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TECHNICAL BRIEF

Triple extraction method enables high quality mass spectrometry-based proteomics and phospho-proteomics for eventual multi-omics integration studies

Virginia Sanchez-Quiles^{1*}, Ming-Jun Shi^{1, 3}, Florent Dingli², Damarys Loew², Isabelle Bernard-Pierrot^{1, #}, François Radvanyi^{1, #} Clémentine Krucker

¹ Institut Curie, PSL Research University, CNRS, UMR144, Equipe Labellisée Ligue contre le Cancer, 75005, Paris, France.

² Institut Curie, PSL Research University, Centre de Recherche, Mass Spectrometry and Proteomics Facility, Paris, France

³ Department of Urology, Beijing Friendship Hospital, Capital Medical University, Beijing, China

joint senior authors

* Corresponding author:

Virginia Sanchez-Quiles

Molecular Oncology, UMR 144 CNRS, Curie Institute.

26, rue d'Ulm. 75248 Paris, France

Tel: +33 156 24 6340; email: virginia.sanchez-quiles@curie.fr

ABBREVIATIONS: **TE_x**: triple extraction; **RPPA**: reverse phase protein array; **ABC**: ammonium bicarbonate; **CAA**: chloroacetamide; **SAX**: strong anion exchange; **HCD**: high energy collision dissociation; **LFQ**: label free quantification; **NSAF**: normalized spectral abundance factor

KEYWORDS: phospho-proteomics, shotgun proteomics, triple extraction, urea extraction

Total number of words: 3544 (from abstract to references, included)

Abstract

Large-scale multi-omic analysis allows a thorough understanding of different physiological or pathological conditions, particularly cancer. Here, an extraction method simultaneously yielding DNA, RNA and protein (thereby referred to as “triple extraction”, TEx) was tested for its suitability to unbiased, system-wide proteomic investigation. Largely proven efficient for transcriptomic and genomic studies, we aimed at exploring TEx compatibility with mass spectrometry-based proteomics and phospho-proteomics, as compared to a standard urea extraction. TEx is suitable for the shotgun investigation of proteomes, providing similar results as urea-based protocol both at the qualitative and quantitative levels. TEx is likewise compatible with the exploration of phosphorylation events, actually providing a higher number of correctly localized sites than urea, although the nature of extracted modifications appears somewhat distinct between both techniques. These results highlight that the presented protocol is well suited for the examination of the proteome and modified proteome of this bladder cancer cell model, as efficiently as other more widely used workflows for mass spectrometry-based analysis. Potentially applicable to other mammalian cell types and tissues, TEx represents an advantageous strategy for multi-omics on scarce and/or heterogenous samples.

The quantitative, system-wide analysis of DNA, RNA, proteins and isoforms through omic techniques has currently become an essential strategy in cellular and tissue molecular characterization for a wide range of diseases, namely cancer. While in the past those large-scale approaches have been considered separately, the current and future tendencies rely on integrative studies for a thorough understanding of biological systems. The inclusion of proteomics in the frame of comprehensive research is of high importance, as proteins are the functional entities of the cell and targets of most of the available drugs currently used for treatment. Indeed, the combination of large scale mass spectrometry(MS)-based proteomics, together with transcriptomics and/or genomics has proven successful for a deeper characterization of different tumor types (Forget et al., 2018; Zhang et al., 2014). RNA transcripts do not steadily predict corresponding protein abundances (Zhang et al., 2014), while RNA/protein imbalance could show characteristic and distinct direction within different tumor subgroups (Forget et al., 2018). Hence, proteomics provides an improved portrayal of biology, shedding new light beyond the gene-based understanding.

One of the critical issues when working with human cancer specimens is intratumor heterogeneity. Very adjacent sections of tumors should be utilized for paralleled analysis to reliably trust their subsequent correlation. However, the compartmentalization of the original sample leads to reduced amounts used for each technique, point particularly problematic in small tissues or with low number of transformed cells. Moreover, separate extractions increase experimental deviations and time for sample preparation. A single protocol for the extraction of DNA, RNA and protein from the very same input material aids to overcoming these issues and improving integrative analysis.

Usually making use of an initial guanidine isothiocyanate denaturing buffer, several methods exist for rendering DNA, RNA and protein from a single same sample. Through the years, our group has widely used a triple extraction strategy (hereby called TEx) based on the original works of Chirgwin (Chirgwin et al., 1979) and Coombs (Coombs et al., 1990). The procedure we have been performing since 1997 (De Medina et al., 1997; Stransky et al., 2006; Bernard-Pierrot et al., 2006; Biton et al., 2014; Sandra Rebouissou et al., 2014; Rochel et al., 2019) makes use of a guanidine isothiocyanate extraction followed by a centrifugation over a cesium chloride gradient for separation of the three types of

macromolecules. Proteins are afterwards recovered through dialysis and purified by lyophilization. In our hands, this method has already proven successful for the study of DNA and RNA, from both cell lines and tumor samples, through different assays including RNA quantification (large scale transcriptome or RT-qPCR), analysis of DNA copy number, sequencing or methylation analysis (S. Rebouissou et al., 2014; Rochel et al., 2019; Stransky et al., 2006; Vallot et al., 2011). Similarly, RPPA studies have previously been performed from TEx-derived proteins from tissues in our group (Calderaro et al., 2014; Neuzillet et al., 2017). Nevertheless, MS-based analysis following guanidine isothiocyanate extraction are less common and, to our knowledge, mostly limited to gel-based 2-D DIGE (Braakman et al., 2015; Grzendowski et al., 2009; Mathieson & Thomas, 2013; Xiong et al., 2011), providing a rather restricted number of identifications and without investigation of phosphorylation.

Here, we explore the suitability of TEx for gel-free MS-based proteomics, together with the conservation of phosphorylated peptides, as compared to urea-extracted counterparts. We employed a bladder cancer cell line, SCaBER, in a 6-plex experimental design (Figure 1A) where each replicate corresponds to approximately 10×10^6 cells at around 70-80% confluency. On one hand, three samples were independently extracted in 8M urea, 50mM ammonium bicarbonate (ABC) supplemented with protease and phosphatase inhibitors. On the other hand, protein from three other replicates were extracted in guanidine isothiocyanate buffer (4M guanidinium thiocyanate, 5mM sodium citrate pH 7, 0.1M β -mercaptoethanol, 0.5% sarkosyl) as described before (Neuzillet et al., 2017), purified by ultracentrifugation over cesium chloride and cleaned through dialysis against 100mM ABC for 24 hours. The protein extracts were subjected to complete freezing at -80°C followed by lyophilization for at least 48 hours. Dried TEx-obtained proteins were resuspended using the very same urea buffer previously mentioned (8M urea, 50mM ABC supplemented with inhibitors). Therefore, all the 6 conditions from urea extraction and TEx were later processed in parallel: quantified simultaneously through BCA (1.5mg protein was subsequently used for each replicate), reduced using 5mM DTT and alkylated with 10mM CAA. Proteins were digested with Trypsin/LysC (Promega) in a 1:100 protease:protein ratio. Peptides were desalted through Sep-Pak C18 cartridges (Waters). Sample volume corresponding to 100ug

peptides of each sample were used for their fractionation through Strong Anion eXchange (SAX) using in-house prepared microcolumns (anion exchange disks, Empore) and a Britton & Robinson buffer at six decreasing pH for elution of peptides (20mM acetic acid, 20mM phosphoric acid, 20mM boric acid at pH range 11, 8, 6, 5, 4, 3)(Wiśniewski et al., 2011). Volume corresponding to 1mg of peptides was used for their phospho-enrichment using Titansphere beads (GL Sciences) as described before (Larsen et al., 2005). Each TiO₂-obtained sample was run thrice on the mass spectrometer.

Peptides were separated and analyzed by nanoLC-MS/MS using a C18 μ -precursor (nanoViper Acclaim PepMapTM 100, Thermo Scientific, Cat: 164942), coupled to an UltiMate 3000 RSLCnano system (Thermo Scientific) and an Orbitrap Fusion mass spectrometer (Q-OT-qIT, Thermo Fisher Scientific). Separation of peptides was performed using solvents A (2% ACN, 0.085% formic acid) and B (100% ACN, 0.085% formic acid) through either linear gradient of 100 min (6 proteome fractions) or three step linear gradients of 211 min (phospho-enriched fractions). Survey MS scans were acquired at a resolution of 120,000, mass range of m/z 400–1500 and a 4×10^5 ion count target. MS/MS was performed by isolation at 1.6Th or 1.2Th (phospho) with the quadrupole, HCD fragmentation with normalized collision energy of 28, and rapid scan MS analysis in the ion trap. MS2 ion count target was 1×10^4 and max injection time was 100ms. Dynamic exclusion duration was set to 30s or 15s (phospho) with a 10ppm tolerance. All raw data have been deposited to the ProteomeXchange Consortium via the Proteomics Identifications (PRIDE) (Vizcaíno et al., 2016) partner repository with identifier PXD011971. Data were acquired using the Xcalibur software (v3.0), analyzed with MaxQuant 1.5.2.8 (Cox & Mann, 2008) and peak lists were searched against human UniProt database 2016.01.21. Label-free quantification (LFQ) was set for the search. Both peptide and protein maximum false discovery rates (FDR) were set to 0.01 based on the target-decoy approach. Perseus, BioVenn web application (www.biovenn.nl/), DAVID (<https://david.ncifcrf.gov/>) and BioInfoMiner (<https://bioinforminer.com>) were used for data interpretation.

Whole proteome analysis resulted in 6697 identified protein groups (excluding reverse sequences, trypsin and contaminants from media supplement serum, FBS). From those, 5532 protein groups were confidently identified (≥ 2 unique peptides across samples, Suppl Table 1). Considering conditions

separately, the number of confident identifications varied from 4530 and 4969 (≥ 2 unique peptides for each), with no differences between extraction methods (Figure 1B). Indeed, majority of protein groups (88.3%) were successfully identified by both protocols (Figure 1C). Confidently quantified protein groups were 5497 considering all conditions (ms count ≥ 2) (Suppl Table 1). When analyzing samples independently, in average 85.3% of identified protein groups were successfully quantified in TEx and 86.8% in Urea (Figure 2D and Suppl Table 1). Furthermore, 79% of proteins were effectively quantified regardless of the processing strategy (Figure 2C). TEx protocol is hence suitable for the quantitative investigation of whole proteome through LC-MS/MS analysis.

As mentioned before, when performing multi-omic analyses, it is important to successfully isolate different macromolecules of good quality from the same original sample through a unique protocol. The integrity and the purity of RNA and DNA were systematically tested. An example is shown in Suppl Table 1 for a set of bladder cancer cell lines, including the SCaBER cell line used in this work for proteomics. Transcriptomic data related to this selection of cell lines can also be found in Suppl Table 1. Nucleic acids resulting from TEx have been shown to be suitable for further studies (Bernard-Pierrot et al., 2006; Biton et al., 2014; De Medina et al., 1997; S. Rebouissou et al., 2014; Rochel et al., 2019; Stransky et al., 2006; Vallot et al., 2011) demonstrating that TEx represents a well-adapted approach for the investigation of different macromolecules.

Concerning the proteomic quantification through LC-MS/MS, the comparison of LFQ intensities of each protein group through the different replicates showed a Pearson correlation intra-extraction always higher than 0.94 (TEx-average: 0.96; Urea-average: 0.95), with coefficients of determination above 0.87 (TEx-average: 0.92; Urea-average: 0.91). When comparing between extraction methods, Pearson correlation ranged between 0.908-0.939, whereas plotted protein data fitted to coefficients of determination higher than 0.8 (Figure 1E and Suppl Table 1). The average relative standard deviation of LFQ intensities was 10.6% for urea and 10.7% for TEx. Importantly, considering the normalized spectral abundance factor (NSAF) for each identified protein, the abundance of extracted molecules by both methods spanned over five orders of magnitude (Figure 1F). TEx proves thus efficient for the simultaneous investigation of both high and low abundant proteins, such the ones encoded by GAPDH

and TTN, respectively. Notably, TEx approach enabled the detection and quantification of molecular characters with a known relevant role or which are known subtype markers in various cancers, including bladder cancer, such as EGFR, ERBB2, CD44, RB1, KRT5, KRT6A, TP63 or TP53. A selection of five identified proteins with LFQ intensities spanning over six orders of magnitude showed that quantification was highly reproducible regardless of the extraction method (Figure 2A).

Considering that most identified and quantified proteins were equally extracted by both protocols (Figure 1C), we expected to enrich similar (gene ontology) GO terms within them. Indeed, best represented cellular localizations were essentially the same, with most proteins mainly obtained from cytoplasm, membrane and nucleoplasm (Figure 2B). Enriched biological processes in both extraction methods related to very general functions of the cell such as adhesion, translation and RNA processing (Figure 2C).

Taken together, these results show that the whole proteome investigation through MS-based analysis might be successfully performed on TEx-derived extracts, as the obtained proteins are qualitatively and quantitatively comparable to those derived from urea extraction.

To corroborate the applicability of TEx for the investigation of post-translational modifications, we combined it with metal oxide affinity chromatography (Larsen et al., 2005). MS analysis resulted in 304105 peptide-spectrum matches detected (FDR 1%), of which 281071 were evidences of phosphorylated ions, demonstrating an enrichment of phospho-peptides higher than 92% (Figure 2D). 17707 phosphorylation sites were identified, excluding reverse sequences and FBS contaminants (Suppl Table 1). Considering the whole dataset, more than 70% of the phosphorylation sites were successfully assigned, with a localization probability of at least 0.7 across all runs (12528 class I sites) (Figure 2E and Suppl Table 1). When taken separately, urea provided 8093 correctly localized sites, while 10354 sites were class I in TEx samples. Of those, 5919 were common sites, identified and successfully assigned in both procedures (Figure 2F). The distributions of modified residues did not show a bias for extraction method, with the majority of class I sites siting on serine residues (86.98% in urea, 86.53%

in TEx), followed by threonine and tyrosine phosphorylations (Thr: 11.97% in urea, 12.22% in TEx; Tyr: 1.05% in urea, 1.25% in TEx) (Figure 2G).

For the quantitative investigation of sites, phosphopeptides were considered as quantified within a replicate when displaying an intensity value in at least 2 out of 3 LC-MS/MS runs. Then, a site was quantified in an extraction protocol if showing a corresponding value in all three replicates of the protocol (such as TEx1, TEx2 and TEx3). Following this highly strict criteria, we obtained 5964 and 5375 quantified phospho-sites in TEx and urea, respectively (Figure 2H and Suppl Table 1). Less than half the total sites (3308 sites, 41.2%) were equally quantified through both processing techniques (Figure 2I). Given that replicates within the same condition share a much higher overlap, the observed results do not seemingly account for variability through different LC-MS runs, but rather for a particular preference of phospho-sites to a certain type of extraction. Indeed, when clustering the sites with a quantification in at least one LC-MS/MS run of our setup (12413 phosphorylated sites, Suppl Table 1) according to their intensities, two main groups are obtained, clearly segregating urea and TEx-derived data (Figure 2J; clustering calculated using Euclidean distance and average linkage). Some sites seem preferentially quantified following one particular extraction, implying that the processing protocols could indeed provide different outputs. When comparing the extracted peptides corresponding to the total proteome, more than 82% of peptides are equally isolated from both protocols (Suppl Table 1); thus, the observed differences at the phosphorylation level more likely respond to different stability of labile modifications through each procedure. Concerning the correlation of our analyses, the associated LFQ intensities showed Pearson values ranging between 0.95-0.99 within extraction methods, underlying the robustness of the performed workflow. Nevertheless, Pearson correlation between different protocols revealed an average of 0.7 (Suppl Table 1), demonstrating a fine correlation of data, although poorer than in proteomes. Hence, again, this may respond to distinct stability of unsteady modifications through each pipeline.

GO enrichment analysis was performed on corresponding modified proteins for each condition. Selecting the 10 most enriched terms in biological processes, most of them were found in both extractions, although specific terms were detected for each condition (Figure 3A). While TEx yielded

phospho-sites related to organization and cell cycle proteins, urea-derived sites were placed in RNA and chromatin-associated proteins. Likewise, even if the majority of the cellular compartments to which the quantified sites belonged were essentially common between urea and TEx (Figure 3B), junction proteins were somewhat more represented in TEx data.

The analysis of linear motifs showed that while the most abundant serine sites are essentially comparable, the most enriched modifications differ between conditions. TEx tends to retrieve basic linear Ser-motifs, containing basic R chains prior to the phosphorylation site, whereas urea most enriched motifs contain acid residues in +1/+2 position (Figure 3C). This ability of extracting modifications of distinctive nature may also be valuable for a more comprehensive vision of samples when combining protocols, particularly in cases when a deeper analysis of sites or an improved understanding of kinases are desired. Using the PhosphoSitePlus database (phosphositeplus.org) as reference list, we detected 941 previously unreported sites within the class I phosphorylations identified in our experiment (12528 sites) (Figure 3D). Interestingly, 33 were phospho-Tyr residues, corresponding to 3.5% of novel sites, proportion three times higher than in the full lists of class I sites (Figure 3D). This data may hence constitute a useful source for the scientific community and future studies.

In summary, TEx proves as efficient as a widely used urea extraction for the mass spectrometry-based investigation of the whole proteome of SCaBER cells, both in a quantitative and qualitative manner, and potentially applicable to further mammalian cell types and tissues. The transferability of our conclusions to systems of other biological origin (non-mammalian cells, plants, fungi) remains to be explored. In our study, the majority of cellular proteins can be obtained and quantified following both TEx or urea extraction, making the implemented protocol presented here a valid strategy for proteomics. Phosphorylations providing a more complete understanding of signaling events, this post-translational modification can be likewise examined through TEx coupled to LC-MS/MS. However, although the amount and quality of phosphorylation sites are comparable between the extraction methods studied here, their nature differ. Urea extraction (or similar standard strategies) might indeed be advantageous for studies focusing exclusively on proteins and isoforms, as the implemented TEx is certainly more time consuming and bears greater protein losses (Suppl Table 1). Nevertheless, TEx represents a major

benefit when multi-omics data must be integrated and especially in the case of scarce and/or heterogenous samples.

FIGURE LEGENDS

Figure 1. Experimental workflow and proteomics from urea and TEx. A) Urea extraction (n=3) and triple extraction (n=3) followed by SAX fractionation for proteomic analysis and TiO₂ for phosphorylation enrichment. B) Identified proteins per replicate. C) Overlap of identified and quantified proteins between extractions. D) Quantified proteins among the identifications, per replicate. E) Exemplified correlation between a urea and a TEx replicate. F) Normalized spectral abundance factor (NSAF) of proteins obtained in each extraction method.

Figure 2. Protein abundances, GO enrichments and phosphorylation analysis. A) Intensities associated to proteins of different abundances across replicates. B, C) GO enrichments calculated for urea and TEx-related samples separately. D) Events detected as phosphorylated or not through mass spectrometry. E) Class I phosphorylation sites (light grey) detected in the whole of our study. F) Overlap of class I modifications between urea and TEx. G) Distribution of sites according to the phosphorylation of Ser, Thr or Tyr residues. H) Quantified phosphorylation sites for each extraction method and independent experiment. I) Overlap of quantified class I phospho-sites between conditions. J) Hierarchical clustering of quantified sites in at least one LC-MS run (grey corresponds to no intensity/quantification).

Figure 3. Nature of extracted phosphorylated sites and novel reported sites. A, B) GO enrichment for terms of Biological Processes and Cellular Compartment, for the proteins bearing sites distinctly quantified through urea and TEx. C) Linear motifs enriched within phosphorylation sites detected in urea, in triple extraction or through both procedures, as performed with Motif-X software. D) Novel sites found in the present work, taking PhosphoSite Plus database as reference.

SUPPLEMENTARY MATERIAL

Supplementary table 1: identified and quantified proteins; identified and quantified phosphorylation sites.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA ACCESSION

Raw data are available on the ProteomeXchange Consortium via the PRIDE partner repository with identifier PXD011971. Reviewers can find the data under the username reviewer_pxd011971@ebi.ac.uk, using the password: 3y4DXGCw.

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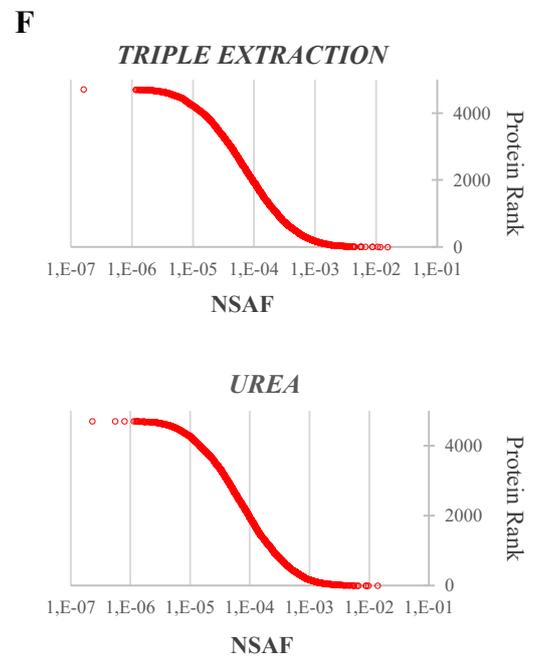
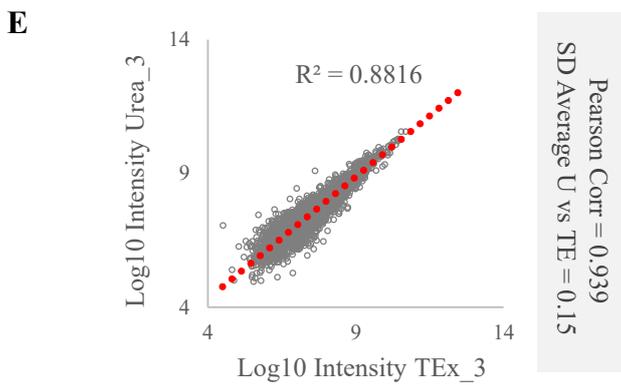
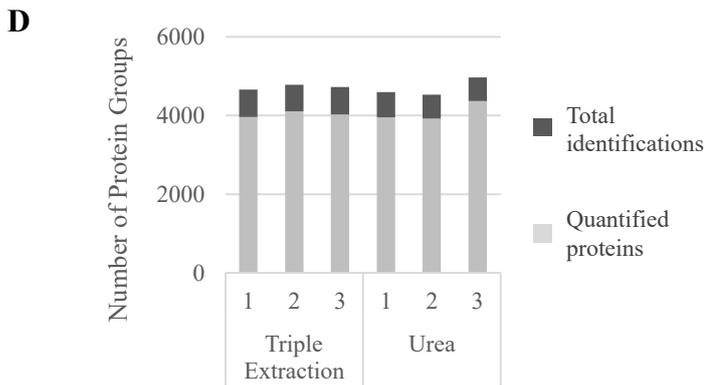
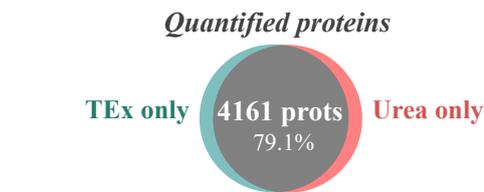
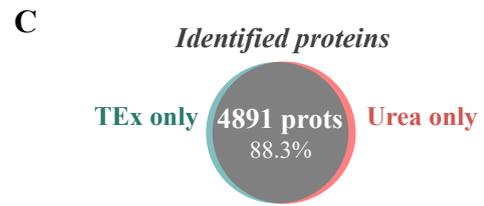
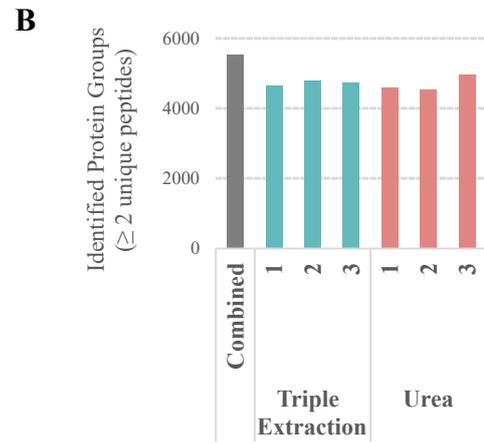
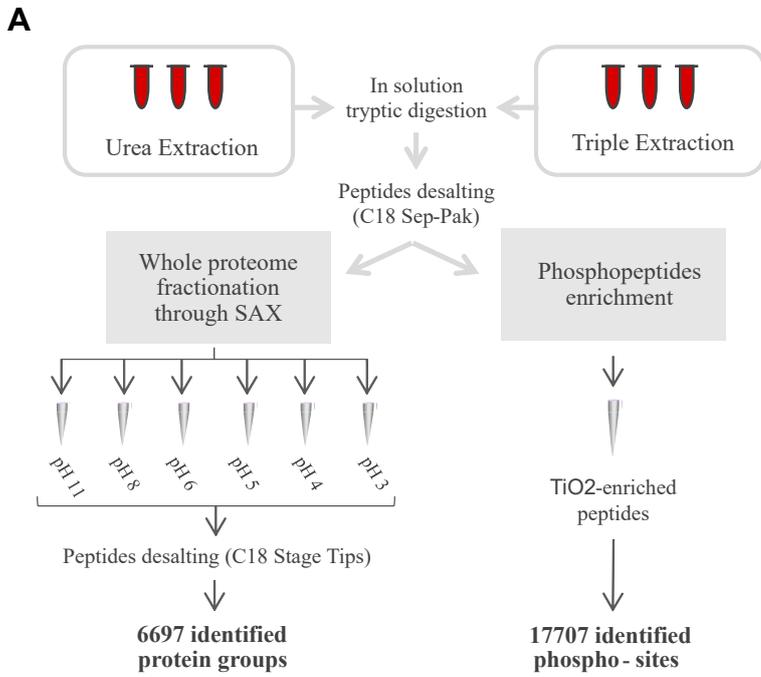
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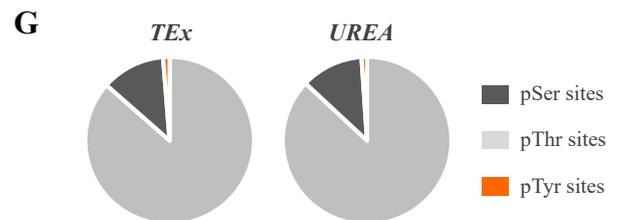
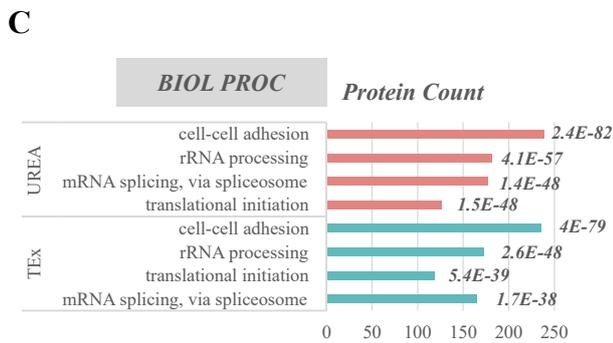
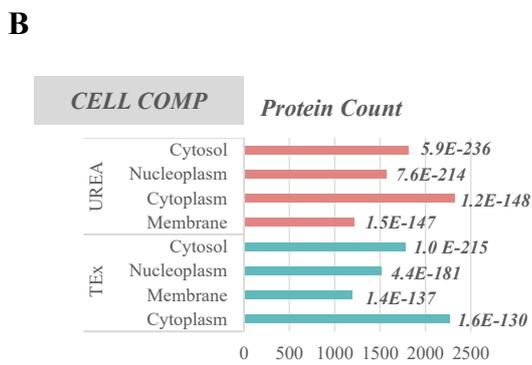
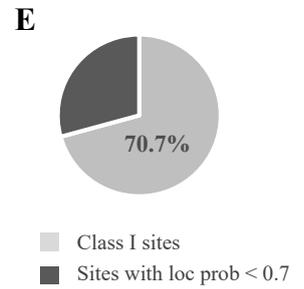
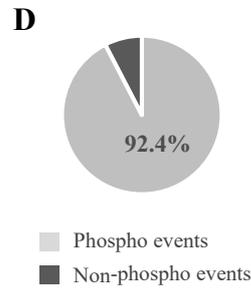
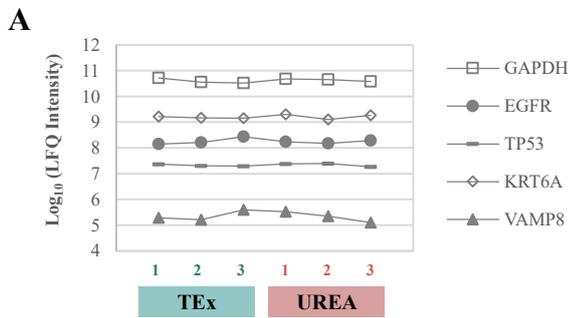
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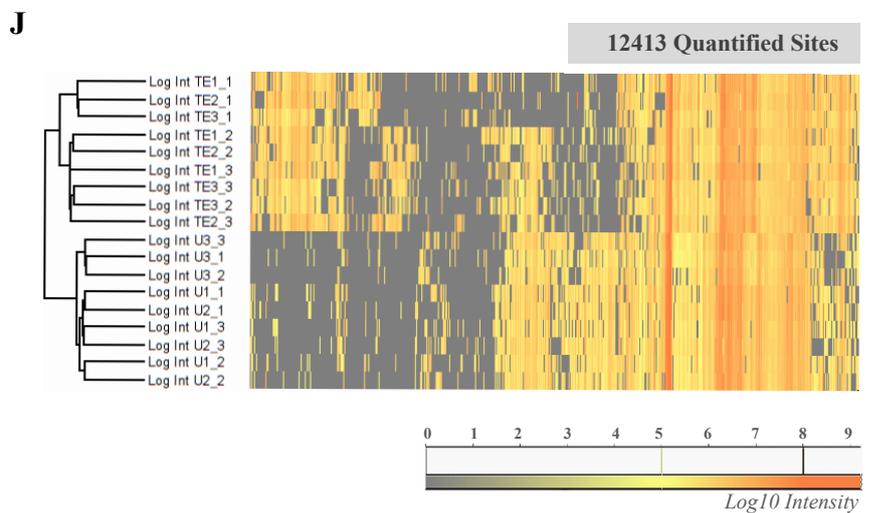
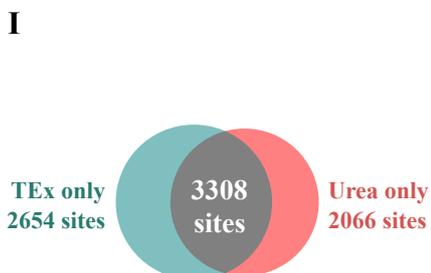




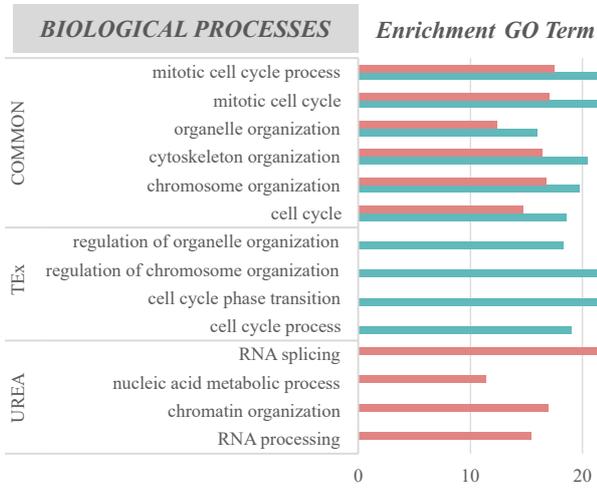
H

Quantified Phospho-Sites (2/3 runs)

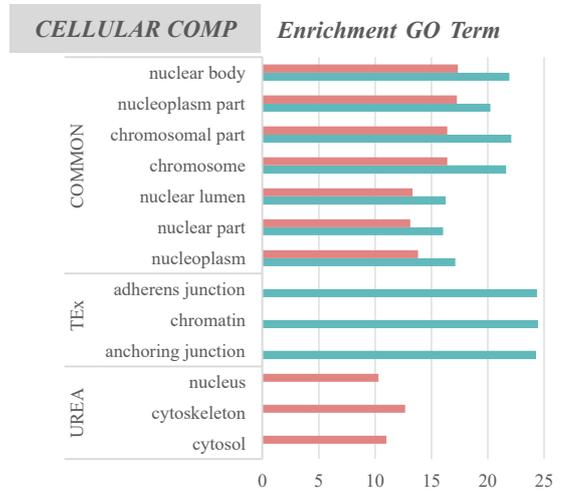
	TEx	Urea
1	7709	6765
2	7289	6530
3	7226	6242
Common	5964	5375



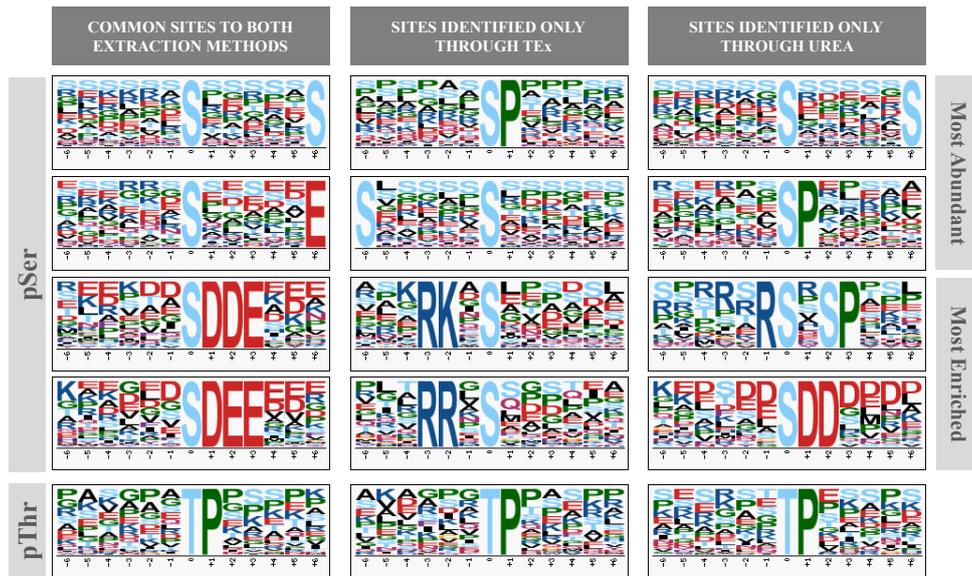
A



B



C



D

	Novel Identified Sites
Ser	633
Thr	275
Tyr	33



Ser-phosphorylated sites
 Thr-phosphorylated sites
 Tyr-phosphorylated sites