# Supplemental data

## Comparison of IMAC materials

A summary of the three methods is shown in Table S1. Briefly, the phosphopeptide sample (1 pmol) was incubated with Ni-NTA (1 µl bed volume), Poros MC 20 (1 µl) or Phos-Select IMAC resin (2 µl) in loading solution for 1 h. Figures S1A-C shows the MALDI MS of the enriched products from the IMAC resins with three different methods. The sequences and phosphorylation sites of observed phosphopeptides are listed in Table S2.

Using conditions optimized for the Ni-NTA resin (loading with 100 mM HAc, washing with 30 % ACN in 100 mM HAc and elution with 20 mg/ml DHB in 50 % ACN and 1 % PA) [1, 2], this material exhibits the best selectivity for phosphopeptide enrichment, with most of the intense peaks representing phosphopeptides (Figure S1A). However, spectra from the Poros MC 20 and Phos-Select enrichments appear to be quite similar, but with a lower intensity of phosphopeptide signals and higher signals from non-phosphopeptides.

Optimized conditions for the Phos-Select resin [3] was also employed to the three IMAC resins (Figure S1C). In these conditions (loading and washing with 50 % ACN in 0.1 % TFA and elution with 20 mg/ml DHB in 50 % ACN in 1 % PA), all the IMAC resins exhibited high selectivity. However, the signals representing multiply phosphorylated peptides dominate the spectra, but only a few peaks from singly phosphorylated peptides can be observed. Thus the high selectivity obtained is at the cost of singly phosphorylated peptides. In the range from *m/z* 2500-3250, the Ni-NTA and Phos-Select resins show slightly higher efficiencies than the Poros MC 20 resin.

The recommended protocol for Poros MC 20 [4] (loading and washing with ACN/MeOH/water (1:1:1, v/v/v) in 0.01 % HAc and elution with 20 mg/ml DHB in 50 % ACN and 1 % PA) was also applied to the three IMAC resins. Although the Ni-NTA enrichment still shows the best selectivity (Figure S1B), the selectivity of all the IMAC resins are worse in comparison to the enrichments conducted under Ni-NTA conditions.

**Optimization of washing solution**

A desirable wash solution maintains the loading of the targeted phosphopeptides and inhibits the non-specific loading of non-phosphopeptides. We chose non-optimized loading conditions to determine the composition of the washing solution. Two loading conditions (100 mM HAc and 100 mM HAc in 80 % ACN) were selected, with the wash solutions containing 100 mM HAc and different concentrations of ACN (0, 30, 60 and 80 %).

It is clear that an efficient washing solution could increase the selectivity, even if the loading is conducted in non-optimized conditions (Figures S4A-B). This observation was not reported by Kokubu *et al.* [3], in which the bound non-phosphopeptides were difficult to remove during the wash step. Although an appropriate wash solution could increase specificity, the overall results were worse than those from earlier experiments using the optimized loading solution. Considerable sample loss occurred when 100 mM HAc was the loading solution and several non-phosphopeptide signals were observed when 100 mM HAc in 80 % ACN was used. As a compromise between specificity and sample loss, the optimized loading solution was also selected as the wash solution.

**Optimization of elution solution**

Imanishi *et al.* compared different elution solutions and demonstrated 5 % PA in 50 % ACN as an efficient elution solution [5]. We also tested the same series of elution solutions from this study: 1 % PA and 50 % ACN, 5 % PA and 50 % ACN, and 5 % ammonia water (Figure S5). We found that the increase in PA (1 to 5 %) increased the signal intensity of multiply phosphorylated peptides and minimized sample loss. Furthermore, the subsequent elution with 5 % PA in 50 % ACN after elution with 5 % ammonia water revealed only one phosphopeptide with low intensity. The different results suggest that an optimized method used for a particular IMAC material cannot be simply applied to other IMAC materials, despite sharing the same phosphopeptide loading mechanism.

An enhanced matrix solution (20 mg/ml DHB in 50 % ACN and 5 % PA) was used as elution solution for MALDI MS, since it was more efficient than a previously reported solution (20 mg/ml DHB in 50 % ACN and 1 % PA) and improved the detection of

multiple phosphopeptide signals [2]. Meanwhile, 5 % ammonia water was chosen as the elution solution for samples analyzed by LC-MS/MS.

**Ratio of sample to resin volume**

The ratio of sample amount to resin volume is an important factor that could affect the efficiency of the IMAC enrichment. To calculate the proper ratio of sample amount to resin volume, four different ratios of sample amount to resin volume (1 pmol phosphopeptides diluted into a 1:50 peptide mixture with 0.5, 1, 1.5 and 2 µl bed volumes). It is clear that most phosphopeptide signals can be obtained with only 0.5 µl of IMAC material (Figure S6). However, the intensity of the mono-phosphopeptide with a missed tryptic cleavage site (*m/z* 1951) increases when the bed volume of IMAC material increased to 1 µl, but no improvements are seen with higher bed volumes. The results suggest that for this phosphopeptide mixture, at least a 1 µl bed volume of IMAC resin is required to minimize sample loss during enrichment. Interestingly, a higher volume of IMAC resin does not lead to low selectivity.

**Effects of incubation time**

Incubation time is also an important factor affecting the efficiency of the IMAC enrichment. Enriched products from six different time points (1, 3, 5, 10, 30 and 60 min) were analyzed (Figure S7A). It is obvious that most phosphopeptide signals can be observed after only 1 min of incubation. However, the signal representing the singly phosphorylated peptide with a missed tryptic cleavage site (*m/z* 1951) increases as the incubation time increases (Figure S7B). This suggests that the required incubation time depends on the sequence and whether it is singly or multiply phosphorylated: less incubation time is needed for phosphopeptides with acidic sequences and/or multiple phosphoryl groups, but longer incubation times are required for singly phosphorylated peptides with non-acidic sequences.

## References:


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3. Kokubu, M.; Ishihama, Y.; Sato, T.; Nagasu, T.; Oda, Y., Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Anal Chem* **2005,** 77, (16), 5144-54.
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5. Imanishi, S. Y.; Kochin, V.; Eriksson, J. E., Optimization of phosphopeptide elution conditions in immobilized Fe(III) affinity chromatography. *Proteomics* **2007,** 7, (2), 174-6.
6. Larsen, M. R.; Thingholm, T. E.; Jensen, O. N.; Roepstorff, P.; Jorgensen, T. J., Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics* **2005,** 4, (7), 873-86.

## Figure legends

**Figure S1. Phosphopeptide enrichment of 1:50 peptide mixtures by three IMAC resins under optimized conditions. A.) MALDI MS spectra of the enriched products obtained by three different IMAC resins performed with the recommended protocol for Ni-NTA material** (loading with 100 mM HAc, washing with 30 % ACN in 100 mM HAc). **B.) MALDI MS spectra of the enriched products obtained by three different IMAC resins performed with the recommended protocol for Poros MC 20 material** (loading and washing with ACN/MeOH/water (1:1:1, v/v/v) in 0.01 % HAc). **C.) MALDI MS spectra of the enriched products obtained by three different IMAC resins performed with the recommended protocol for Phos-Select material** (loading and washing with 50 % ACN in 0.1 % TFA). The sample was incubated with loading solution for 1 h in all tests. All labeled signals indicate phosphorylated peptides from Table S2.

**Figure S2. A.) Phosphopeptide enrichment of 1 pmol trypsin digested α, β-casein with different percentages of ACN in the loading solution. B.) Magnified area from *m/z* 1925 to 1955. C.) Flowthrough of phosphopeptide enrichment of 1 pmol trypsin digested α, β-casein with different percentages of ACN in the loading solution. D.) Magnified area from *m/z* 1925 to 1965.** A concentration of 100 mM HAc was constant in the loading solution and 1 µl bed volume of Fe(III)-IMAC resin used. The sample was incubated with loading solution for 1 h in this test. All labeled signals indicate phosphorylated peptides from Table S2.

**Figure S3. Phosphopeptide enrichment of 1:50 peptide mixtures in different loading solutions.** Various HAc concentrations (25 to 400 mM) in 60 % ACN were used for loading. A 1 µl bed volume of Fe(III)-IMAC resin was incubated with the sample in loading solution for 1 h. All labeled signals indicate phosphorylated peptides from Table S2.

**Figure S4. Evaluation of washing solutions. A) Loading solution was 100 mM HAc, washing solution was labeled in each spectrum; B) Loading solution was 80 % ACN in 100 mM HAc, washing solution was labeled in each spectrum**. A 1 µl bed volume of Fe(III)-IMAC resin was incubated with the sample in loading solution for 1 h in all tests. All labeled signals indicate phosphorylated peptides from Table S2.

**Figure S5. Efficiencies of different elution solutions.** A(1), eluted with 2 μl of 20 mg/ml DHB in 50 % ACN in 1 % PA; A(2), after A(1) was eluted, the column was washed with 5 μl water and then eluted with 2 μl of 20 mg/ml DHB in 50 % ACN in 5 % PA; B(1), eluted with 2 μl of 20 mg/ml DHB in 50 % ACN in 5 % PA; B(2), after B(1) was eluted, the column was washed with 5 μl water and then eluted with 2 μl of 20 mg/ml DHB in 50 % ACN in 5 % PA; C(1), eluted with 2 μl of 20 mg/ml DHB in 50 % ACN in 5 % PA; C(2), after C(1) was eluted, the column was washed with 5 μl water and then eluted with 5 μl of 5 % ammonia water; D(1), eluted with 5 μl of 5 % ammonia water; D(2), after D(1) was eluted, the column was washed with 5 μl water and then eluted with 2 μl of 20 mg/ml DHB in 50 % ACN in 5 % PA. A 1 μl bed volume of Fe(III)-IMAC resin was used and 100 mM HAc in 60 % ACN was employed both as loading and washing solutions. The sample was incubated with loading solution for 1 h in these tests**.** All labeled signals indicate phosphorylated peptides from Table S2.

**Figure S6. Phosphopeptide enrichment of 1:50 peptide mixtures with different bed volumes of IMAC material.** A 100 mM HAc in 60 % ACN solution was employed as loading and washing solutions. The sample was incubated with loading solution for 1 h in all tests**.** All labeled signals indicate phosphorylated peptides from Table S2.

**Figure S7. A.) Phosphopeptide enrichment of 1:50 peptide mixtures with different incubation times; B.) Magnified area from *m/z* 1825 to 1965.** A 1 μl bed volume of Fe(III)-IMAC resin was used and 100 mM HAc in 60 % ACN was employed as loading and washing solutions in this test**.** All labeled signals indicate phosphorylated peptides from Table S2.

**Figure S8. MALDI MS/MS spectra of the peptides at *m/z* 1816.7, 1896.6, 2939.0, 3190.1 and 3270.1.**

**Table S1. Recommended loading and washing solutions for various IMAC resins**

| | Ni-NTA | Poros MC 20 | Phos-Select |
|---|---|---|---|
| **Loading solution** | 100 mM HAc | 0.01 % HAc in 1:1:1 ACN/MeOH/water | 0.1 % TFA in 50 % ACN |
| **Washing solution** | 30% ACN in 100 mM HAc | 0.01 % HAc in 1:1:1 ACN/MeOH/water | 0.1 % TFA in 50 % ACN |
| **Reference** | Stensballe *et al.* [1] | Ndassa *et al.* [4] | Kokubu *et al.* [3] |

**Table S2. Overview of the phosphopeptides obtained by tryptic digestion of α -casein S1 (α-S1) and S2 (α-S2), and β -casein (β-C) observed in enrichment experiments (adapted from Larsen *et al.*[6]).**

| Peptide sequence | Number of phosphate groups | *m/z* | |
|---|---|---|---|
| | | $[M + H]^+$ | $[M + 2H]^{++}$ |
| EQLpSTSEENSK, α-S2, 141–151 | **1** | 1331.53 | |
| EQLpSTpSEENSK, α-S2, 141–151 | **2** | 1411.50 | |
| TVDMEpSTEVFTK, α-S2, 153–164 | **1** | 1466.61 | |
| TVD[Mo]EpSTEVFTK, α-S2, 153–164 | **1** | 1482.61 | |
| EQLpSTpSEENSKK, α-S2, 141–152 | **2** | 1539.62 | |
| VPQLEIVPNpSAEER, α-S1, 121–134 | **1** | 1660.79 | |
| YLGEYLIVPNpSAEER, α-S1, 104–119 | **1** | 1832.83 | |
| DIGpSEpSTEDQAMEDIK, α-S1, 58–73 | **2** | 1927.69 | |
| DIGpSEpSTEDQA[Mo]EDIK, α-S1, 58–73 | **2** | 1943.69 | |
| YKVPQLEIVPNpSAEER, a-S1, 119–134 | **1** | 1951.95 | |
| FQpSEEQQQTEDELQDK, β-C, 33–48 | **1** | 2061.83 | 1031.42 |
| NVPGEIVEpSLpSpSpSEESITR, β-C, 7–25 | **4** | 2352.85 | |
| NTMEHVpSpSpSEEpSIISQETYK, α-S2, 17–36 | **4** | 2618.90 | |
| NT[Mo]EHVpSpSpSEEpSIISQETYK, α-S2, 17–36 | **4** | 2634.89 | |
| VNELpSKDIGpSEpSTEDQAMEDIKα-S1, 52-73 | **3** | 2678.01 | |
| Q*MEAEpSIpSpSpSEEIVPNpSVEAQK, α-S1, 74–94 | **5** | 2703.89 | |
| QMEAEpSIpSpSpSEEIVPNpSVEAQK, α-S1, 74–94 | **5** | 2720.91 | |
| NTMEHVpSpSpSEEpSIISQETYKQ, α-S2, 17–37 | **4** | 2747.10 | |
| NT[Mo]EHVpSpSpSEEpSIISQETYKQ, α-S2, 17–37 | **4** | 2763.01 | |
| ELEELNVPGEIVEpSLpSpSpSEESITR, β-C, 17–40 | **4** | 2966.16 | |

| | | | |
|---|---|---|---|
| NANEEEYSIGpSpSpSEEpSAEVATEEVK, α-S2, 61–85 | **4** | 3008.01 | |
| NANEEEYpSIGpSpSpSEEpSAEVATEEVK, α-S2, 61–85 | **5** | 3087.99 | |
| RELEELNVPGEIVEpSLpSpSpSEESITR, β-C, 16–40 | **4** | 3122.27 | 1561.64 |

Lowercase 'p' represents a phosphoryl group
[Mo] represents methionine oxidation
* represents pyroglutamylation on the N-terminal glutamine

**Table S3. Summary of phosphorylation sites and phosphopeptides from 20 µg of mouse cell lysate**

**Table S4. Summary of phosphorylation sites and phosphopeptides from 50 µg of *Drosophila melanogaster* cell lysate**