of loose $TiO_2$ resin with a manufacturer reported binding capacity of 25.9 $\mu$g/mg resin was combined from five purchased columns. *Danio rerio* (zebrafish) husbandry and morpholino induced knock-down of GRK5 is described in Supporting Information. All other regents were purchased at the highest available purity from commercial sources.

**Protein Extraction and Digestion.** Dechorionated and deyolked embryos were lysed in 50 mM ammonium bicarbonate (pH 8.0) containing 0.4% RapiGest SF and phosphatase inhibitors (1 mM KF, 1 mM $NaVO_4$) by three 10s pulses of burst sonication intermitted by cooling on ice for 30 s. Samples were centrifuged and supernatants were reduced with 5 mM DTT at 60 °C for 20 min and alkylated with 10 mM iodoacetamide at room temperature in the dark for 45 min. Trypsin was added to final ratio (w/w) of 1:50 enzyme-to-protein and digestion was performed at 37 °C for 18 h. All samples were spiked with trypsin digested α-casein at 30 fmol α-casein per 1 $\mu$g of zebrafish lysate and frozen at −80 °C until phosphopeptide enrichment was performed.

**Phosphopeptide Enrichment.** $TiO_2$ spin columns were packed using 200 $\mu$L gel-loading pipet tips with a 1 mm × 1 mm 15−45 um X-4900 UHMW Porex (Fairburn, GA) plug. The resin bed was compressed three times by adding 10 column volumes of 20% acetonitrile, 5% $NH_4OH$ (pH 10.5) followed by centrifugation at 200 rcf for 1 min. The column was then re-equilibrated by adding 200 $\mu$L of 80% acetonitrile, 1% TFA (pH 2.5) (wash buffer A) followed by centrifugation at 200 rcf for 1 min.

For $TiO_2$ enrichment condition optimization, five aliquots each of 20, 100, 200, or 400 $\mu$g wild-type zebrafish lysate were brought to dryness using vacuum centrifugation and resuspended in wash buffer A with either 0, 50, 100, 200, or 300 mg/mL MassPrep Enhancer. Three additional aliquots of 350 $\mu$g wild-type zebrafish lysates in wash buffer A and 200 mg/mL MassPrep Enhancer were prepared for reproducibility studies and two additional 1000 $\mu$g aliquots spiked with 25 fmol/$\mu$g or 75 fmol/$\mu$g bovine alpha-casein in wash buffer A and 200 mg/mL MassPrep Enhancer were prepared for quantitative spiking experiments. Samples were loaded into a $TiO_2$ spin column containing 7.7 mg or 35.8 mg of resin and centrifuged at 100 rcf for 2 min for enrichment condition and reproducibility experiments or spiking experiments, respectively. The unbound flow through was re-applied to the top of the column and centrifuged a second time. Bound peptides were washed once with wash buffer A containing the same amount of MassPrep Enhancer as in the sample and then twice with wash buffer A alone. Remaining peptides were eluted twice with 100 $\mu$L 20% acetonitrile, 5% $NH_4OH$ (pH 10.5), acidified to pH 3.5 with neat formic acid and brought to dryness using vacuum centrifugation.

For GRK5 knock-down experiments, two separate 200 $\mu$g aliquots of lysate from each treatment group was resuspended in wash buffer A with100 mg/mL MassPrep Enhancer were applied to a $TiO_2$ spin column containing 3.85 mg of $TiO_2$ resin and brought through the same phosphopeptide enrichment protocol as described above.

**Nanoflow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS).** Samples were resuspended in 20 $\mu$L 2% acetonitrile, 0.1% formic acid, 50 mM citric acid (pH 3.0) and injected onto a Waters NanoAquity UPLC equipped with a 1.7 $\mu$m BEH130 $C_{18}$ 75 $\mu$m i.d. × 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 4 $\mu$L injection, peptides were trapped for 5 min on a 5 $\mu$m Symmetry $C_{18}$ 180 $\mu$m i.d. × 20 mm column at 20 $\mu$L/min in 99.9% A. The analytical column was then switched in-line and the mobile phase was held for 5 min at 5% B before applying a linear elution gradient of 5% B to 40% B over 90 min at 300 nL/min.

MS data for all $TiO_2$ enrichment optimization and evaluation experiments were acquired on a Waters Synapt HDMS G1 mass spectrometer using two different acquisition strategies. For qualitative only acquisitions, the instrument was operated in data-dependent acquisition (DDA) mode with a precursor MS scan from $m/z$ 50 to 1990, followed by 3 MS/MS product ion scans from $m/z$ 50 to 1990 with a charge-state dependent CID energy setting. For qualitative/quantitative acquisitions, the instrument was operated in a data-independent analysis (DIA) high-energy/low-energy alternative scanning ($MS^E$) mode with a 0.9 s low-energy precursor MS scan from $m/z$ 50−1990 followed by a high-energy MS scan from $m/z$ 50−1990 with a CID energy set to ramp from 15 v to 40 v. The duty cycle of this acquisition was approximately 2 s and allowed a minimum of 10 low-energy MS spectra to be acquired across a 20 s chromatographic peak. This improvement in duty cycle inherent in DIA compared with DDA significantly improves the quantitative rigor and reproducibility of the analysis. To increase coverage of lower abundant precursor ions, a 120 s dynamic exclusion list was employed for all DDA acquisitions. For both acquisitions, a separate LC channel with 200 fmol/$\mu$L Glu-1-Fibrinopeptide in 50% acetonitrile/0.1% formic acid flowing at 500 nL/min was referenced every 30 s through a nano lock-spray interface.

MS data from GRK5 knock-down experiments were acquired on a Thermo LTQ-Orbitrap XL mass spectrometer using two different acquisition strategies For all acquisitions, the instrument was set to acquire a precursor MS scan from $m/z$ 400−2000 with $r$ = 60,000 at $m/z$ 400 and a target AGC setting of 1e6 ions. MS/MS spectra for the top 10 precursor ions were acquired in the LTQ for qualitative only acquisitions and the top 5 or top 2 precursor ions were acquired in the LTQ or FT, respectively, for qualitative/quantitative acquisitions. For all experiments, fragmentation occurred in the LTQ linear ion trap with a CID energy setting of 35% and a dynamic exclusion of 60 s for previously fragmented precursor ions.

**Qualitative Peptide Identification.** DTA files from each LC-MS analysis were submitted to Mascot (Matrix Science, Boston, MA) searches against a forward-reverse NCBI *Danio rerio* protein database appended with bovine α, β, and γ casein protein entries.

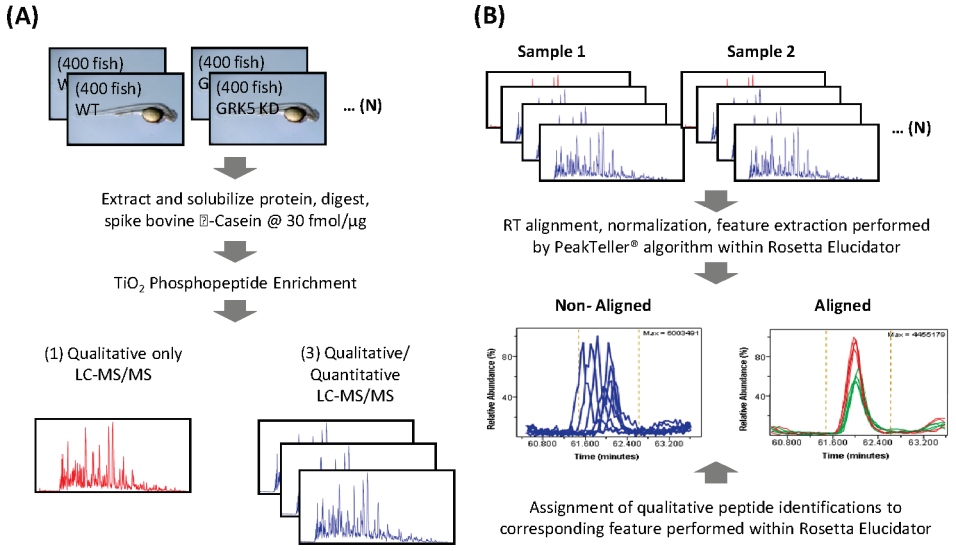


**Figure 1.** General overview of (A) sample preparation and data acquisition strategy and (B) data analysis workflow utilized in the label-free quantitative phosphoproteomic analysis of WT vs GRK5 knock-down zebrafish embryos.

Search tolerances of 20 ppm precursor and either 0.04 or 0.8 Da product ions were applied for Synapt HDMS data files or LTQ-Orbitrap files, respectively. Spectra were searched using trypsin specificity with up to two missed cleavages. Carbamidomethylation (+57.0214 Da on C) was a fixed modification, whereas oxidation (+15.9949 Da on M) and phosphorylation (+79.9663 Da on STY) were considered as variable modifications. Peptide FDR was determined by adjusting the Mascot peptide ion score threshold to allow a 1% occurrence of peptides from reverse protein entries. All qualitative data are available at ProteomeCommons.org as described in Supporting Information.

## RESULTS AND DISCUSSION

**Label-Free Phosphopeptide Quantitation Workflow.** *General Label-Free Quantitation Strategy Using Rosetta Elucidator.* A generalized overview of the workflow utilized in these experiments is illustrated in Figure 1. To assess $TiO_2$ enrichment performance and quantitative reproducibility, digested lysates were spiked with 30 fmol of bovine alpha-casein digest per 1 $\mu$g of lysate prior to enrichment. Samples were then subjected to a single qualitative only acquisition and three qualitative/quantitative acquisitions intended to either maximize the number of qualitative identifications or provide a quantitatively accurate description of the peptide's chromatographic elution profile, respectively. Although qualitative peptide identifications were generated from both types modes of acquisitions, only peak areas from qualitative/quantitative acquisitions were used for quantitative data interpretation.

Label-free quantitation and integration of qualitative peptide identifications was performed using Rosetta Elucidator (v 3.3, Rosetta Inpharmatics, Seattle, WA). Following chromatographic retention time alignment of all LC-MS data within an experiment using the PeakTeller algorithm, quantitation of all detectable features (an individual isotopomer within a precursor ion's charge state envelope) in the precursor MS spectra was performed by measuring either peak area under curve (Synapt HDMS data) or peak height (LTQ-Orbitrap data) measurements of the corresponding extracted ion chromatograms.[19] Qualitative identifications assigned to a specific feature were also projected to features of the same retention time and $m/z$ across all LC-MS analysis, regardless if the peptide was qualitatively identified in the other runs, in a method analogous to the Smith et al. AMT approach.[10] Fold-change values between WT and GRK5 knock-down treatment groups were calculated on the peptide level from the averages of the sum of all features associated with the precursor ion within a biological replicate. To account for slight differences in total peptide loading between injections, all of the features within an LC-MS analysis were subjected to a robust mean normalization of all of the feature intensities which excluded the highest and lowest 10% of the signals.

**Optimizing Global Phosphorylation Enrichment.** *Effects of $TiO_2$ Enrichment Condition on Global Peptide Composition.* Of critical importance to any quantitative label-free phosphoproteomics platform is establishing an effective and reproducible $TiO_2$ enrichment prior to LC-MS analysis, as each sample must be enriched independently. Phosphopeptide enrichments are known to be dependent on a number of factors including peptide-to-resin capacity ratios,[20,21] pH of the sample buffer,[22,23] and the use of various buffer additives (such as 2,4-dihyroxybenzoic acid (DHB)) to reduce binding of nonphosphorylated peptides.[24−27] The influence of these variables was observed in a pilot phosphopeptide enrichment of 2 pmol of α-casein with a ProteaTip $TiO_2$ tip using the manufacturers supplied buffers versus a generalized buffer system (80% acetonitrile, 50 mg/mL modifier, 1% TFA) previously described.[24] Although nine unique phosphorylated peptides were identified following both protocols, six nonphosphorylated peptides were identified using the manufacturers protocol identified compared to only one nonphosphorylated peptide using the generalized buffer system protocol identified (data not shown).

Although the effects of varying resin capacity and buffer modifies have been individually described, the codependence of these variables has not been investigated in detail. To maximize enrichment specificity and yield of recovered phosphopeptides, a matrix of twenty different enrichment conditions varying in peptide-to-resin capacity ratios and MassPrep Enhancer concentrations was generated. For each enrichment condition, digested


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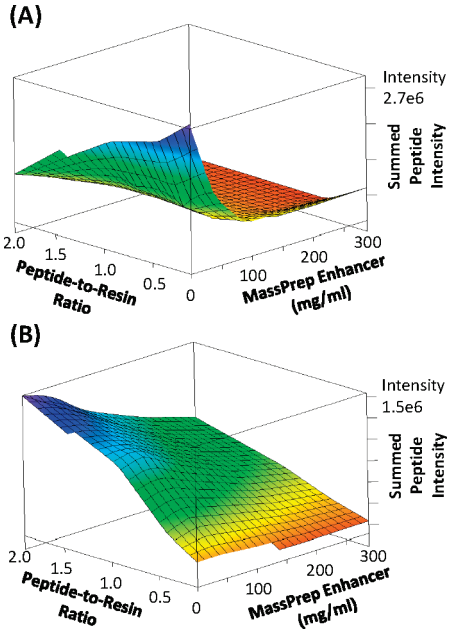


**Figure 2.** Three-dimensional surface plots of the summed areaunder-curve extracted ion chromatograms intensities for all (A) unique nonphosphorylated peptides ($n$ = 665) or (B) unique phosphorylated peptides ($n$ = 205) from WT zebrafish embryo lysates subjected to twenty unique $TiO_2$ enrichment conditions.

wild-type zebrafish lysate was applied to a 200 $\mu$g binding capacity $TiO_2$ spin column and an identical enrichment and elution sequence was employed (see Materials and Methods). Samples from each point in the matrix were subjected to the label-free quantitative workflow described above on a Synapt HDMS. To assess the composition of individual peptides or groups of peptides as a function of enrichment condition, the intensities of individual peptides or summed intensities of groups of peptides were plotted on a three-dimensional surface plot with peptide-to-resin capacity ratio and MassPrep Enhancer concentration axis. This strategy was preferred over total spectral counts as measuring the abundance of each peptide across all conditions allows for a more accurate description of the effectiveness of each enrichment condition on a per peptide basis.

The influence of varying peptide-to-resin capacity ratios and MassPrep Enhancer concentration on total phosphorylated and nonphosphorylated peptide intensities are illustrated in Figure 2. Consistent with observations that DHB decreases nonphosphorylated peptide binding, addition of 50 or 100 mg/mL of MassPrep Enhancer reduced the summed intensity of nonphosphorylated peptides by 68% or 82% at a peptide-to-resin ratio of 1:1, respectively, with a negligible reduction observed with higher MassPrep Enhancer concentrations (Figure 2A). Reductions in the abundance of nonphosphorylated peptides up to 76% were also observed by increasing the peptide-to-resin capacity ratio from 0.1:1 to 2:1 in the absence of MassPrep Enhancer, evidently due to the increased abundance of phosphorylated peptides in the mixture out competing nonphosphorylated peptides for $TiO_2$ binding sites.

Although the most effective reduction in the abundance of nonphosphorylated peptides was obtained by simultaneously increasing both MassPrep Enhancer and peptide-to-resin capacity ratios, these conditions did not provide the highest total yield of phosphorylated peptides (Figure 2B). While it was not surprising that increasing the peptide-to-resin capacity resulted in higher abundances of phosphorylated peptides, it was observed that the addition of 50 or 100 mg/mL of MassPrep Enhancer reduced the summed intensity of phosphorylated peptides by 7% or 25%, respectively, at a peptide-to-resin capacity ratio of 2:1. This is an important consideration when performing $TiO_2$-based phosphopeptide enrichments from sample matrices such as tissue which typically yield low amounts of total phosphorylated peptides per wet weight of tissue, as not analyzing a full column load (1 $\mu$g target amount per injection) will reduce the number of signals which are identified in the label-free experiment.

To assess enrichment specificity, the summed intensity of the phosphorylated peptides were calculated as a percentage of the total summed peptide intensity (Figure 3). The global specificity for all phosphopeptides varied from 6% at a peptide-to-resin capacity ratios of 0.1:1 and 0 mg/mL MassPrep Enhancer up to 80% at a peptide-to-resin capacity ratios of 2:1 and 300 mg/mL MassPrep Enhancer (Figure 3A). Generally, higher concentrations of MassPrep Enhancer increases the enrichment specificity, however the gain in specificity with more than 100 mg/mL MassPrep Enhancer was minimal for all peptide-to-resin ratios. Surprisingly, an increase in global phosphopeptide enrichment specificity by as much as 64% was observed by simply increasing the ratio of peptide-to-resin capacity from 0.1:1 to 2:1. MassPrep Enhancer seems to have modest effects on overall specificity at high and low Peptide-to-Resin ratios (10% and 14%, respectively) while having the greatest effect at intermediate loading ratios (40% improvement). These data suggest conditions of peptide-to-resin ratios of 2:1 with 50 mg/mL to 200 mg/mL MassPrep Enhancer provide optimal enrichment conditions to provide relatively high specificity and absolute phosphopeptide intensity (Supporting Information, Table S-1).

To investigate if these conditions resulted in a biased enrichment of subsets of phosphorylated peptides, the enrichment specificity of unique singly ($n$ = 164) and unique multiply phosphorylated ($n$ = 41) peptides were plotted (Figure 3 B−C). The enrichment specificity distributions for these subsets of phosphopeptides clearly indicated a differentiation in optimal enrichment conditions for singly- and multiply phosphorylated peptides, with higher MassPrep Enhancer concentrations and peptide-to-resin capacity ratios favoring the enrichment of multiply phosphorylated peptides. Even under conditions where MassPrep Enhancer concentrations had a small effect on overall specificity (2:1 peptide-to-resin), it has a large apparent effect on the relative composition of singly and multiply phosphorylated species. Example phosphorylated peptides from nuclear casein kinase and cyclin-dependent kinase substrate-1 identified in its singly- and doubly phosphorylated form (SSPKDEEEAE[pS]-PAEDEEEDEVEK and S[pS]PKDEEEAE[pS]PAEDEEEDEVEK) clearly demonstrates that the optimal enrichment condition for the doubly phosphorylated version was shifted toward higher amounts of MassPrep Enhancer (Supporting Information, Figure S-1). These data suggest that sequential $TiO_2$ enrichments at different enrichment conditions could be used to effectively select for distinct subpopulations of phosphorylated peptides from a single sample without requiring a change of $TiO_2$ column hardware. Such a strategy may represent a viable fractionation approach to increase phosphopeptide coverage. Although no single enrichment condition was found to be optimal for both singly- and multiply phosphorylated peptides, all proceeding

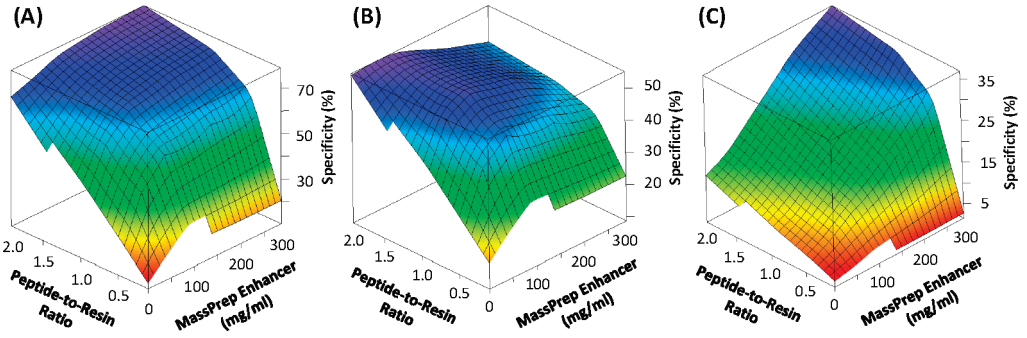


**Figure 3.** Phosphorylated peptide $TiO_2$ enrichment specificity across a twenty-point matrix of enrichment conditions. Specificity was determined by calculating the percentage of the summed area-under-curve extracted ion chromatogram intensities for (A) all unique phosphopeptides ($n$ = 205), (B) phosphopeptides containing 1 phosphate ($n$ = 164), or (C) phosphopeptides containing more than 1 phosphate ($n$ = 41) as a part of the summed area-under-curve extracted ion chromatogram intensities for all identified peptides ($n$ = 871).

experiments utilized peptide-to-resin capacity ratios of 2:1 and 100 mg/mL MassPrep Enhancer which we found provided excellent enrichment specificity for phosphopeptides (~75%) and robust quantitative reproducibility (Supporting Information, Table S-1).

*Quantitative Reproducibility and Accuracy of $TiO_2$ Enrichment.* Quantitative reproducibility of the $TiO_2$ enrichment was determined by subjecting three 350 $\mu$g aliquots of wild-type zebrafish embryo lysate to enrichments using different $TiO_2$ spin columns. Variation of phosphopeptide intensity values between replicate injections (analytical variation) and between individual $TiO_2$ enrichments (analytical + $TiO_2$ enrichment variation) was determined by generating coefficient of variation (% CV) distributions of the quantitative values for all identified phosphorylated peptides (Figure 4A). Approximately 80% of the peptides had an analytical variation below 20%, with median CVs of as 7.6%, 6.7%, and 6.6%. Variation across all three $TiO_2$ enrichments resulted in approximately 80% of the phosphorylated peptides having CVs less than a 33% with a median CV of 23.5%, indicating that the variation from the $TiO_2$ enrichment itself was approximately 16%.

To determine if specific subsets of peptides were correlated with higher variation between $TiO_2$ enrichments, CV distributions were recalculated using nonphosphorylated peptides ($n$ = 88), singly phosphorylated peptides ($n$ = 58), or multiply phosphorylated peptides ($n$ = 42) (Figure 4B). These distributions clearly indicate that enrichment variability is higher for nonphosphorylated peptides (median CV of 44%) as compared to singly- and multiply phosphorylated peptides (median CVs of 23.4% and 19.7%, respectively). Moreover, these reproducibility metrics also showed a very weak correlation as a function of phosphopeptide intensity, as the average CV of the phosphopeptides in the lowest quartile of intensity was 11.0%, and CVs for the second, third, and fourth quartiles were 16%, 9.4%, and 8.7% respectively. These results demonstrate that $TiO_2$ enrichment can be performed reproducibly and as such enables this portion of the protocol to be compatible with downstream label-free quantitation, however nonphosphorylated peptides which are nonspecifically enriched should generally not be considered in quantitative experiments.

The ability of the $TiO_2$ enrichment to accurately measure a change in phosphopeptide abundance was investigated by spiking 1000 $\mu$g of wild-type zebrafish lysate with 25 fmol or 75 fmol of predigested bovine α-casein peptide per $\mu$g of lysate (Table 1).

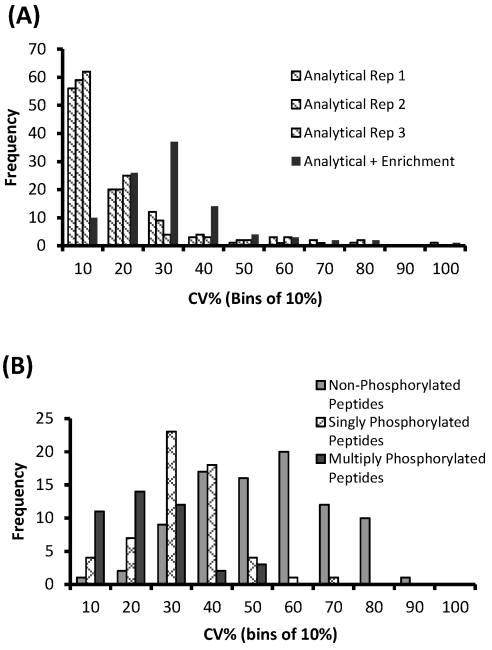


**Figure 4.** Analytical and TiO2 enrichment variation from three 350 ug WT zebrafish embryo lysates subjected to independent $TiO_2$ enrichments followed by triplicate LC-MS/MS analysis. (A) Coefficient of variation (CV%) distributions for all phosphorylated peptide extracted ion chromatogram intensities ($n$ = 99) for each set of analytical replicates (average median CV 7.0%) or across all three $TiO_2$ enrichments (median CV 23.5%). (B) Coefficient of variation distributions of nonphosphorylated ($n$ = 88, median CV 44.0%), singly phosphorylated ($n$ = 57, median CV 23.4%), and multiply phosphorylated ($n$ = 42, median CV 19.7%) peptide intensities across all three $TiO_2$ enrichments.

Consistent with the results from the analytical + $TiO_2$ enrichment variation experiment, the average measured fold-change across all non-casein (background) phosphorylated peptides between the two conditions was 0.95 with an average RSD of 23.8%. Measured fold changes for three unique phosphorylated α-casein peptides (one singly phosphorylated peptides was identified in two different charge states) varied from 2.89 to 3.57, which an average fold-change of 3.11 and an RSD of 10.2%. The measured fold-change across all casein phosphopeptides therefore deviated <5% from the theoretical 3-fold measurement, which is well within the determined analytical + $TiO_2$ enrichment variability of this workflow.

**Table 1. Accuracy of Quantitative Enrichment Assessed by Spiking 1000 μg WT Zebrafish Embryo Lysates with 25 fmol Bovine α-Casein/μg Lysate or 75 fmol Bovine α-Casein/μg Lysate and Subjecting to $TiO_2$ Enrichment Followed by Triplicate LC-MS/MS Analysis[a]**

| peptide | intensity 25 fmol/ug | CV % | intensity 75 fmol/ug | CV % | fold change |
|---|---|---|---|---|---|
| $[VPQLEIVP\ NS^*AEER]^{2+}$ | 54824 | 1.9 | 158448 | 1.7 | 2.9 |
| $[YKVPQLEIV\ PNS^*AEER]^{2+}$ | 32659 | 16.5 | 116737 | 22.0 | 3.6 |
| $[YKVPQLEIV\ PNS^*AEER]^{3+}$ | 10032 | 8.6 | 30623 | 13.6 | 3.1 |
| $[DIGS^*ES^*T\ EDQAMEDIK]^{2+}$ | 3514 | 3.6 | 10297 | 5.5 | 2.9 |

[a] Average analytical coefficient of variations (CV%) were 9.2% and average measured fold increase across three unique phosphorylated peptides (* = site of modification) was 3.11 fold (R.S.D 10.2%).

**Quantitative Analysis of GRK5 Knock-down Zebrafish.** To demonstrate the utility of the workflow on a more biologically complex system, a differential analysis of wild-type zebrafish embryos and those injected with a morpholino (MO) targeted against the closest homologue of mammalian G-protein coupled receptor kinase 5 (GRK5) was conducted. GRK5 is one of the seven members of the family of G protein-coupled receptor kinases, which were originally identified as the kinases phosphorylating and thus desensitizing G protein-coupled receptors.[28] Across all LC-MS analyses, 719 unique phosphorylated peptides from 449 proteins were identified at a peptide false discovery rate of 1.1%. The specificity of phosphopeptide enrichment varied between 87.4% to 94.8%, with an average enrichment specificity of 90.2%. The median analytical variation of phosphopeptide intensity values across all analysis was 7.9% RSD and the median analytical + $TiO_2$ enrichment variation was 20.3% RSD for wild-type embryos and 21.9% RSD for GRK5 knock-down embryos (Supporting Information, Figure S-2). Seven unique casein phosphorylated peptides (three singly phosphorylated, two doubly phosphorylated, and two triply phosphorylated) spiked into each sample prior to enrichment yielded an average analytical variations of 5.1% RSD and an average analytical + TiO2 enrichment of 17.7% RSD variations across all samples (Supporting Information, Table S-2).

Of the identified phosphorylated peptides, 60% contained a single phosphate, 32% contained two phosphates, and 8% contained three or more phosphates, consistent with the distributions predicted from the enrichment condition matrix (Supporting Information, Figure S-3). Of the 719 unique phosphorylated peptides, 24 were down-regulated and 27 were up-regulated with a minimum absolute fold-change of 2.0 and a *p*-value of less than 0.05 as a function of GRK5 knock-down (Supporting Information, Table S-3). Manual kinase motif searches of phosphorylated residues from the up-regulated group of phosphopeptides revealed a preference for serine residues neighbored by acidic residues (17 of the 27 contained the motif [pS/T]D/E) and indicate acidophilic serine/threonine kinases such as casein kinases and GSK3 may become activated in response to GRK5 knock-down (Supporting Information, Figure S-3). It remains to be tested if these specific proteins are in fact direct targets of GRK5 or if the phosphorylation of these residues is due to activation of other acidophilic kinases from alternative signaling pathways.

## CONCLUSIONS

This work described a quantitatively robust label-free phosphoproteomic workflow capable of analyzing relatively small amounts (100s of micrograms) of sample directly from any biological matrix, including human tissue and biological fluids. Traditionally, label-free strategies have had limited utility in global quantitative phosphoproteomics because of the requirement to prepare and enrich multiple samples independently. We therefore focused on establishing a reproducible single-step $TiO_2$ based enrichment strategy aimed at reducing the binding of non-phosphorylated peptides while maximizing the total signal intensity of phosphorylated peptides. From this work we determined that optimal conditions of 100 mg/mL MassPrep Enhancer and 2:1 peptide-to-resin binding capacity could provide excellent enrichment specificity for phosphopeptides and robust quantitative reproducibility. Interestingly, our results indicated that even with the same $TiO_2$ column, significantly different enrichment specificities between 6% and 80% could be obtained simply by changing the initial loading buffers or total loading amounts. One potential advantage of this could be in selecting for specific subpopulations of phosphorylated peptides with a potential for increased depth of phosphoproteome coverage through replicate enrichments at different conditions. Although the number of qualitative identifications in the GRK5 knock-down study is lower than many global phosphoproteomics reports, the samples were not subjected to additional orthogonal fractionations reducing the total analysis time to two hours per replicate. This is important factor when considering extrapolating the approach to larger biological cohorts and multifaceted experimental designs in which many samples would need to be analyzed in a realistic timeline.

## ASSOCIATED CONTENT

**Supporting Information.** Additional material as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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