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Sequential enrichment using metal oxide affinity chromatography (SMOAC) to enhance phosphoproteome coverage for quantitative proteomic analysis

Authors
Jae Choi, Ryan Bomgarden, Bhavin Patel, Leigh Foster, Sergei Snovida, John C. Rogers

Thermo Fisher Scientific, Rockford, IL, USA

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Introduction

Phosphorylation is a critical protein post-translational modification that can modulate protein activity, abundance, interaction, and localization. Recent advances in mass spectrometry (MS) instrumentation have led to the development of powerful methods for proteome-wide phosphorylation analysis in complex biological samples. However, due to the relative low abundance of phosphorylation, phosphopeptide affinity chromatography is required to selectively enrich phosphopeptides from unmodified peptides.

Metal oxide affinity chromatography (MOAC) and immobilized metal ion affinity chromatography (IMAC) are two of the most common phosphopeptide enrichment methods. As each method isolates different phosphopeptide sequences, it has been proposed that there is currently no single method that can enrich all of the peptides in the phosphoproteome.[1,2] Therefore, researchers have explored the use of multiple enrichment strategies to ensure a more complete enrichment of the phosphoproteome. One approach is the use of sequential enrichment with immobilized metal affinity chromatography, or SIMAC. In the SIMAC method, the sample is first enriched using

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Fe-NTA IMAC, followed by TiO_2 resin MOAC (Figure 1A).[3] The main benefit of this approach is that two distinct phosphopeptide fractions are derived from the same sample, which reduces the amount of initial sample required compared to parallel enrichment methods.

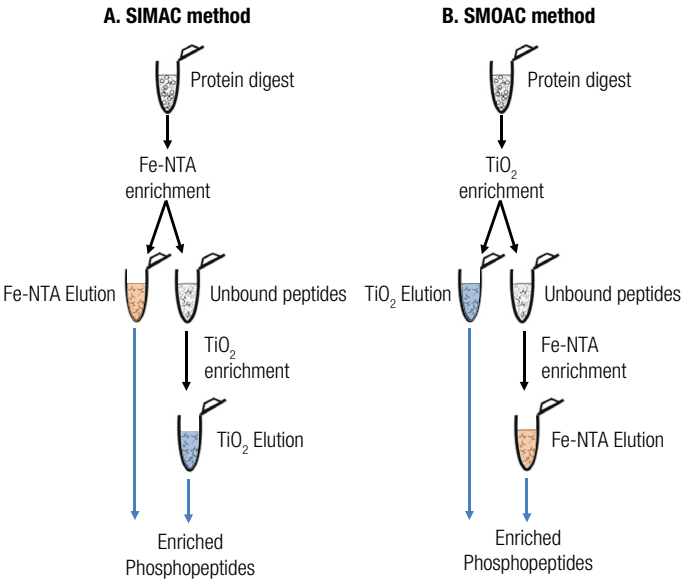

Figure 1. A) Sequential enrichment using immobilized metal affinity chromatography (SIMAC) for phosphopeptide enrichment and B) sequential enrichment using metal oxide affinity chromatography (SMOAC) for phosphopeptide enrichment

Recently, we optimized the protocol and buffer formulations in the Thermo Scientific™ High-Select™ Fe-NTA and TiO_2 Phosphopeptide Enrichment Kits to greatly improve phosphopeptide yield and specificity from complex protein digests. When used in parallel, each kit provides highly efficient phosphopeptide enrichment with less than 45% overlap in unique phosphopeptide sequences. To determine an optimal sequential enrichment method using these kits, we evaluated both the SIMAC method and a new double enrichment strategy called sequential metal oxide affinity chromatography, or SMOAC (Figure 1B), in which phosphopeptides are first enriched by TiO_2 resin followed by enrichment with Fe-NTA resin. In addition, we evaluated the SMOAC method in combination with high pH reversed-phase fractionation for enhanced phosphoproteomic coverage and enrichment of Tandem Mass Tag™ (TMT™)-labeled phosphopeptides for quantification of phosphorylation network dynamics in HeLa cells under various stimulation conditions.

Experimental

Cell culture and treatment

HeLa S3 cells (ATCC™ CCL-2.2) were cultured in suspension using Gibco™ S-MEM/glutamate/10% FBS media, and adherent HeLa (ATCC™ CCL-2) were cultured using Gibco™ DMEM with high glucose/10% fetal bovine serum/1X penicillin-streptomycin.

For general phosphopeptide enrichment workflow optimization studies, HeLa suspension cells were treated with 2.5 µg/mL nocodazole for 16 h to achieve homogeneous mitotic arrest. For differential phosphoproteome mapping, adherent HeLa cells were serum starved for 1 h or 24 h in DMEM/0.1% charcoal-stripped FBS before treatment for 15 min with 100 ng/mL hIGF-1, 50 ng/mL hEGF, 50 ng/mL hPDGF, 10% FBS, or 400 nM TPA for 15 min. Additional conditions for cells without starvation included no treatment and 100 ng/mL nocodazole for 24 h.

Sample preparation

Approximately 1.7 x 10^8 nocodazole-arrested HeLa cells were harvested and washed with PBS. Cells were lysed in 4 mL of 100 mM TEAB/8 M Urea (P/N 90114 and 29700) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (P/N 78442) and sonicated using a microtip probe with 10 s pulses at 6 watts for 3 min total. Protein concentration was measured using the Thermo Scientific™ Pierce™ 660 nm Protein Assay Kit (P/N 22662). The lysate was reduced with 10 mM DTT (P/N 20291) for 30 min at 60 °C, alkylated for 30 min with 30 mM iodoacetamide (P/N 90034) at room temperature, and quenched with 30 mM DTT at room temperature for 15 min. The lysate was diluted two-fold with MS-grade water (P/N 51140) before digestion with Thermo Scientific™ Pierce™ Lys-C Protease (P/N 90051) (1:50 w/w) at 37 °C for 2 h with gentle shaking. After 2 h, the Lys-C digest was diluted five-fold with 100 mM TEAB and digested using Thermo Scientific™ Pierce™ Trypsin Protease (1:50 w/w) overnight (P/N 90057). Protein digests were acidified with TFA and desalted using solid phase extraction clean-up either by Sep-Pak™ tC18 vacuum columns (P/N WAT043425) or Thermo Scientific™ Pierce™ Peptide Desalting Spin Columns (P/N 89852) according to manufacturer instructions. Final peptide concentration was measured by Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay (P/N 23275).

For TMT reagent-based protein and phosphopeptide quantitation, equivalent amounts of HeLa cells (~2 × 10^7) from 11 different treatment conditions were processed using Thermo Scientific™ Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (P/N 84840) and Halt phosphatase inhibitor during lysis. The digested samples were measured using the Thermo Scientific™ Pierce™ Fluorometric Peptide Assay (P/N 23290) before labeling with Thermo Scientific™ TMT10plex™ (P/N 90406) reagents and Thermo Scientific™ TMT11-131C according to manufacturer's instructions. TMT11-131C was used to label an equivalent portion of each sample to create a pooled sample for TMT reporter quantitation ratio normalization. Aliquots of each labeled sample were mixed in equal mass ratios before sample desalting (as described above), phosphopeptide enrichment, and fractionation.

Phosphopeptide enrichment and fractionation

For standard phosphopeptide enrichment, 1 mg of HeLa tryptic digest was subjected to the Thermo Scientific™ High-Select™ TiO_2 Phosphopeptide Enrichment kit (P/N A32993) or Thermo Scientific™ High-Select™ Fe-NTA Phosphopeptide Enrichment kit (P/N A32992) according to manufacturer's protocol.

For the SMOAC method, 1 mg of HeLa tryptic digest was subjected to the High-Select TiO_2 Phosphopeptide Enrichment kit according to manufacturer's protocol. The TiO_2 eluent was saved for either MS analysis or fractionation as described below. The TiO_2 flow-through (FT) and wash fractions were pooled, and the phosphopeptides were enriched using the High-Select Fe-NTA Phosphopeptide Enrichment kit according to manufacturer's protocol. Both TiO_2 and Fe-NTA eluents were fractionated using the Thermo Scientific™ Pierce™ High pH Reversed-Phase Fractionation kit (P/N 84868) according to manufacturer instructions. All eluents were quantitated by the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide assay (P/N 23275) and analyzed by the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer.

LC-MS and data analysis

For the LC-MS analysis, 1 µg of sample was injected onto a 50 cm Thermo Scientific™ EASY-Spray™ C18 LC column (2 µm particle size) to separate peptides with a 5–25% acetonitrile gradient over 180 min at a flow rate of 300 nL/min. Spectra were acquired on a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ Mass Spectrometer at top speed using the following parameters: FTMS full scan at resolution of 120,000 @ *m/z* 200, AGC 4e5, maximum injection time of 50 ms followed by FT MS2 scans at 0.7 isolation, 50,000 resolution, HCD 35% collision energy, AGC 1e5, maximum injection time of 120 ms. For data analysis, the Thermo Scientific™ Proteome Discoverer™ 2.2 software with SEQUEST®HT search engine was used. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 0.02 Da was utilized. Carbamidomethylation (+57.021 Da) for cysteine and TMTsixplex (+229.162 Da) for lysine and *N*-terminus residues were used as fixed modifications with methionine oxidation (+15.996 Da) and phosphorylation (+79.966 Da, T, Y, S) used as variable modifications. Data was searched against a Swiss-Prot™ human database with a 1% FDR using Percolator with phosphoRS for site localization. A custom TMT11plex quantification method was used to calculate the reporter ratios and apply isotopic correction factors.

Results and discussion

SIMAC and SMOAC for sequential phosphopeptide enrichment

Fe-NTA IMAC and TiO_2 MOAC resins are the two most common affinity resins used for phosphopeptide enrichment. Although each affinity method is capable of enriching phosphopeptides with high efficiency and specificity, there are differences in the phosphopeptide sequences enriched using each method in parallel. To determine the optimal workflow for sequential enrichment, we evaluated both SIMAC and SMOAC for phosphopeptide enrichment using 1 mg of nocodazole-treated HeLa tryptic digest. Elution fractions from either Fe-NTA or TiO_2 enrichment alone resulted in ~14,000 unique phosphopeptides for each method with high enrichment specificity of 97% and 93%, respectively (Figure 2A). Sequential enrichment of the Fe-NTA resin flow through fraction using TiO_2 resin using the SIMAC method only resulted in ~2,900 additional phosphopeptide identifications. In contrast, sequential enrichment of the TiO_2 resin flow through using Fe-NTA resin and the SMOAC method resulted in ~13,300 additional phosphopeptide identifications.

These results demonstrate that the SMOAC method enriches significantly more phophosphopeptides than the SIMAC method using the High-Select Fe-NTA and TiO_2 Phosphopeptide enrichment kits. Surprisingly, the SIMAC method actually enriched 20% fewer phosphopeptides than Fe-NTA and TiO_2 resins in parallel, whereas the SMOAC method identified 21% more unique phosphopeptides (Figure 2B & 2C). Although more starting material was used for parallel enrichment (i.e. 1 mg per column for 2 mg total), the increased identifications from the SMOAC method suggest that more starting material does not result in more phosphopeptide identifications for a single LC-MS analysis. A more likely explanation is that the 2-fold higher binding capacity and slightly higher specificity of our Fe-NTA columns resulted in fewer phosphopeptides present in the flow through for sequential TiO_2 enrichment. Therefore, TiO_2 enrichment before Fe-NTA using the SMOAC method is the preferred order for sequential enrichment using the High-Select kits.

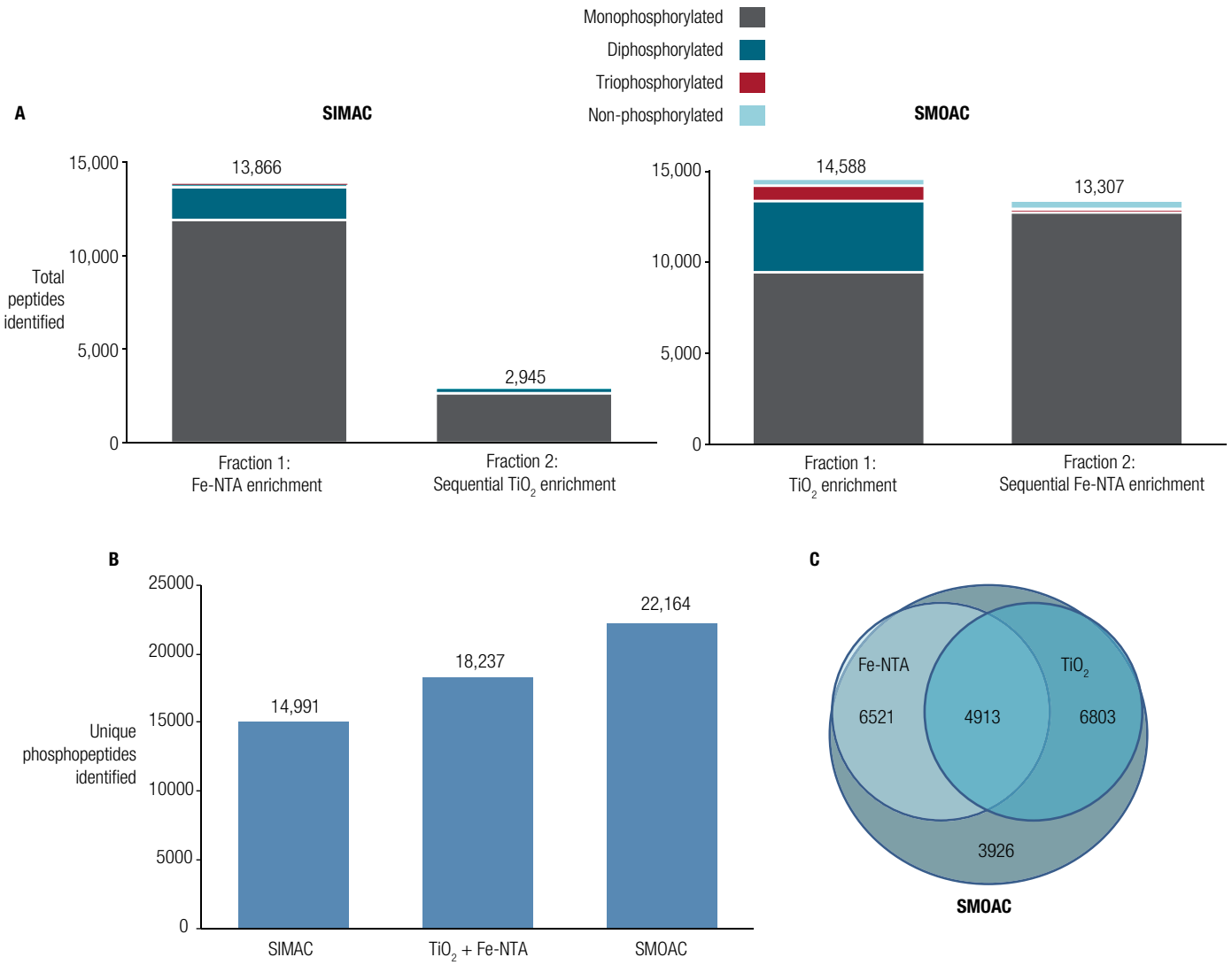

Figure 2. Evaluation of SIMAC and SMOAC for phosphopeptide enrichment. A) Total number of peptides identified following sequential enrichment using High-Select Fe-NTA and High-Select TiO_2 resins (ie. SIMAC or SMOAC). B) Number of unique phosphopeptides identified following enrichment using each kit separately in parallel (Fe-NTA + TiO_2) or sequentially (SIMAC or SMOAC). The graph represents combined unique phosphopeptides from both elution fractions. C) The Venn diagram showing overlap in unique phosphopeptide identifications.

High pH fractionation of phosphopeptides for increased phosphoproteome coverage

For deep phosphoproteome analysis, it is often necessary to further reduce the sample complexity, either before or after phosphopeptide enrichment. Two orthogonal peptide fractionation methods commonly used to reduce peptide sample complexity are strong cation exchange (SCX) and high pH reversed-phase chromatography.[4] Of these, high pH reversed-phase fractionation is growing in popularity as it is orthogonal to low pH analytical LC-MS separations and does not require an additional desalting step before LC-MS analysis. We evaluated this latter approach using a step-wise gradient with acetonitrile to fractionate enriched phosphopeptides in a high pH reversed-phase spin column. Before loading onto the high pH reversed-phase columns, both elution fractions from the SMOAC enrichment method were combined, dried, and reconstituted in 0.1% TFA. LC-MS analysis of the nine different fractions resulted in 32,584 unique phosphopeptides, which is >10,000 more phosphopeptides than using the SMOAC method without fractionation.

The depth of the phosphoproteome analysis achieved using the SMOAC method in conjunction with high pH reversed-phase fractionation enabled identification of both novel and previously reported phosphorylation sites for the CDC25 class of proteins. CDC25 proteins are serine/threonine and tyrosine dual-specificity phosphatases that remove inhibitory phosphate residues from targeted cyclin-dependent kinases during the cell cycle. Multiple kinases phosphorylate CDC25 at Thr-48, Thr-67, Ser-122, Thr-130, Ser-168, and Ser-214 for its activation.[5,6] TiO_2 enrichment identified a novel site at Ser-15 on CDC25C, and further enrichment using the SMOAC method identified Thr-48, Ser-168, and Ser-263 (Table 1). The SMOAC method plus fractionation revealed numerous additional phosphorylation sites for CDC25 family members, demonstrating the power of fractionation for increased phosphoproteomic coverage.

Multiplex quantification of the phosphoproteome

Although sequencing phosphopeptides and determining phosphorylation site localization is necessary for characterizing the phosphoproteome, measuring changes in peptide phosphorylation status is essential for understanding the function of various phosphorylation modifications in a protein subjected to various conditions or over time. TMT reagents have become the method of choice for multiplexed, relative quantification of protein samples.[7] Some advantages of TMT-based, multiplexed relative quantification include higher multiplexing capability (up to 11), reduced overall LC-MS analysis time, fewer missing quantitative values, and higher reproducibility among samples.

TMT workflows have also become a powerful platform for large scale, simultaneous phosphoproteome site identification and quantitation.[8-10] To determine how our phosphopeptide enrichment methods enhanced TMT phosphopeptide quantification, we prepared

Table 1. Phosphorylation sites identified in the CDC25 protein family from nocodazole-treated HeLa cells following phosphopeptide enrichment. Phosphopeptides were enriched with the High-Select TiO_2 Phosphopeptide Enrichment kit followed by sequential enrichment of the flow through and wash fractions with High-Select Fe-NTA Phosphopeptide Enrichment kit (SMOAC method). SMOAC-enriched peptides were then subjected to fractionation with the High pH Reverse-Phase Fractionation Kit.

	TiO_2 enrichment	SMOAC method	SMOAC plus fractionation
CDC25A		**Ser-18** (LLFAcsPPPASQPVVK) **Ser-88** (MGSSESTDSGFcLDsPGPLDSK) **Ser-261** (LFDsPSLcSSSTR)	**Ser-88** (MGSSESTDSGFcLDsPGPLDSK) **Ser-116** (LLGcsPALK) **Ser-126** (SHSDsLDHDIFQLIDPDENK) **Ser-261** (LFDsPSLcSSSTR) **Ser-321** (AHETLHQSLSLAsSPK)
CDC25B	**Ser-375** (sLcHDEIENLLDSDHR)	**Ser-375** (sLcHDEIENLLDSDHR)	**Ser-160** (LLGHsPVLR) **Ser-249** (MEVEELsPLALGR) **Ser-353** (sVTPPEEQQEAEEPK) **Ser-375** (sLcHDEIENLLDSDHR)
CDC25C	Ser-15 (EEGSsGSGPSFR)	**Thr-48** (DTSFTVcPDVPRtPVGK) **Ser-168** (YLGsPITTVPK) **Ser-263** (TVsLcDITITQMLEEDSNQGHLIGDFSK)	**Ser-15** (EEGSsGSGPSFR) **Thr-48** (DTSFtVcPDVPRtPVGK) **Thr-67** (FLGDSANLSILsGGTPK) **Ser-168** (YLGsPITTVPK) **Ser-214** (SGLYRsPSMPENLNRPR) **Ser-263** (TVsLcDITITQmLEEDSNQGHLIGDFSK)

protein digests from HeLa cells grown in 10 different experimental conditions. Peptides were labeled with the TMT reagents prior to TiO_2 enrichment, SMOAC phosphopeptide enrichment, or SMOAC plus high pH reversed-phase fractionation (Figure 3). Our optimized SMOAC plus high pH reversed-phase fractionation workflow resulted in the identification of over 33,000 phosphopeptides, including ~24,000 quantified phosphopeptides with highly confident site localization (confidence score >90) (Table 2). Furthermore, this comprehensive phosphoproteomics analysis enabled quantitation of phosphorylation changes for multiple signaling pathway proteins under the different experimental treatment conditions.

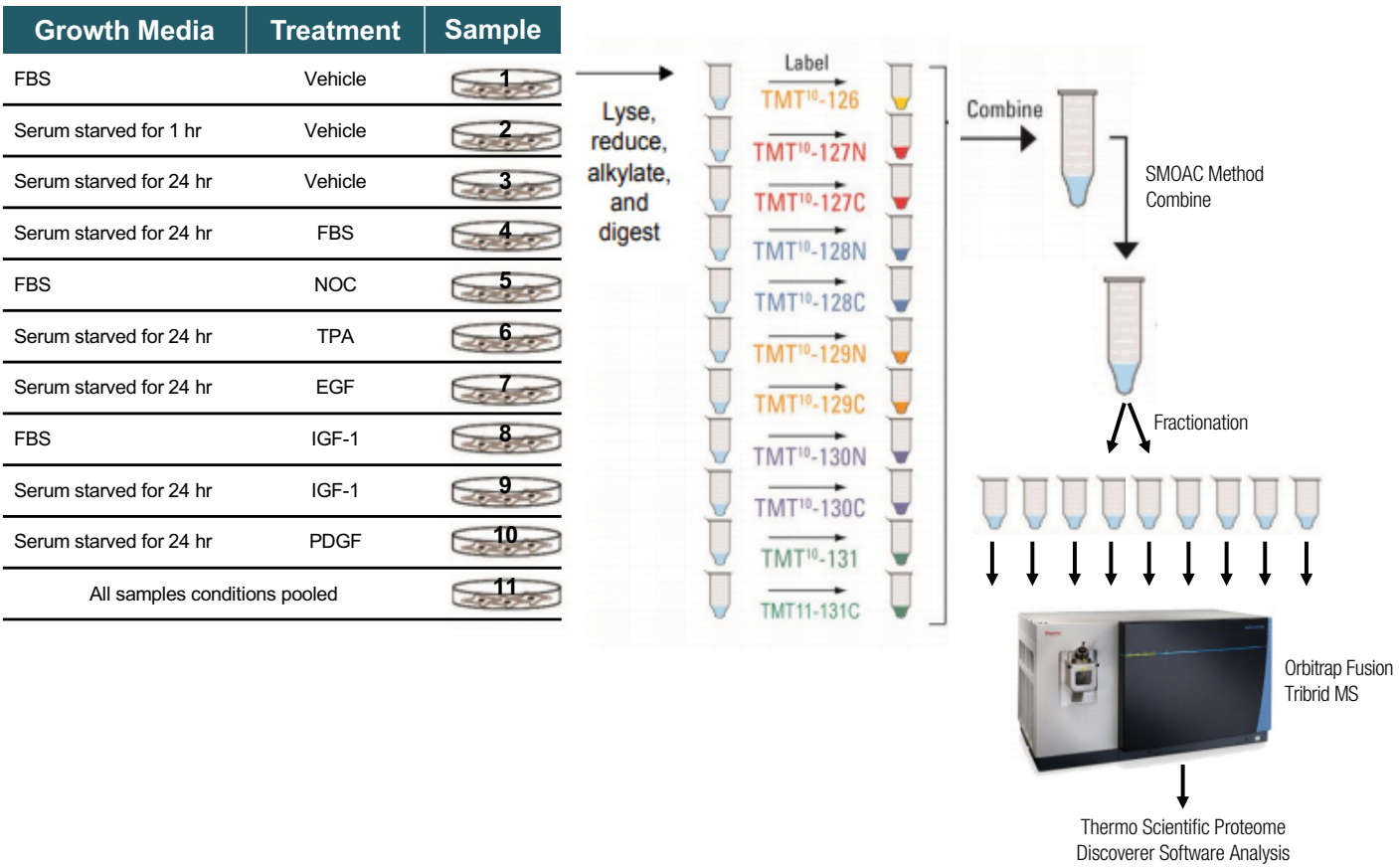

Figure 3. Schematic of TMT reagent sample preparation, phosphopeptide enrichment, and fractionation followed by LC-MS analysis

Table 2. Summary of protein groups, unique peptides, unique phosphopeptides identified, and quantified phosphopeptides from 11plex TMT-labeled samples

	TiO_2 enrichment	SMOAC	SMOAC plus fractionation
Protein Groups	3,831	5,288	5,763
Unique Peptides	21,596	32,936	42,431
Unique Phosphopeptides	14,572	28,211	33,135
Quantified phosphopeptides	11,100	19,137	23,983

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Conclusion

To enable deep phosphoproteome sequencing, a novel approach called SMOAC was developed, in which phosphopeptides are sequentially enriched using Thermo Scientific High-Select TiO_2 and Fe-NTA Phosphopeptide Enrichment Kits. Utilization of both kits in conjunction provided increased phosphopeptide identification in comparison to each enrichment alone. Deeper phosphoproteome profiling was further enabled by fractionation using the Pierce High pH Reversed-Phase Fractionation Kit. This kit reduced the overall complexity of the samples and provided greater coverage of the phosphoproteome for both unlabeled and TMT-labeled peptides. Incorporating the TMT workflow enabled quantification of nearly 24,000 phosphopeptides in eleven different samples, providing a powerful tool for large-scale phosphoproteomic analysis.

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