

Technical Note

Multi-laboratory experiment PME11 for the standardization of phosphoproteome analysis



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ABSTRACT

Global analysis of protein phosphorylation by mass spectrometry proteomic techniques has emerged in the last decades as a powerful tool in biological and biomedical research. However, there are several factors that make the global study of the phosphoproteome more challenging than measuring non-modified proteins. The low stoichiometry of the phosphorylated species and the need to retrieve residue specific information require particular attention on sample preparation, data acquisition and processing to ensure reproducibility, qualitative and quantitative robustness and ample phosphoproteome coverage in phosphoproteomic workflows. Aiming to investigate the effect of different variables in the performance of proteome wide phosphoprotein analysis protocols, ProteoRed-ISCI and EuPA launched the Proteomics Multicentric Experiment 11 (PME11). A reference sample consisting of a yeast protein extract spiked in with different amounts of a phosphomix standard (Sigma/Merck) was distributed to 31 laboratories around the globe. Thirty-six datasets from 23 laboratories were analyzed. Our results indicate the suitability of the PME11 reference sample to benchmark and optimize phosphoproteomics strategies, weighing the influence of different factors, as well as to rank intra and inter laboratory performance.

Many aspects of cell biology are regulated by reversible protein phosphorylation networks that involve thousands of phosphorylation events. In the last decade multiple methods have been developed to identify and quantify involved phosphorylation sites, and their modulation and dynamics under physiological and pathological conditions. Global post-translational modification analysis based on cutting edge mass spectrometry technology has emerged as the premier tool in many laboratories worldwide to investigate the complexity of signaling pathways and their crosstalk [1–3].

In 2016 the Spanish Proteomics Network ProteoRed-ISCI proposed the PME11 multi-laboratory experiment as part of the EuPA Standardization Initiative. The aim was to evaluate the performance and reproducibility of phosphopeptide enrichment procedures and to test the usefulness of phosphopeptide mixture standards to set up, monitor, and troubleshoot phosphopeptide analysis pipelines. The reference samples analyzed in the study (PME11-A1, A2, A3) consisted of a yeast tryptic digest (125 µg of a C-18 purified peptide digest), spiked-in with three different concentrations (100, 250 and 500 fmol) of a mixture of 20 human phosphopeptide standards (Phosphomix 1 and 2 from Sigma-Aldrich, (product reference MSPL1 and MSPL2, Table 1), containing light isotopes. Each participant laboratory received two aliquots of each of the three samples (SUPP INFO 1&2), that were distributed in dry ice, lyophilized from a water-acetonitrile mixture. One additional vial PME11-B, containing 2 pmol of each of the corresponding isotopically labeled heavy Phosphomix standard peptides (Sigma-Aldrich MSP1H and MSP2H) was distributed in dried form for ulterior quantitative analyses. Upon reception participants were indicated to re-dissolve the samples in the appropriate buffer for the enrichment procedure selected. Then, enriched phosphopeptides were analyzed by LC-MS/MS (three replicates) following the recommended guidelines (10 to 30% of the enriched sample and 60 min 0–35% acetonitrile gradient). Analysis of pre-enriched samples was also recommended. Detailed descriptions of the experimental settings, reference sample and analysis guidelines were provided to the participants (SUPP INFO 1&2).

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Recently, a related study conducted by several laboratories in the frame of the MS Resource Pillar of the HUPO Human Proteome Project has been reported [4]. In this study, a standard set of 94 phosphopeptides and their nonphosphorylated counterparts, mixed in a neat sample and a yeast background were analyzed. Unlike the HUPO study samples, the samples proposed in the present study allowed for the assessment of the enrichment of the endogenous yeast phosphopeptides, in conditions and amounts similar to a real sample. Besides, the spiked-in phosphopeptide standards were provided in isotopically labeled and unlabeled form, allowing for assessment not only of targeted phosphopeptide analysis, but also to estimate the yield of the enrichment procedures used.

Under the coordination of ProteoRed-ISCI, 36 datasets were received from 23 laboratories (Table 2) distributed across Europe -Spain, France, Switzerland, United Kingdom, and Sweden- and USA. Individual reports including experimental details and results were prepared by each participant in the template specifically design for this experiment. Additionally, MS/MS files (mgf format) were also submitted to the coordination unit for their centralized processing and integration, which will be described elsewhere. Some laboratories provided various datasets that corresponded to different analytical pipelines, which allowed the specific evaluation of the experimental conditions tested as the user and instrument used in these cases were the same. Shotgun analysis results were used to evaluate the general performance of each laboratory in terms of number of yeast phosphopeptides identified, efficiency of the enrichment procedure (phosphopeptides/total peptides ratio) and detection of spiked-in phosphopeptide standards.

In light of the dispersion of the analytical conditions used by the participating labs, a comprehensive statistical analysis may have limitations. Nevertheless, several outcomes are worth to be discussed taking into consideration the interlaboratory nature of the present experiment. Samples were processed following different protocols in eight different mass spectrometers as summarized in Table 2 and supplementary information.

A first clear outcome is that intra-laboratory reproducibility is in general very good, as shown by the error bars in the graph in Fig. 1A, with a median %CV between triplicate analysis of 9.16% (Table 2). It has to be remarked that these correspond to triplicate experiments, including the enrichment step and the LCMS analysis.

Regarding inter-laboratory comparison, the number of

Table 1
Phosphopeptide standard description.

Sequence	PhosphoMix #	phosphosite 1	phosphosite 2
ADEPSSEESDLEIDK	1,6	S5	
ADEPSSEESDLEIDK	1,7	S6	S9
ADEPSSEESDLEIDK	2,6	S9	
ELSNSPLRENSFGSPLEFR	1,9	S5	S14
ELSNSPLRENSFGSPLEFR	2,9	S3	S5
ETQSPEQVK	2,3	T2	
EVQAEQPSSSSPR	1,5	S10	
FEDEGAGFEESSETGDYEEK	1,8	S12	
HQYSDYDYHSSEK	2,7	Y8	S12
LGPGRLPTFFPTSECTSDVEPDTR	2,10	T12	
LPQETAR	2,1	T5	
NTPSQHSLSIQHSRPER	2,8	S4	S9
RDSLGTYSR	1,3	T6	
RSYSRSR	1,2	Y3	S4
RYSSRSR	2,2	S3	S4
SPTEYHEPVYANPFYRPTTPQR	1,10	Y10	T19
SRSPSPELNK	2,5	S1	S5
TKLITQLRDAK	1,4	T1	T5
VIEDNEYTAR	2,4	Y7	
VLHSGSR	1,1	S6	

phosphopeptides identified in the different experiments spans a wide range, with an average value of 1026, (Fig. 1A, B and Table 2). One of the main factors that explains this wide range is of course the technical capability of the different instruments used. To roughly estimate the contribution of this factor, normalized values have been calculated (black points in the graph) using as normalization factor the ratio between the reported number of total peptides in the analysis of the pre-enrichment sample for each experiment (Table 2), and the average values for all the experiments. Using this normalization to “compensate” for instrument performance, the inter-laboratory %CV for the number of phosphopeptides decreases from 66% to 36% (Fig. 1B).

Other factors accounting for this variability would certainly include the enrichment protocol used, as well as the parameters used for data processing and database searches, but also reflect the different expertise of the different laboratories. This is apparent when comparing the results from laboratories using the same type of enrichment and identical instrument (see for example L14 vs L09, L28 vs L15, or L13 vs L20, in Fig. 1A).

The amount of sample analyzed (Table 2) is also a factor that influences the result, as is well illustrated by data from L23 with around 500 phosphopeptides detected upon processing 10% sample in an Orbitrap XL (L23-1 and -4) and about 1000 and 2000 identifications when 2% and 20% sample were processed (L23-2 and -3, -5 and -6 respectively) in an Orbitrap Fusion Lumos respectively (Fig. 1A and Table 2).

The enrichment selectivity (Fig. 1C) spans from 15 to 90%. Overall, there is no clear correlation between the observed selectivity and the number of phosphopeptides identified in each of the experiments, influenced, as discussed, by many other factors.

TiO₂ was the preferred enrichment method, representing more than 80% of the analyses and resulted in higher enrichment selectivity (above 50%) compared to those obtained with IMAC (below 40%). This is also the case when comparing TiO₂ versus IMAC enrichment data from the same laboratory, such as data from L23 and L13. These results seem to be in agreement with previous data reporting increased selectivity of

TiO₂ compared to metal chromatography [5]. It has been also reported that IMAC enrichment would favor the identification of poly-phosphorylated peptides [6]. In the results gathered in this study, no significant differences have been observed in this respect (data not shown). However, the small number of IMAC analysis, together with the variety of protocols and instruments used, precludes a general conclusion. Combination of two enrichment steps, either TiO₂-TiO₂ or TiO₂-IMAC, increased the enrichment efficiency notwithstanding the total number of phosphopeptides identified (comparing data obtained in the same instrument in different labs), as deduced from L25 data (Fig. 1A and B).

Data from TiO₂ and IMAC enrichment for L23 and L13, but also data from L28, where different sample/TiO₂ matrix ratios were assayed (see Table 2), exemplify a general trend where higher selectivity of the enrichment step would result in a significant increase in the number of phosphopeptides detected. This behavior would be consistent with a “masking” effect of the presence of a higher ratio of non-phosphorylated peptides with respect to phosphopeptides in the enriched sample.

The enrichment chromatography format did not have any systematic effect either in the number of phosphopeptides detected or in the enrichment capacity; the observed variations result from inter-operator variability.

Detection of phosphopeptide standards relied on an enrichment step, no matter the amount of standard spiked on the yeast extract (aprox. 100, 50 or 20 fmol on column). The frequency of detection defined as the proportion of laboratories detecting a given peptide in three samples, was above 60% for most phosphopeptides (12/20 labs), around 50% in five cases while three phosphopeptides were not detected in any lab, likely due to their small size and highly hydrophilic nature, preventing their retention in the C18 precolumn (Fig. 2). No significant differences were observed in terms of phosphomix standard detection in regard of the different instruments or enrichment methods used. The phosphomix peptide standards are in general readily observable, even at the lowest concentration assayed, with the exceptions described, and so can be useful for quantitative purposes to measure the yield of a particular

Table 2

Datasets gathered in the study, experimental settings, and summary of the main results. Datasets coded with the same L number correspond to experiments performed in the same laboratory using different enrichment or LCMS analysis conditions.

CODE	Affinity Enrichment type	Support	Ratio TiO2/sample w/w	MS instrument	Enriched sample amount loaded in LCMS (%)	# peptides Pre-enriched sample	# Phosphopeptides ¹	Std Dev. # Phosphopeptides ¹	%CV # Phosphopeptides ¹
L06	TiO2	Stage Tip SpinTip column	6	5600 TTOF	30	5755	823	59.2	7.2
L12	TiO2	Stage Tip SpinTip column	N/A	5600 TTOF	25	5666	679	28.4	4.2
L07	TiO2	Stage Tip SpinTip column	6	5600 TTOF Amazon	25	11,008	741	199.3	26.9
L16	TiO2	Stage Tip SpinTip column	8	Speed ETD	20	1461	141	19.1	13.5
L21_1	TiO2	Stage Tip SpinTip column	6	Orbitrap XL	30		606	101.9	16.8
L23_1	TiO2	Stage Tip SpinTip column	6	Orbitrap XL	10		539	39.7	7.4
L14_1	TiO2	Stage Tip SpinTip column	6	OT Fusion	15	9073	856	112.8	13.2
L14_2	TiO2	Batch SpinTip column	24	OT Fusion	15	9024	716	150.9	21.1
L09	TiO2	Batch SpinTip column	N/A	OT Fusion	20		102	24.5	24.0
L28_1	TiO2	Batch SpinTip column	0.3	Lumos	17	23,851	2675		
L23_3	TiO2	Batch SpinTip column	6	OT Fusion Lumos	20	12,402	2486	83.8	3.4
L19	TiO2	Batch SpinTip column	N/A	Lumos	20	16,524	2295	54.7	2.4
L28_2	TiO2	Batch SpinTip column	40	OT Fusion Lumos	17	23,851	2184		
L28_3	TiO2	Batch SpinTip column	6	OT Fusion Lumos	17	23,851	2161		
L10	TiO2	Batch SpinTip column	6	OT Fusion Lumos	20	17,286	2062	57.4	2.8
L23_2	TiO2	Batch SpinTip column	6	OT Fusion Lumos	2	10,181	1104	26.7	2.4
L15	TiO2	Batch SpinTip column	6	OT Fusion Lumos	10	5636	768	34.9	4.6
L02_2	TiO2	Stage Tip -Gly	5	OT Velos	25	5813	1333	144.7	10.9
L13_1	TiO2	Stage Tip	6	OT Velos	20	5299	1184	56.7	4.8
L05	TiO2	Stage Tip	24	OT Velos	30	6846	1051	136.6	13.0
L04	TiO2	Stage Tip	8	OT Velos	30		599	67.6	11.3
L03	TiO2	Stage Tip	6	OT Velos	20	4194	586	206.0	35.1
L02_1	TiO2	Stage Tip- DHB	2	OT Velos	25	5813	549	92.9	16.9
L20	TiO2	Magnetic Beads	N/A	OT Velos	10	4527	427	57.8	13.5
L08_1	TiO2	Stage Tip	5	Q-Exactive	25	6231	1412	26.4	1.9
L08_2	TiO2	Stage Tip	5	Q-Exactive	25	6231	1407	56.6	4.0
L29	TiO2	Batch	24	Plus	16	4774	647	56.1	8.7
L30	TiO2	Stage Tip	6	Synapt G2	10		930	92.6	10.0
L21_2	TiO2	Stage Tip	6	Synapt	30		437	3.1	0.7
L25_1	TiO2/TiO2	Batch	6	5600 TTOF	25	5271	623	58.5	9.4
L25_2	TiO2/IMAC	Batch/Phos Select	6	5600 TTOF	25	5271	830	74.1	8.9
L17	IMAC	PhosSpinTrap		OT Velos	30	7653	667	175.2	26.3
L13_3	IMAC	Phos Select		OT Velos	20	5299	476	39.1	8.2
L13_2	IMAC	Phos Select		OT Velos	20	5299	408	15.9	3.9
L23_4	IMAC	Phos Select		Orbitrap XL	10		366	20.4	5.6
L23_6	IMAC	Phos Select		OT Fusion Lumos	20	12,402	2114	236.5	11.2
L23_5	IMAC	Phos Select		OT Fusion Lumos	2	10,181	972	110.8	11.4

1- Number of phosphopeptides identified in the enriched sample. Average of triplicate analysis, Std. Dev. and %CV shown when available.

enrichment experiment.

In conclusion, the use of different protocols, instruments and operators provides a wide scenario of experimental conditions that is optimal to prove the suitability of the reference material here described for inter- and intra-lab protocol benchmarking, indicating strengths, weaknesses, and guidance for optimization (Stage-Tip vs batch, sample/medium

ratio). Overall, we propose that the use of a standardized reference material in a multi-lab study is a useful resource for technology testing as has been extensively demonstrated [7–10] and provide excellent references to set up protocols and rank the performance of individual labs, contributing to the democratization of sophisticated proteomics pipelines under standardized conditions. We think that the results here

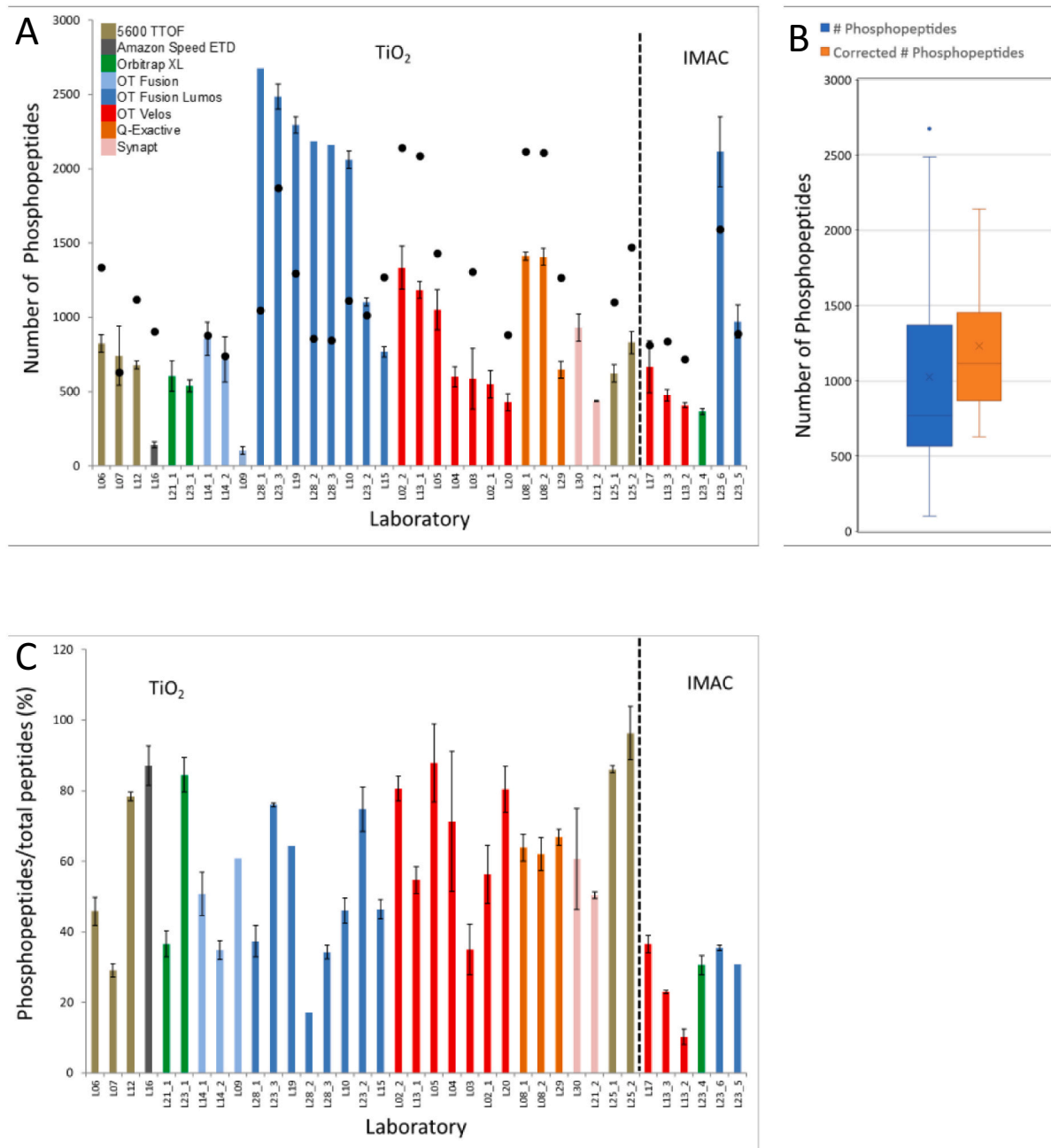


Fig. 1. Results of the analysis of PME11 samples reported by the different laboratories participating in the study. A) Number of phosphopeptides from each analysis. Each bar represents the average number reported, the error bars being the standard deviation of triplicate analysis performed in the same laboratory (when available). Columns are colored according to the MS instrument used for the analysis, as indicated in the legend. Results are grouped by the type of affinity enrichment used (TiO₂, IMAC). L25-2 corresponds to a two step sequential enrichment TiO₂-IMAC. The black points indicate the corrected number of phosphopeptides weighed by instrument performance (see text). B) Box and whisker plot summarizing the raw and weighed number of phosphopeptides data. C) Selectivity of the phosphopeptide enrichment measured as the % of phosphopeptides in the enriched sample. Results are shown in the same order as in Fig. 1A.

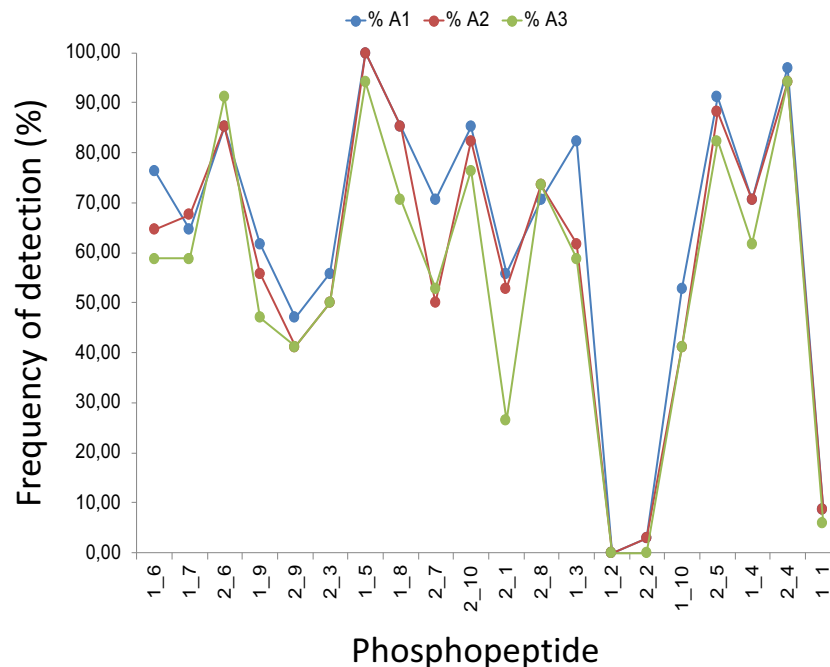


Fig. 2. Results of the identification of the Phosphomix peptide standards. Frequency of the identification of each of the phosphomix standards in the analysis performed by all the laboratories, for each of the three standard samples, containing different amounts of the standards: A1 (100), A2 (250), A3 (500) fmol/125 μ g of initial yeast digest.

described demonstrate that the standard proposed in this study is a suitable reference material for the assessment and optimization of phosphoproteomic analysis and certainly provide valuable information to dig deeper into the pros and cons of phosphoproteomics workflows.

Data availability

No

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2021.104409>.

References

- [1] M.A. Jünger, R. Aebersold, Mass spectrometry-driven phosphoproteomics: patterning the systems biology mosaic, *Wiley Interdiscip. Rev. Dev. Biol.* 3 (1) (2014 Jan-Feb) 83–112, <https://doi.org/10.1002/wdev.121>. Epub 2013 Jul 2, 24902836.
- [2] J.V. Olsen, M. Mann, Status of large-scale analysis of post-translational modifications by mass spectrometry, *Mol. Cell. Proteomics* 12 (12) (2013 Dec), <https://doi.org/10.1074/mcp.O113.034181>, 3444–52. Epub 2013 Nov 1, 24187339, PMC3861698.
- [3] W. Qiu, C.A. Evans, A. Landels, T.K. Pham, P.C. Wright, Phosphopeptide enrichment for phosphoproteomic analysis - a tutorial and review of novel materials, *Anal. Chim. Acta* 1129 (2020 Sep 8) 158–180, <https://doi.org/10.1016/j.aca.2020.04.053>. Epub 2020 Apr 28, 32891386.
- [4] M.R. Hoopmann, U. Kusebauch, M. Palmblad, N. Bandeira, D.D. Shteynberg, L. He, B. Xia, S.H. Stoychev, G.S. Omenn, S.T. Weintraub, R.L. Moritz, Insights from the first phosphopeptide challenge of the MS resource pillar of the HUPO human proteome project, *J. Proteome Res.* 19 (12) (2020 Dec 4) 4754–4765, <https://doi.org/10.1021/acs.jproteome.0c00648>. Epub 2020 Nov 9. PMID: 33166149; PMCID: PMC8204901.
- [5] X. Yue, A. Schunter, A.B. Hummon, Comparing multistep immobilized metal affinity chromatography and multistep TiO₂ methods for phosphopeptide enrichment, *Anal. Chem.* 87 (17) (2015 Sep 1) 8837–8844, <https://doi.org/10.1021/acs.analchem.5b01833>. Epub 2015 Aug 11, 26237447. PMC4766865.
- [6] T.E. Thingholm, M.R. Larsen, Sequential elution from IMAC (SIMAC): an efficient method for enrichment and separation of mono- and multi-phosphorylated peptides, *Methods Mol. Biol.* 1355 (2016) 147–160, https://doi.org/10.1007/978-1-4939-3049-4_10. 26584924.
- [7] V. Vialas, N. Colomé-Calls, J. Abian, K. Aloria, G. Alvarez-Llamas, O. Antúnez, J. M. Arizmendi, M. Azkargorta, S. Barceló-Batlloiri, M.G. Barderas, F. Blanco, J. I. Casal, V. Casas, C. de la Torre, E. Chicano-Gálvez, F. Elortza, G. Espadas, J. M. Estanyol, J. Fernandez-Irigoyen, P. Fernandez-Puente, M.J. Fidalgo, M. Fuentes, M. Gay, C. Gil, A. Hainard, M.L. Hernaez, N. Ibarrola, A.T. Kopylov, A. Lario, J. A. Lopez, M. López-Lucendo, M. Marcilla, A. Marina-Ramírez, G. Marko-Varga, L. Martín, M.I. Mora, E. Morato-López, J. Muñoz, M.A. Odena, E. de Oliveira, I. Orera, I. Ortea, C. Pasquarello, K.B. Ray, M. Rezelí, I. Ruppen, E. Sabidó, M.M. S. Del Pino, J. Sancho, E. Santamaría, J. Vazquez, M. Vilaseca, F. Vivanco, J. J. Walters, V.G. Zgoda, F.J. Corrales, F. Canals, A. Paradela, A multicentric study to evaluate the use of relative retention times in targeted proteomics, *J. Proteome* 152 (2017 Jan 30) 138–149, <https://doi.org/10.1016/j.jprot.2016.10.014>. Epub 2016 Oct 29, 27989941.
- [8] A. Campos, R. Díaz, S. Martínez-Bartolomé, J. Sierra, O. Gallardo, E. Sabidó, M. López-Lucendo, J. Ignacio Casal, C. Pasquarello, A. Scherl, C. Chiva, E. Borrás, A. Odena, F. Elortza, M. Azkargorta, N. Ibarrola, F. Canals, J.P. Albar, E. Oliveira, Multicenter experiment for quality control of peptide-centric LC-MS/MS analysis - A longitudinal performance assessment with nLC coupled to orbitrap MS analyzers, *J. Proteome* 127 (Pt B) (2015 Sep 8) 264–274, <https://doi.org/10.1016/j.jprot.2015.05.012>. Epub 2015 May 14, 25982386.
- [9] J.P. Albar, F. Canals, Standardization and quality control in proteomics, *J. Proteome* 95 (2013 Dec 16) 1–2, <https://doi.org/10.1016/j.jprot.2013.11.002>. 24275454.
- [10] S.M. Mische, N.C. Fisher, S.M. Meyn, K. Sol-Church, R.L. Hegstad-Davies, F. Weis-Garcia, M. Adams, J.M. Ashton, K.M. Delventhal, J.A. Dragon, L. Holmes, P. Jagtap, K.E. Kubow, C.E. Mason, M. Palmblad, B.C. Searle, C.W. Turck, K. L. Knudtson, A review of the scientific rigor, reproducibility, and transparency studies conducted by the ABRF research groups, *J. Biomol. Tech.* 31 (1) (2020 Apr) 11–26, <https://doi.org/10.7171/jbt.20-3101-003>. PMID: 31967995; PMCID: PMC6959150.