**In Situ Sample Processing Approach (iSPA) for Comprehensive Quantitative Phosphoproteome Analysis**

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**ABSTRACT:** Current sample preparation protocols for quantitative phosphoproteome analysis are tedious and time-consuming. Here, a facile in situ sample processing approach (iSPA) is developed by using macroporous Ti(IV)-IMAC microspheres as the preparation “beds”, where all sample preparation procedures including the enrichment of phosphoproteins, tryptic digestion of proteins, enrichment, and isotope labeling of phosphopeptides are performed in situ sequentially. As a result of the in situ processing design and the seamless procedures, extra steps for desalting and buffer exchanging, which are always required in conventional approaches, are avoided, and the sample loss and contamination could be greatly reduced. Thus, better sensitivity and accuracy for the quantitative phosphoproteome analysis were obtained. This strategy was further applied to differential phosphoproteome analysis of human liver tissues with or without hepatocellular carcinoma (HCC). In total, 8548 phosphorylation sites were confidently quantified from three replicate analyses of 0.5 mg of human liver protein extracts.

**KEYWORDS:** in situ sample processing approach, phosphoprotein enrichment, on-beads digestion, dimethyl labeling, solid phase labeling, phosphoproteome quantification, human liver, hepatocellular carcinoma

**INTRODUCTION**

Protein phosphorylation, as one of the most important post-translational modifications (PTMs), plays important roles in regulating biological processes such as signal transduction and cell division, growth, differentiation and apoptosis.¹ About 30% of cellular proteins can be phosphorylated during the cell cycle, and abnormal protein phosphorylation events are always accompanied by many diseases, such as cancers, diabetes, chronic inflammation, and neurodegeneration.² Comprehensive identification and quantification of protein phosphorylation is helpful for understanding biological processes and for aiding disease diagnosis.³⁻⁵ Compared with non-phosphoproteins, the abundance of phosphoproteins is much lower, and for bottom-up phosphoproteomics the detection of phosphopeptides is much less sensitive than for non-phosphopeptides due to the low ionization efficiency in MS. Therefore, direct identification of phosphopeptides in a proteome digest by LC−MS/MS analysis is almost impossible. Thus, selective enrichment of phosphoproteins or phosphopeptides before MS analysis is vital for the phosphorylation analysis.⁶

Recently, there are many methods that have been developed for the phosphopeptides enrichment, such as strong cation/anion exchange chromatography (SCX/SAX), immobilized metal ion and metal oxide affinity chromatography (IMAC and MOAC), etc.⁷⁻¹⁰ Thousands of phosphopeptides could be routinely identified in one LC−MS/MS analysis using these methods. However, the sample preparation procedures of these enrichment methods are often performed in solution, and for the quantitative phosphoproteome analysis, an additional sample preparation step, i.e., stable isotope labeling, is required. Because of the incompatibility of buffers used in these different procedures including digestion, labeling, and phosphopeptide enrichment, buffer exchanges are always required. The multiple steps of desalting and solution transfer processes may lead to serious sample loss and contamination, which will compromise the performance for phosphoproteomics analysis. Additionally, because of the tedious sample preparation processes, the conventional approach is very time-consuming and typically costs over 2 days for the sample preparation.⁶ Moreover, because of the complexities of mammalian tissue proteomes and their low phosphorylation level, the proteome-wide identification and quantification of protein phosphorylation from mammalian tissues is still a challenge.³,¹¹

Thus, new strategies with optimized sample preparation procedures and reduced preparation time to improve the...
sensitivity, coverage, and accuracy of the phosphoproteome quantitative analysis, especially for the analysis of mammalian tissue samples, are urgently need. Recently, a method using sequential enrichment of phosphoproteins and phosphopeptides for phosphoproteome analysis has been reported, in which high enrichment efficiency was achieved. However, the enriched phosphoproteins were eluted from the beads followed by multiple steps of sample preparation including solvent exchange, digestion, desalting, lyophilization, and phosphopeptide enrichment. Similar to the conventional method, it is also tedious, has low sensitivity, and has not been used in quantitative phosphoproteome analysis.

Recently, our group developed macroporous Ti$^{4+}$-IMAC microspheres for highly specific phosphopeptides enrichment, which exhibited better performance than common methods such as Fe$^{3+}$-IMAC, TiO$_2$, or ZrO$_2$. Because of its macroporous characteristics (average pore diameter of ca. 110 nm and porosity of 58.34%) and the strong interaction between Ti$^{4+}$ and phosphate groups, we found that it is also able to selectively enrich intact phosphorylated proteins from complex biological samples. Herein, an in situ sample processing approach (iSPA) was developed for large-scale quantitative phosphoproteome analysis by using these macroporous Ti$^{4+}$-IMAC microspheres as the preparation "beds", where all sample preparation procedures including the enrichment of phosphoproteins, tryptic digestion of proteins, enrichment, and isotope labeling of phosphopeptides were performed sequentially on the beads (Scheme 1).

As a result of these seamless procedures, extra steps for desalting and buffer exchange used in conventional quantitative phosphoproteome analysis could be avoided, and the total sample preparation time could be reduced from over 2 days to 5 h. Moreover, the sample loss and unexpected contamination were greatly reduced, and accuracy for the quantitative analysis of phosphoproteome was improved. Applying the merits above, this iSPA strategy was further used for quantitative phosphoproteome analysis of human liver tissues with and without hepatocellular carcinoma (HCC). Finally, 8548 phosphorylation sites were reliably quantified from three replicate analysis of 0.5 mg of human liver protein extracts.

### EXPERIMENTAL SECTION

#### Chemicals and Materials

Formic acid (FA) and sodium cyanoborohydride (NaBH$_3$CN) were provided by Fluka (Buchs, Germany). Acetonitrile (ACN, HPLC grade) was purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were purchased from Sigma (St. Louis, MO). Fused silica capillaries with 75 μm i.d. and 200 μm i.d. were obtained from Polymicro Technologies (Phoenix, AZ). All water used in these experiments was prepared using a Mill-Q system (Millipore, Bedford, MA). Ti$^{4+}$-IMAC microspheres were prepared in our lab with the method described by Yu et al.21

#### Protein Sample Preparation

Standard protein mixtures were prepared by mixing α-casein, β-casein, and BSA in the ratio of 1:1:10. Adult female C57 mice were purchased from Dalian Medical University (Dalian, China). The hepatocellular carcinoma (HCC) and normal human liver tissues were provided by Second Affiliated Hospital of Dalian Medical University.
of Dalian Medical University (Dalian, China), and the utilization of human tissues complied with guideline of Ethics Committee of the hospital. Informed consent was obtained from patients enrolled in this study. The normal human liver tissues were non-tumorous liver tissues ≥2 cm outside the hepatic hemangiomas removed by surgical operation. The liver tissues have been checked by histopathological examination to exclude the presence of invading or microscopic metastatic tumor cells. The HCC tissues were obtained from advanced stage HCC patients and were removed by the surgical operation. As described in our previous work, the liver tissues were lysed in a homogenization buffer, consisting of 8 M urea, 1% v/v Triton X-100, 65 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 10 μL of protease inhibitor cocktail for 1 mL of homogenized buffer, phosphatase inhibitors (1 mM NaF, 1 mM Na2VO4, 1 mM C6H5NaO2P, 10 mM Na2O2P2O7), and 40 mM Tris-HCl at pH 7.4. The protein concentration was determined by Bradford assay. In our study, the same amount of normal samples were precipitated by chloroform/methanol mixed together, respectively. Then, the proteins in the HCC or normal samples were precipitated by chloroform/methanol precipitation. After washing with methanol, the pellets were resuspended in denaturing buffer containing 50 mM Tris/HCl (pH 7.4) and 8 M urea, and the protein concentration was determined again by Bradford assay.

**In Situ Sequential Phosphoprotein and Phosphopeptide Enrichment**

As a typical example of the iSPA strategy, 250 μg of mouse liver proteins in 250 μL of 8 M urea and 50 mM Tris-HCl (pH 7.4) was first reduced by 10 mM DTT at 60 °C for 30 min and alkylated by 20 mM iodoacetamide in the dark at room temperature for 30 min. Then 2.5 mg of Ti4+-IMAC microspheres was added (the optimal material/protein ratio is 10:1, enlarged ratios will not result in more the phosphopeptides bound on the beads). After digestion, the Ti4+-IMAC microspheres suspension was centrifuged at 20,000 g for 3 min, and supernatant was removed. A protein level washing was subsequently performed by using 300 μL of 8 M urea and 50 mM Tris-HCl (pH 7.4). Then the phosphoproteins absorbed on Ti4+-IMAC microspheres were resuspended in 300 μL of 100 mM ammonium bicarbonate buffer (pH 8.2) and digested by adding trypsin with 1:20 trypsin/protein ratio at 37 °C for 2 h. After digestion, the Ti4+-IMAC microspheres suspension was acidified and centrifuged to remove the supernatant, and a peptide level washing was performed by using 300 μL of 50% ACN, 6% TFA, and 200 mM NH4HCO3. The phosphopeptide bound on the beads were subjected to in situ isotope dimethyl labeling or directly eluted by 100 μL of NH4HCO3 (10%, v/v) for phosphopeptide identification.

**In Situ Isotope Dimethyl Labeling of Phosphopeptides**

After the protein and peptide level enrichment, Ti4+-IMAC beads with captured phosphopeptides were resuspended in 300 μL of 100 mM TEAB (triethylammonium bicarbonate, pH ∼6) and labeled with dimethyl labeling reagents. The labeling is based on the reaction of the amines with formaldehyde to generate a Schiff base, which is rapidly reduced by cyanoborohydride sodium (NaBH4CN) in the reaction buffer. The labeling procedure was just like the free solution labeling approach. Briefly, 40 μL of CH3O (4%, v/v) was added into the sample solution, and then 40 μL of freshly prepared NaBH4CN (0.6 M) was added. The resultant mixture was incubated for 1 h at room temperature, and the excess labeling reagents were consumed by adding 16 μL of NH3·H2O (10%, v/v) for 30 min. For quantitative analysis, two or three samples were labeled with different isotopic dimethyl labels (CH3O (4%, v/v) and NaBH4CN (0.6 M) for light label, CD3O (4%, v/v) and NaBH4CN (0.6 M) for intermediate label, and 13CD3O (4%, v/v) and NaBD4CN (0.6 M) for heavy label). After on-bead labeling, they were combined, acidified, and subsequently centrifuged to remove the supernatant. The labeled phosphopeptides on the beads were eluted by 100 μL of NH3·H2O (10%, v/v) and finally centrifuged at 20,000g for 3 min. The supernatant containing phosphopeptides was collected and lyophilized for later LC–MS analysis.

**Online RP-SCX-RP Multidimensional Separation and Mass Spectrometry Analysis**

The lyophilized phosphopeptides were resuspended in 0.1% FA. The automated sample injection and multidimensional separation using the RP-SCX-RP system was constructed as previously described, and the RP segment of the RP-SCX biphasic column was used as the sample loading column to reduce the sample loss. The redisolved phosphopeptide sample was loaded onto the biphasic column’s RP segment, and then a 120 min RP gradient LC–MS/MS (as described below) was applied. There are two purposes of this procedure: the phosphopeptides retained on the RP segment could be transferred to the SCX segment, and in the meantime, the phosphopeptides that did not retain on SCX segment could be loaded directly onto the RPLC column for MS analysis (termed as 0 mM fraction). Then a series stepwise elution with salt concentrations of 2.5, 60, 160, 300, 400, and 1000 mM NH4AC (pH 2.7) was used to elute phosphopeptides from SCX monolithic column to an in-house packed 75 μm i.d. and 15 cm length C18 separation column (3 μm, 120 Å, Daiso). Each salt step lasted 10 min followed by a 15 min equilibrium phase with 0.1% FA in water.

LC–MS/MS analysis was performed using a quaternary surveyor MS pump (Thermo, San Jose, CA) and LTQ-Orbitrap Velos mass spectrometer (Thermo, San Jose, CA). For the RPLC separation, 0.1% FA in water and in acetonitrile were used as mobile phases A and B, respectively, and the flow rate was adjusted to ∼300 nL/min after splitting. The 200 min gradient elution was performed with a gradient of 0−3% B in 5 min, 3−25% B in 145 min, 25−35% B in 10 min, 35−80% B in 3 min, 80% B in 7 min, 80−100% B in 3 min, and 100% B for 27 min. Other gradients for 1D LC–MS/MS analysis were similar to the 200 min gradient by increasing or decreasing the time of 3−25% B gradient as needed. The LTQ-Orbitrap Velos mass spectrometer (Thermo, San Jose, CA) was operated in data-dependent MS/MS acquisition mode. Full mass scan performed on the Orbitrap analyzer was acquired from m/z 400 to 2000 (R = 60,000 at m/ z 400). The 20 most intense ions from the full scan were selected for fragmentation via collision induced dissociation (CID) in the LTQ (relative collision energy for CID was set to 35%). The dynamic exclusion function was set as follows: repeat count 2, repeat duration 30 s, and an exclusion duration of 60 s.

**Protein Identification and Quantification**

All MS/MS spectra were searched using MaxQuant version 1.1.1.36 with the exception that the global comparative phosphoproteomic analysis of hepatocellular carcinoma (HCC) and normal human liver tissues were searched using...
the newest MaxQuant version 1.3.0.05 against a composite International Protein Index (IPI) database (IPI mouse 3.87 (59534 entries) and IPI human 3.87 (91464 entries) were used for mouse liver and human liver, respectively).21 Carbamidomethylation on cysteine was set as a fixed modification, whereas oxidation on methionine and phosphorylation on serine, threonine, and tyrosine as well as light and intermediate dimethylation on lysine and peptide amino termini were set as the variable modifications. Trypsin was set as the specific proteolytic enzyme with up to two missed cleavages allowed. The mass tolerance for the precursor ion was set to 15 ppm (new version 6 ppm) and 0.8 Da (new version 0.5 Da) for the fragment ion. Phosphopeptides with the false discovery rate (new version 6 ppm) and 0.8 Da (new version 0.5 Da) for the precursor ion was set to 15 ppm (new version 6 ppm) and 0.8 Da (new version 0.5 Da) for the fragment ion. Phosphopeptides with the false discovery rate \( \leq 0.01 \) were accepted for protein identification and quantification. Also, a minimum PTM score for phosphorylation site identification was set as 30, and for phosphorylation site quantification the minimum localization probability was at 0.5. All other parameters were as the default settings in MaxQuant.

Results and Discussion

Performance of Phosphoprotein Enrichment by Macroporous Ti\(^{4+}\)-IMAC Microspheres

Enrichment of phosphoproteins from complex biological sample is the first step for the iSPA strategy. There have been some phosphoproteins enrichment methods reported with good performance by using Al(OH)\(_3\), mesoporous TiO\(_2\), Fe\(^{3+}\)-IMAC and Zn\(^{2+}\)-bound phos-tag agarose, etc.22–25 In principle, our homemade macroporous Ti\(^{4+}\)-IMAC microspheres also has this enrichment capability. To evaluate its enrichment performance, a standard testing protein mixture containing \( \alpha \)-casein, \( \beta \)-casein, and BSA at a ratio of 1:1:10 (w/w) was subjected to Ti\(^{4+}\)-IMAC enrichment as described in the Experimental Section. The adsorbed proteins were directly eluted by 10% NH\(_4\)OH and analyzed by SDS-PAGE to determine the phosphoprotein enrichment efficiency. Based on the change of intensity for protein bands before and after Ti\(^{4+}\)-IMAC enrichment (Figure 1), it was found that 81.2 \( \pm \) 1.3% of phosphoproteins (\( \alpha \)-casein and \( \beta \)-casein) were captured on Ti\(^{4+}\)-IMAC, while only 12 \( \pm \) 0.4% of non-phosphoproteins (BSA) were captured. Clearly Ti\(^{4+}\)-IMAC microspheres could be used to enrich phosphoproteins.

We then investigated the phosphoprotein enrichment performance of Ti\(^{4+}\)-IMAC with more complex protein samples extracted from mouse liver. The amounts of proteins adsorbed on the beads and presented in the supernatant were determined by BCA assay. It was found that over 70% of the proteins were removed after the enrichment by using Ti\(^{4+}\)-IMAC beads (Figure 2A). The proteins in the supernatant and adsorbed on beads were digested by trypsin, respectively. Subsequently, phosphopeptides enriched from the above two digests were both analyzed by 1D LC−MS/MS. As shown in Figure 2B, over 800 unique phosphopeptides were identified from the enriched protein fraction, while less than 200 unique phosphopeptides were identified from the proteins in the supernatant fraction and a majority of (60%) these phosphopeptides were also included in the enriched protein fraction (Figure 2C). Even though the enriched proteins account for only 30% of total proteins, more than 90% of the phosphopeptides were identified from this fraction. Moreover, it was observed that 95% of phosphopeptide intensity in LC−MS of the enriched protein fraction was higher than that in the supernatant fraction (2-fold higher for 77% of phosphopeptides). Therefore, it can be concluded that most of the phosphoproteins were enriched onto the Ti\(^{4+}\)-IMAC beads, while a majority of the non-phosphoproteins were depleted. Thus, this method significantly reduces the complexity of the samples for phosphoproteome analysis.

Optimization of Digestion Time for iSPA Strategy

After phosphoproteins were captured onto the Ti\(^{4+}\)-IMAC beads, the next step was to digest the proteins absorbed on the beads. As the digestion was performed on the Ti\(^{4+}\)-IMAC beads, we are not sure how long the digestion should be. Thus, we first investigated the effect of digestion time on the phosphoproteome analysis in the iSPA strategy. For comparison, an experiment with the conventional solution-based method was performed in a similar way. As shown in Figure 3, the number of unique phosphopeptides identified increased substantially by extending the digestion time from 30 min to 1 h in the iSPA strategy, and there was also a small increase up to 4 h digestion. However, further extending digestion time led to a slight decrease in the number of the phosphopeptides identified beyond 4 h. This data indicated that longer digestion time was not necessary for the iSPA strategy. A similar change tendency for the number of identified phosphopeptides over digestion time was observed in the conventional solution-based method. This indicated that the potential steric hindrance for this in situ digestion has no significant effect on the digestion speed. If not otherwise stated, on-beads digestion of 2 h was performed for all of the following experiments.

Performance of Phosphopeptides Enrichment

A benefit from the one-step design and the seamless procedures, the iSPA strategy is expected to provide better phosphoproteome coverage versus that of conventional strategy. We compared the phosphoproteome analysis coverage for three methods: the iSPA strategy, a control method, and the solution-based conventional method. In the iSPA strategy, the phosphoproteins were always adsorbed on the Ti\(^{4+}\)-IMAC beads, whereas in the control method, the enriched phosphoproteins were first eluted from the Ti\(^{4+}\)-IMAC beads and then subjected to trypsin digestion in solution followed by Ti\(^{4+}\)-IMAC enrichment of phosphopeptides, which was in

![](image-url)

Figure 1. SDS-PAGE of proteins enriched from a protein standard mixture of BSA (non-phosphoprotein), \( \alpha \)-casein, and \( \beta \)-casein (phosphoprotein) at the ratio of 10:1:1.
accordance with other methods for sequential enrichment of phosphoproteins and phosphopeptides. For all the three methods, the same amount of proteins from mouse liver was used as the sample. As shown in Figure 4A, compared with the conventional in-solution approach, no more phosphopeptides were identified by using the control method despite its higher enrichment specificity, while about 50% more phosphopeptides could be identified by using the iSPA strategy due to its low sample loss and high sensitivity. The above data clearly confirmed that the one-step design significantly improved the phosphoproteome analysis coverage. The iSPA strategy was also found to have high enrichment specificity of 84.7 ± 0.7%, which is slightly lower than the conventional method (88.8 ± 3.1%), while as the p-value obtained from the t test of the enrichment specificity of the above method is 0.11, they do not have significant difference. Much higher enrichment specificity (97.5 ± 1.5%) could be obtained in the control method, where the enriched phosphoproteins were eluted and transferred to the new beads and centrifuged tube. However, probably because severe sample loss occurred in the conventional and the control methods, phosphoproteome analysis coverage was much lower. We further compared the size of the phosphoproteins between the conventional method and iSPA strategy (Figure 4B), and no difference was observed. The above data demonstrated that, even start with enriching the phosphoprotein, the iSPA strategy has no bias on protein size and is able to provide comprehensive analysis of the phosphoproteome.

Further investigation was carried out to compare the sample loss between solution-based conventional method and iSPA strategy. One aliquot of mouse liver proteins was processed with conventional enrichment strategy, and the enriched phosphopeptides were labeled with light isotope reagents (Supplementary Method 1), while another aliquot of the same sample was processed with iSPA strategy and labeled with heavy isotope reagents. The two samples were pooled together for 1D LC−MS/MS analysis. As shown in Figure 4B, 80% of the quantified phosphopeptides exhibited higher abundance in iSPA strategy and 40% of them with ratios higher than 2. Clearly iSPA strategy has less sample loss than the conventional strategy, and thus better phosphoproteome coverage could be obtained.
Performance of Quantitative Phosphoproteome Analysis by iSPA Strategy

After on-beads trypsin digestion, the phosphopeptides were still stuck on the Ti⁴⁺-IMAC beads. As the solution for dimethyl labeling is compatible with Ti⁴⁺-IMAC phosphopeptide enrichment, the in situ stable isotope labeling can also be performed on the beads (Supplementary Method 2, Table S1). After LC–MS/MS analysis of the in situ labeled phosphopeptides from 50 μg of mouse liver proteins, it was found that 776 (99.7%) of the 778 identified phosphopeptides could be successfully labeled with dimethyl groups. Among them, 770 (99.0%) phosphopeptides were N-termini labeled and 561 (99.3%) of the total 565 lysine residues in the phosphopeptides were fully labeled, indicating that high labeling efficiency of phosphopeptides was achieved by this in situ labeling onto the Ti⁴⁺-IMAC beads.

To evaluate the quantification reproducibility of this iSPA strategy, two aliquots of proteins from mouse liver (250 μg) were processed by iSPA strategy in parallel as described above. After light and heavy dimethyl labeling on beads, the two samples were pooled and analyzed by LC–MS/MS. Then the data was searched by MaxQuant, and the result showed that more than 99% of the quantification ratios were within a 2-fold change, while over 90% of the quantified ratios were distributed between 0.8- and 1.2-fold ranges. For comparison, quantitative phosphoproteome analysis using conventional in-solution labeling methods was performed as previously reported. It was found that 99% of quantified ratios were in the range of <2-fold. However, the ratio distribution was wider than that of iSPA strategy, with less than 80% of the quantified ratios distributed in a 0.8-1.2-fold range, which indicated that high quantification reproducibility was achieved by the iSPA strategy (Supplementary Figure S1).

There are mainly two factors that contribute to the superior performance of the iSPA strategy: First, high abundance non-phosphoproteins could be removed, which would be in favor of the phosphopeptides enrichment from the complex mammalian tissue samples by using Ti⁴⁺-IMAC. Second, the iSPA strategy integrated all of the sample preparation procedures in one tube with Ti⁴⁺-IMAC beads, which could effectively avoid sample loss and contamination and increase the recovery of the phosphopeptides enrichment. Therefore, high reproducibility...
and accuracy could be achieved for quantitative phosphoproteome analysis by using the iSPA strategy.

**Quantitative Phosphoproteome Analysis of HCC and Normal Liver Tissues**

Hepatocellular carcinoma (HCC) is one of the major liver diseases with a high fatality rate, and over a million people die of this cancer every year. As protein phosphorylation plays important roles in the pathologic processes of HCC, quantification of site-specific phosphorylation change could provide valuable insight to elucidate the mechanism of HCC. Many studies on the phosphoproteome of HCC by using human liver tissues have been reported, yet the quantification coverage of the HCC phosphoproteome is still very limited due to the poor detection sensitivity of conventional methods.27,28

Herein, the iSPA strategy was utilized for large-scale quantitative phosphoproteome analysis of HCC and normal human liver tissues. In total, 6260 unique phosphorylation sites corresponding to 2260 phosphoproteins were identified from one 2D LC–MS/MS analysis of 0.5 mg of protein extracts of HCC and normal human liver tissues (Supplementary Table S4), and 5350 phosphorylation sites that exhibited localization probability over 0.5 were quantified from 0.5 mg of protein extracts of HCC and normal human liver tissues (Supplementary Table S5).

To assess the repeatability of iSPA strategy for quantitative phosphoproteome analysis, another two independent technical replicates including one reverse labeling experiment were further performed; 5883 and 5829 unique phosphorylation sites were identified, and among them 4811 and 4975 could be quantified, respectively (Supplementary Tables S2, S4, and S5). Additionally, 2955 phosphorylation sites were quantified in both replicate 1 and replicate 2. As shown in Figure 5A, the scatter plots for the log2 ratio demonstrated good reproducibility of the quantification (89.38% of phosphopeptides quantified with RSD < 30%; the average RSD was 13.81%, Figure 5B) and that it could fairly match the generally accepted SILAC methods for quantitative phosphoproteome analysis (84.57% and 82.33% of phosphopeptides were quantified with RSD < 30% in Monetti’s and Mayya’s studies,29,30 with average RSDs of 15.66% and 17.52%, respectively). Similar results could also be obtained by comparison between replicate 1 and replicate 3, where the phosphopeptides isotope labeling was reversed for HCC and normal human liver tissues (Supplementary Figure S2 and Tables S3 and S5). The above results indicated that better reproducibility could be obtained in quantitative phosphoproteome analysis by using the iSPA strategy.

As shown in Figure 6A and B, 10,086 phosphorylation sites were identified in the three technical replicate (7943 sites with localization probability >0.75, 2523 sites with localization probability between 0.75 and 0.25), of which 8548 phosphorylation sites were quantified, by using the iSPA strategy (Supplementary Table S4 and S5). These data indicated that the phosphoproteome was characterized in unprecedented depth from a limited amount of protein. The obtained data set provided a comprehensive picture of phosphoproteome changes in HCC. In these three replicate analyses, 4260 phosphorylation sites were quantified at least twice. If the average quantification ratio out of a 2-fold change with RSD <50% was considered as the threshold of phosphorylation regulation in HCC oncogenesis, then 671 phosphorylation sites were found to be up-regulated, while 539 phosphorylation sites were found to be down-regulated (Supplementary Table S5). It should be mentioned that the significant change of the ratios for phosphosites may because the abundance of the proteins was significantly different between the two samples. To find the significant down/up-regulated phosphosites, the ratios deter-
mined above must be normalized with protein ratios determined by non-phosphopeptides. The huge number of phosphosites quantified indicated the excellent performance of this new method for quantitative phosphoproteome analysis.

**CONCLUSIONS**

In this study, an iSPA strategy was developed for quantitative analysis of a phosphoproteome by using Ti⁴⁺-IMAC beads as the adsorbents. This strategy integrated all of the sample preparation procedures, including phosphoprotein enrichment, tryptic digestion, phosphopeptide enrichment, and isotope labeling, into one tube. The desalting and solvent transfer procedures in conventional strategies are eliminated, which further led to better sample recovery, better quantification accuracy, and less time consumption. Furthermore, this strategy was successfully applied to the comprehensive quantitative analysis of phosphorylation in human liver tissue, and a total number of 8548 unique phosphorylation sites were confidently quantified from a total of 1.5 mg of human liver protein extracts, which is the largest quantitative data set of phosphoproteome from human liver to date. This method is readily applicable to analysis of other human tissues or other samples.

**ASSOCIATED CONTENT**

Supporting Information

Supplementary figures, tables, and methods as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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