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Optimizing Workflows for LC/MS Analysis of Co-Immunoprecipitated Protein Complexes – "Soap Opera (tions)"

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Editorial

The ability to define specific, and often dynamic protein-protein associations within interactomes is critical to advancing an understanding of cell signal transduction pathways, drug design and the actions of therapeutic agents [1]. Standard approaches to assessing protein-protein interactions commonly have involved the retrieval of target proteins ("bait") plus partner protein co-immunoprecipitates; the isolation of partner proteins from 1D or 2D gels; and the identification of retrieved partners via LC-MS/MS [2]. The use of in vivo biotinylation [3,4] or HALO tagging of target / bait proteins [5,6] can further extend the detection of protein partnerships [7]. Limitations, however, can include the need for multi-step workflows, relatively large-scale preparations, and/or the construction of labeled baits. Shotgun approaches including MudPIT (multidimensional protein identification technologies) [8] also continue to be advanced that can bypass SDS-PAGE, and in certain formats avoid the need for elution of immune complexes from adsorbent gels. In RIME (Rapid Immunoprecipitation Mass Spectrometry) co-IP LC/MS, for example, immune complexes are proteolyzed from Ig or Ig- protein A/G/L beads and are analyzed directly by LC/MS [9,10]. Beyond this, in silico procedures also have recently been developed to guide cell lysis and/or subcellular fraction extraction methods, and co-IP workflows [11,12]. For LC-MS/MS data analysis, advanced algorithms and reference databases also continue to be developed (e.g., DIA/SWATH) [13]. During the earlier fundamental steps of cell lysate preparation and target/bait plus partner protein immunoprecipitation, the choice of detergents also becomes a point of central importance. This relates to the need to solubilize, but not disrupt protein complexes, while avoiding detergent incompatibilities with LC/MS. In a context of cell signal transduction, membrane proteins additionally can be key components (including transmembrane receptors), and this brings further attention to detergent considerations [14]. In particular, this includes a requirement to retain the solubility of hydrophobic proteins, while also maintaining protein-protein partnerships [15,16]. One non-ionic detergent frequently suggested as a potentially advantageous choice for this challenge is octyl beta-D-glucoside (OBG). This is based on OBG's effectiveness in solubilizing and retrieving membrane proteins [17,18], and OBG's exceptional property of rapid micelle disassembly upon dilution or dialysis [17,19]. In published studies, however, comparably few examples exist for OBG's use in cell lysis and co-IP (compared, for example, with Triton-X-100, Igepal, DOC, CHAPS). This includes systems that use FLAG-epitope tagged target/bait proteins. In a workflow context, a challenge also exists during immune complex isolation for replacing OBG (an LC-MS incompatible detergent) with an LC-MS compatible detergent that continues to preserve solubilized co-IP complexes. For LC-MS compatible detergents, new options are emerging [11,16,20]. Most continue to be ionic and/or strong detergents, several of which are proving to be useful in improving protein yields in urea and/or DOC extracts [11]. Certain, however, are zwitterionic (including "Silent PPS") [11] and therefore are attractive to consider as candidate detergents for processing membrane protein co-IP complexes prior to elution (e.g., in RapiGest), and LC/MS,. Here, we suggest that for co-IP LC/MS experiments involving membrane or membrane associated proteins, cell lysates and IP complexes might effectively be prepared by initially using OBG (CMC ~25 mM). Removal of OBG can then be via exchange, upon IP complex washing, in PPS (CMC ~0.1%) followed by the elution of partnered proteins in RapiGest (or a similar LC-MS compatible ionic detergent). Subsequent steps of denaturation in urea, peptide hydrolysis, and LC-MS can then advance. To provide initial proof-of-principle for this concept, we have tested this approach using FLAG-epitope tagged protein tyrosine phosphatase PTPN18 [21] stably expressed in hematopoietic progenitor UT7epo cells [22]. These studies (see Figure 1A for workflow) demonstrate: 1/ effective lysis of cells using 25 mM OBG (100 mM NaCl, 50 mM NaPi, pH 7.9) together with the efficient IP of FLAG-PTPN18 in OBG (as compared directly to Triton-X-100 at 0.5%) (Figure 1B); and 2/ excellent retention of FLAG-PTPN18 binding to anti-FLAG beads during washes with PPS (0.1%) with the efficient elution and recovery of FLAG-PTPN18 in RapiGest (0.25%) (Figure 1C). Alternatively, certain approaches are available for removing LC-MS incompatible detergents [23-25]. These, however, can lead to substantial loss of protein (and LC-MS signals), and residual detergent contamination. One aim of the present editorial therefore is to suggest that the sequential use of OBG and PPS in LC-MS experiments should improve membrane protein extraction, better preserve co-IP complexes, lessen sample processing, improve LC-MS signals, and further extend insight into protein-protein partnerships. Direct testing of this suggested workflow is ongoing, including those involving EPO receptor associated interactions [26,27].

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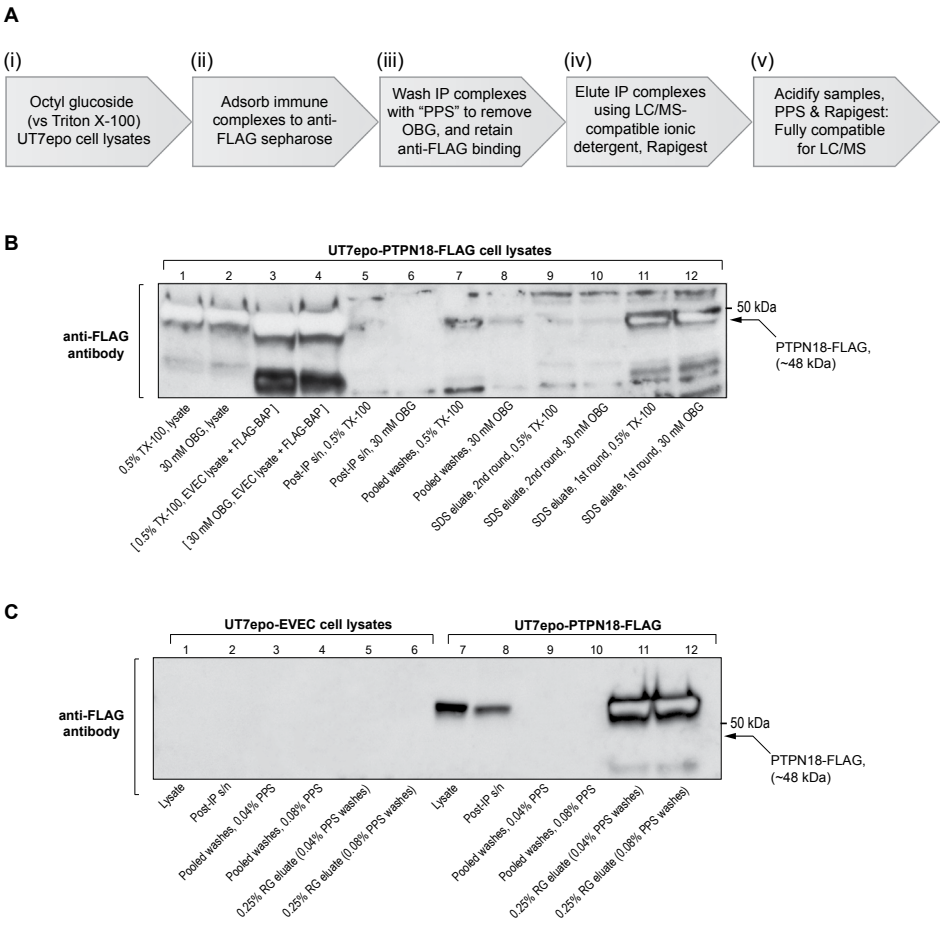

Figure 1: Sequential use of octyl beta-D-glucoside, PPS and Rapigest detergents to generate LC/MS compatible cell lysates and co-immunoprecipitated protein complexes. **1A)** Outlined workflow for: (i) the use of octyl beta-D-glucoside ("OBG") for cell lysate preparations (as assessed in parallel vs Triton X-100, "TX100"); (ii) retrieval of FLAG-protein immune complexes in OBG (*vs.* TX100); (iii) replacement of OBG, and preservation of IP complexes, using zwitterionic PPS Silent Surfactant ("PPS"); (iv) use of "Rapigest SF" (RG) to elute IP complexes with subsequent sample acidification to hydrolyze PPS and RG (v). Using these conditions, analysis and identification of target proteins and binding partners can then advance directly via LC/MS. **1B)** OBG provides for effective cell lysis, and efficient immunoprecipitation of FLAG epitope tagged bait proteins. In initial experiments, protein lysates were extracted using OBG or TX100 from UT7epo cells stably expressing FLAG epitope tagged Protein Tyrosine Phosphatase PTPN18, and from control UT7epo cells transduced with a corresponding empty vector (UT7epo-EVEC cells). Specifically, UT7epo-PTPN18-FLAG and UT7epo-EVEC cells were lysed in 30 mM OBG or 0.5% TX-100 in 150 mM NaCl, 20 mM HEPES, pH 7.8 for 10 minutes at 4°C (with protease and phosphatase inhibitors). An aliquot of lysate from each extraction was denatured and analyzed by western blotting for PTPN18-FLAG (lanes 1 & 2). (As a control for western blotting, BAP-FLAG protein also was added to analyzed cell lysates, lanes 3 & 4). Lysates were then pre-cleared with isotype-control IgG-agarose beads (1 hour, 4°C). Cleared supernatants were then incubated with anti-FLAG-agarose beads (4 hours, 4°C). Samples were centrifuged and supernatants were analyzed as unbound fractions (lanes 5 & 6). Antigen bound anti-FLAG beads were next washed four times in the surfactant used in cell lysis (i.e., either 30 mM OBG or 0.5% TX-100). Wash volumes were pooled, and analyzed by western blotting (lanes 7 & 8). Washed immune complexes were then eluted from anti-FLAG beads in 2% SDS, 20 mM Tris pH 6.8 (80°C for 10 minutes), and levels of eluted PTPN18-FLAG protein were determined (lanes 11 & 12). A second round of elution was also performed to test for possible residually bound PTPN18-FLAG (lanes 9 & 10). **1C)** Use of PPS to wash bait protein immune complexes and replace octyl beta-glucoside without disrupting anti-FLAG antibody binding. For cell lysates prepared from UT7epo-EVEC and UT7epo-PTPN18-FLAG using OBG (lanes 1 & 7), and processed through immunoprecipitations as in "B" above, but by comparison with PPS Silent Surfactant to replace OBG during immune complex wash steps (0.04% and 0.08% PPS concentrations tested). Post- IP bead wash samples (bead supernatants) are lanes 2-4, and 8-10. Subsequently, PTPN18-FLAG complexes were eluted from beads using 0.25% Rapigest (lanes 5-6, and 11-12).

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