Journal of Visualized Experiments

Label-Free Immunoprecipitation Mass Spectrometry Workflow for Large-scale Nuclear Interactome Profiling

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60432R1
Full Title:	Label-Free Immunoprecipitation Mass Spectrometry Workflow for Large-scale Nuclear Interactome Profiling
Section/Category:	JoVE Biochemistry
Keywords:	mass spectrometry; proteomics; Subcellular fractionation; Immunoprecipitation; Interactome; Bioinformatics,
Corresponding Author:	William Old University of Colorado Boulder Boulder, CO UNITED STATES
Corresponding Author's Institution:	University of Colorado Boulder
Corresponding Author E-Mail:	William.Old@colorado.edu
Order of Authors:	Steven Guard
	Christopher Ebmeier
	William Old
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Boulder, CO USA

TITLE:

Label-Free Immunoprecipitation Mass Spectrometry Workflow for Large-scale Nuclear Interactome Profiling

AUTHORS AND AFFILIATIONS:

Steven E. Guard[1], Christopher C. Ebmeier[1], William M. Old[1,2]

[1]Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO, USA

[2]Linda Crnic Institute for Down Syndrome, University of Colorado School of Medicine, Aurora, CO, USA

Corresponding Author:

William M. Old (william.old@colorado.edu)

Email Addresses of Co-authors:

Steven E. Guard (steven.guard@colorado.edu)

Christopher C. Ebmeier (christopher.ebmeier@colorado.edu)

KEYWORDS:

mass spectrometry, proteomics, subcellular fractionation, immunoprecipitation, interactome, bioinformatics, workflow

SUMMARY:

Described is a proteomics workflow for identifying protein interaction partners from a nuclear subcellular fraction using immunoaffinity enrichment of a given protein of interest and label-free mass spectrometry. The workflow includes subcellular fractionation, immunoprecipitation, filter aided sample preparation, offline cleanup, mass spectrometry, and a downstream bioinformatics pipeline.

ABSTRACT:

Immunoaffinity purification mass spectrometry (IP-MS) has emerged as a robust quantitative method of identifying protein-protein interactions. This publication presents a complete interaction proteomics workflow designed for identifying low abundance protein-protein interactions from the nucleus that could also be applied to other subcellular compartments. This workflow includes subcellular fractionation, immunoprecipitation, sample preparation, offline cleanup, single-shot label-free mass spectrometry, and downstream computational analysis and data visualization. Our protocol is optimized for detecting compartmentalized, low abundance interactions that are difficult to identify from whole cell lysates (e.g., transcription factor interactions in the nucleus) by immunoprecipitation of endogenous proteins from fractionated subcellular compartments. The sample preparation pipeline outlined here provides detailed instructions for the preparation of HeLa cell nuclear extract, immunoaffinity purification of endogenous bait protein, and quantitative mass spectrometry analysis. We also discuss methodological considerations for performing large-scale immunoprecipitation in mass

spectrometry-based interaction profiling experiments and provide guidelines for evaluating data quality to distinguish true positive protein interactions from nonspecific interactions. This approach is demonstrated here by investigating the nuclear interactome of the CMGC kinase, DYRK1A, a low abundance protein kinase with poorly defined interactions within the nucleus.

INTRODUCTION:

The human proteome exhibits vast structural and biochemical diversity through the formation of stable multisubunit complexes and transient protein-protein interactions. Accordingly, the identification of interaction partners for a protein of interest is commonly required in investigations to unravel molecular mechanism. Recent advances in affinity purification protocols and the advent of high-resolution fast-scanning mass spectrometry instrumentation have enabled easy mapping of protein interaction landscapes in a single unbiased experiment.

Protein interaction protocols commonly employ ectopic expression systems with affinity-tagged fusion constructs to identify protein interactions without requiring high-quality antibodies recognizing a protein of interest[1,2]. However, epitope tag-based methods have several drawbacks. Physical interactions with the epitope may lead to the detection of nonspecific copurifying proteins[3]. Additionally, fusion of these epitope tags to the N- or C-terminal of a protein may block native protein-protein interactions, or disrupt protein folding to promote non-physiological conformations[4]. Furthermore, ectopic expression systems typically overexpress the bait protein at supraphysiological concentrations, which can result in the identification of artifactual protein interactions, particularly for dosage-sensitive genes[5]. To circumvent these issues, the endogenous bait protein can be immunoprecipitated along with associated interacting prey proteins, assuming availability of a high-quality antibody that recognizes the native protein.

Provided here is an interaction proteomics workflow for detecting the nuclear interactome of an endogenous protein using the CMGC protein kinase DYRK1A as an example. Disruption of DYRK1A copy number, activity level, or expression can cause severe intellectual disability in humans, and embryonic lethality in mice[6–9]. DYRK1A exhibits dynamic spatiotemporal regulation[10], and compartmentalized protein interactions[11,12], requiring approaches capable of detecting low abundance interaction partners specific to different subcellular compartments.

This protocol employs cellular fractionation of human HeLa cells into cytosol and nucleoplasm fractions, immunoprecipitation, sample preparation for mass spectrometry, and an overview of a bioinformatic pipeline for evaluating data quality and visualizing results, with R scripts provided for analysis and visualization (**Figure 1**)**.** Proteomics software packages used in this workflow are all freely available for download or can be accessed through a web interface. For additional information on software and computational methods, in-depth tutorials and instruction are available at the links provided.

PROTOCOL:

NOTE: All buffer compositions and protease mixtures are outlined in **Table 1**.

1. Preparation of cells

NOTE: A starting material of 1−10 mg nuclear lysate per replicate is desired for this immunoprecipitation mass spectrometry (IP-MS) approach. Cell quantities will be given for 1 mg of nuclear immunoprecipitations in triplicate plus triplicate controls.

1.1. If using an adherent cell line, grow the cells to 90% confluency in 3 x 15 cm dishes per replicate prior to harvesting.

NOTE: It is recommended to perform a minimum of three replicate immunoprecipitations for bait and control conditions. This protocol will assume the use of 'beads only' controls, which control for abundant nonspecific interactions with the beads, starting at section 4. Other types of controls may be useful. These are described in depth in the discussion section.

1.1.1. Wash plates 2x with phosphate buffered saline (PBS) and trypsinize cells using 3 mL of 0.25% trypsin per 15 cm plate. Spin down the cells at 1,200 x *g* for 5 min and decant the trypsin.

1.2. For suspension cells, grow to a similar scale/density to achieve 70−80 mg of total protein.

1.2.1. Pellet cells at 1,200 x *g* and 4 °C for 5 min. Decant the media carefully.

NOTE: Pellets can be combined during this step to enable efficient processing using the large-scale subcellular fractionation outlined in section 2.

1.3. Wash the cell pellet 2x with PBS + 5 mM $MgCl_2$ supplemented with protease inhibitors (PIs) and phosphatase inhibitors (PhIs) (see **Table 1**).

NOTE: Cell pellets may be flash frozen in liquid nitrogen and stored at -80 °C until ready for fractionation.

2. Preparation of nuclear extract

NOTE: Protease and phosphatase inhibitors should be added to the fractionation buffers within 30 min of use.

2.1. If frozen, thaw the cell pellets for 15 min in 1x pellet volume of cold Buffer A + PIs/ PhIs. Place the cell pellet on a nutator at 4 °C to aid in resuspension while thawing. Otherwise, resuspend the cell pellet from step 1.3 into 1x pellet volume Buffer A + PIs /PhIs.

2.1.1. Pellet at 2,000 x *g* and 4 °C for 10 min. Decant the buffer.

2.2. Suspend the cells with 5x the packed cell volume with Buffer A and incubate on ice for 20 min.

2.3. Pellet at 2,000 x *g* and 4 °C for 10 min. Decant the buffer and resuspend with 2x original packed cell volume Buffer A + PIs/ PhIs and dounce ~7x with "A"/loose pestle.

2.4. Centrifuge the lysate for 10 min at 2,000 x *g* and 4 °C.

2.5. Carefully pipette off the supernatant and flash freeze with liquid nitrogen. Store the lysate at -80 °C. The supernatant from this step is the cytosolic subcellular fraction.

NOTE: The nuclear pellet can be saved during this step by flash freezing with liquid nitrogen and storing at -80 °C

2.6. Resuspend the pellet with 0.9x pellet volume of Buffer B + PIs/ PhIs and mix on a nutator for 5 min at 4 °C.

2.7. To lyse the nuclei, dounce 20x with a tighter pestle "B".

2.8. Mix the nuclear lysate on a nutator for 30 min at 4 °C so that it is homogenous.

2.9. Centrifuge the nuclear lysate for 30 min at 21,000 x *g* at 4 °C. Pipette off the supernatant and save as a soluble nuclear protein extract.

NOTE: Nuclease treatment of the resulting nuclear pellet allows for the recovery of a chromatin-associated protein fraction.

2.10. Dialyze the soluble nuclear extract against Buffer C + PIs for 3 h at 4 °C.

2.10.1. Cut an appropriate length of 24 mm width dialysis tubing with an 8 kDa molecular weight cut off. Clamp one side of the tubing and load nucleoplasm into the tube. After loading the lysate, clamp the other end and submerge into a clean glass container containing Buffer C + PIs.

2.11. Centrifuge the dialyzed nuclear extract/nucleoplasm at 21,000 x *g* at 4 °C for 30 min. Aliquot 3x 20 µL volumes of nuclear extract for fractionation validation by western blot. The nuclear extract used for IP-MS analysis can be aliquoted and flash frozen in liquid nitrogen and stored at -80 °C, if needed.

3. Validation of subcellular fractionation

3.1. Complete a protein assay to determine the protein concentration of the nuclear lysate. A bicinchoninic acid protein assay provides sufficient sensitivity for downstream application.

3.2. Load 20 µg of both the cytosolic and nuclear fractions on an SDS-PAGE gel for the western blot analysis as previously described[13]. Skip lanes when loading to avoid mischaracterization of a sample.

3.3. Probe the western blot for p84 (THOC1) as a nuclear marker, and GAPDH as a cytosolic marker. Determine the extent of fractionation by the ratio of cytosolic marker in the nuclear fraction and vice versa.

NOTE: Antibodies for other nuclear and cytosolic markers may be used.

4. Immunoprecipitation of endogenous nuclear bait protein

NOTE: It is recommended to use low retention tubes from this point on. This will reduce the nonspecific binding to the tubes during sample handling and avoid unnecessary loss of sample. Additionally, ensure that LCMS grade H_2O is used to prepare buffers for the remaining steps.

4.1. Prepare a protein A/G bead mixture for each replicate by combining 12.5 µL of bead volume for both protein-A and protein-G in microcentrifuge tubes. Store the bead stocks as a slurry containing 20% ethanol. Determine the concentration of beads within the slurry %(v/v) and pipette the necessary volume using a pipette tip that has been cut on the tip to ensure that the beads can enter the tip.

4.2. Wash the protein A/G bead mixture 2x with 300 µL of IP Buffer 1. Spin the beads at 1,500 x *g* at 4 °C for 1 min and decant buffer.

4.3. Prepare the antibody-protein A/G beads: To bind the antibody to the beads, add 300 µL of IP Buffer 1 and 10 µg of the desired antibody. Allow the bead/antibody mixture to rock on a nutator at 4 °C overnight. For bead-only controls, do not add any antibody.

NOTE: A total of 10 µg of antibody per replicate can be used as a starting point, but the exact amount will need to be optimized for each individual antibody and scale of the lysate used in the experiment

4.4. Thaw the nuclear lysates from step 2.10 in a water bath and aliquot appropriate volumes into low retention microcentrifuge tubes for 1 mg protein input per replicate.

4.4.1. Spin the lysate at 16,000 x *g* for 30 min and transfer the supernatant to a new tube.

4.4.2. Add 1 µL of benzonase (250 units/µL) per 1 mg of the nuclear lysate and rock on a nutator at 4 °C for 10–15 min.

4.5. Prepare beads for preclearing the lysate. Add 12.5 µL of each protein A and protein G beads to 1.5 mL low retention tubes as in step 4.1. Wash 2x with IP Wash Buffer 1 + PIs and decant the buffer.

4.6. Add 1 mg of the nuclear lysate to the beads from step 4.5. Incubate while rocking on a nutator for 1 h at 4 °C.

4.6.1. Centrifuge precleared lysates at 1,500 x *g* and 4 °C for 1 min.

4.6.2. While nuclear lysates are incubating with beads in step 4.5.1, wash the antibody-protein A/G beads 2x with IP Buffer 1 + PIs. Centrifuge at 1,500 x *g* and 4 °C for 1 min and decant the buffer.

4.7. Transfer the precleared nuclear lysate from step 4.6.1 onto the antibody-protein A/G beads. Incubate while rocking on a nutator at 4 °C for 4 h. Centrifuge following the incubation at 1,500 x *g* and 4 °C for 1 min.

4.8. Transfer the supernatant into tubes labeled as the flow through for each replicate.

4.9. Wash the antibody-protein A/G beads with 1 mL of IP Buffer 2 + PIs. Centrifuge at 1,500 x *g* and 4 °C for 1 min, decant buffer, and repeat for a total of 3x.

4.10. Wash the beads 2x with 1 mL of IP Buffer 1+ PIs centrifuging as in the previous step. Ensure that all buffer is removed after the last wash.

4.11. Elute 2x with 20 µL of 0.1 M glycine (pH 2.75) for 30 min each. Ensure that the tubes is rocking during the incubation with the elution buffer. Spin at 750 x *g* and 4 °C for 1 min and pipette off the supernatant after each 30 min incubation.

NOTE: While the low pH glycine method described here elutes most bait proteins, some antibody-antigen interactions require more stringent buffer conditions.

4.12. Flash freeze eluates in liquid nitrogen and store at -80 °C.

5. Sample preparation

NOTE: Insulin spiked into the immunoprecipitation elution samples aids in the recovery of proteins during trichloroacetic acid (TCA) precipitation and sample processing, which is important for low abundance endogenous bait proteins.

5.1. Thaw the eluates at room temperature if frozen.

5.1.1. Place the samples on ice and add 10 µL of 1.0 mg/mL insulin for every 100 µL of eluate. Vortex and then immediately add 10 µL of 1% sodium deoxycholate. Vortex the sample again and add 30 µL of 20% TCA followed by one final vortex.

5.1.2. Incubate the samples on ice for 20 min, then centrifuge at 21,000 x *g* at 4 °C for 30 min.

5.1.3. Aspirate the supernatant and add 0.5 mL of acetone that has been prechilled to -20 °C. Vortex and then spin at 21,000 x *g* and 4 °C for 30 min. Repeat this step.

5.1.4. Aspirate the supernatant and air dry the pellet remaining in the bottom of the tube.

5.2. Prepare the sample for mass spectrometry using a modified Filter Aided Sample Prep (FASP) method, optimized for reducing sample handling, as outlined below[14].

5.2.1. Resuspend the protein pellet from step 5.1.4 with 30 µL of SDS Alkylation Buffer (see **Table 1**). Incubate the sample on a 95 °C heat block for 5 min. Let it cool at room temperature for 15 min before preceding to the next step.

5.2.2. Add 300 µL of UA solution and 30 µL of 100 mM TCEP to each sample. Load this solution onto a 30k centrifugal filter. Spin the centrifugal filter at 21,000 x *g* at room temperature for 10 min.

NOTE: The bait protein and its putative interactors should be bound to the filter at this point. However, the flow through may be kept, in case there is a problem with the filter.

5.2.3. Wash the filter with 250 µL of UA and centrifuge at 21,000 x *g* for 10 min. Decant the flow through and repeat for a total of 3x.

5.2.4. Wash the filter with 100 µL of 100 mM Tris pH 8.5 and centrifuge at 21,000 x *g* for 10 min. Decant the flow through and repeat for a total of 3x.

5.2.5. Add 3 µL of 1 µg/µL Lys C resuspended in 0.1 M Tris pH 8.5. Fill the filters up to the 100 µL mark and allow to digest for 1 h at 37 °C while rocking on a nutator.

5.2.6. Add 1 µL of 1 µg/ µL MS grade trypsin. Mix gently and allow for the trypsin to incubate with the sample overnight at 37 °C while rocking on a nutator.

5.2.7. Centrifuge at 21,000 x *g* for 20 min to elute the peptide from the filter.

NOTE: Multiple rounds of centrifugation may be required to recover all the eluate. If this is not done, there is a potential for severe sample loss.

5.3. Desalt the peptides using C18 spin columns. Follow the protocol provided by the manufacturer.

5.4. Resuspend the lyophilized peptide in 7 µL of 0.1% TFA in 5% acetonitrile. Sonicate the sample for 3 min to ensure that the peptides have been resuspended. Spin down at 14,000 x *g* for 10 min.

5.5. Transfer the resuspended peptide into an appropriate sample vial to for loading onto the liquid chromatography–mass spectrometry (LC/MS) system.

6. LC/MS system suitability

NOTE: Due to the small scale and generally lower abundance of protein from affinity-purified samples, it is critical that the LC/MS platform operates at a maximal sensitivity and robustness.

6.1. Add 1 mL of LC/MS grade formic acid to 1 L of LC/MS grade water for mobile phase A, and add 1 mL of LC/MS grade formic acid to 1 L of LC/MS grade acetonitrile for mobile phase B.

6.2. Prepare or install a 75 µm fused-silica capillary column packed with <2 µm reversed-phase C18 resin that is ≥250 mm in length. Best results will be had with a direct injection of samples into the column.

6.3. Purge the Ultra Performance Liquid Chromatography (UPLC) system with fresh mobile phases. With a C18 column installed, establish a stable flow rate and electrospray with a suitable emitter (i.e., 20 µm id x 360 µm od pulled to a 10 µm tip). Maintain the column at 40−60 °C.

6.4. Test the overall LC/MS system performance by injecting a complex quality control standard, such as 100−200ng of a HeLa whole cell lysate tryptic digest. Elute with a suitable gradient for a complex sample (i.e., 2−3 h gradient elution time). Establish a baseline system performance of the peptide and protein identifications.

NOTE: For best results, 3,000−5,000 or more protein identifications from 20,000−35,000 unique peptides will provide optimal performance for experimental samples.

6.5. For routine LC/MS system suitability, inject 100−200 fmol or less of a single protein digest standard, such as Bovine Serum Albumin (BSA). Elute with a short gradient (i.e., 20−30 min).

NOTE: Multiple injections of a protein digest will help establish baseline LC/MS system performance, and repeat injection after each IP-MS sample provides a measure of system performance throughout the experiment and allows for the detection of instrument drift, which can bias label-free experiments. A baseline of the select individual peak intensities and peak shapes will inform on MS, LC, and column performance.

6.6. To avoid overloading the analytical column, load a small portion (15−30% of the total) of an experimental sample onto the column and separate using a gradient suitable for complex samples (i.e., 2−3 h). If the number of protein identifications is unsatisfactory, load all of the sample onto the column.

6.7. Run a single protein digest standard in between samples to monitor LC/MS system performance and sample carryover. Multiple standards may be required to reduce sample carryover depending on your samples.

7. Data Processing

7.1. Download the proteomics software package MaxQuant found at

https://www.maxquant.org/.

NOTE: This will be used to process the RAW MS data file from step 6.6 into data tables of protein IDs, gene names, and quantitative values associated with the identification of these for downstream analysis.

7.1.1. Select **Load** within the **Input Data** subheader of the **Raw Data** tab. Open the file location where the MS raw files are stored and select raw files for each MS/MS run.

7.1.2. Click on the **Group-Specific** tab and select **Digestion**. Within the enzyme list select **LysC** and click on the right arrow to add this enzyme into the list that will be used in the search. Next, select **Instrument** and ensure that the correct instrument type appears in the drop-down list at the top of the screen. Leave other group-specific search parameters on standard settings.

7.1.3. Click on the **Global Parameters** tab and select **Sequences**. Add the appropriate FASTA file for the taxonomy that will be used in this search. Peptides will not be assigned properly if this is not done. For the human proteome, download the FASTA file from UniProt at https://www.uniprot.org/help/human_proteome.

7.1.4. Within the **Global Parameters** tab, click on **Protein Quantification**. Within the **Peptides for Quantification** drop-down menu, select **Unique + Razor**.

NOTE: MaxQuant offers alternate quantitation of proteins through intensity-based absolute quantification (iBAQ) and label-free quantitation (LFQ). However, peptide count information is sufficient for the downstream analysis in this protocol[15].

7.1.5. In the bottom left of the MaxQuant interface, select the number of processors to be used for the search. This will directly affect the length of time required for the run, so select as many as possible for this). Click **Start** in the bottom left of the screen to start the run. Select the **Performance** tab at the top of the screen to view the progress of the search.

7.2. When the run has completed, open the proteingroups.txt file in Perseus, a proteomics computation platform, or other spreadsheet program to view the data[16].

7.2.1. Use Perseus to remove common contaminants and hits to reversed protein sequences. Follow the detailed Perseus documentation at http://www.coxdocs.org/doku.php?id=perseus:user:use_cases:interactions.

NOTE: Opening the proteingroups.txt file in analysis software (e.g., Excel) will automatically corrupt certain gene and protein names.

7.3. Analyze experimental data using the Contaminant Repository for Affinity Purification (CRAPome). Register an account at this repository http://crapome.org/ and follow the tutorial as needed[17,18].

7.3.1. Use the **Analyze Your Data** workflow found on the CRAPome homepage. Select external controls that correspond to the affinity purification system used in this interaction experiment.

NOTE: These controls can be used to calculate a second fold change enrichment that is useful for detecting common contaminants.

7.3.2. Generate an input file from the proteingroups.txt output from MaxQuant using Perseus or a suitable spreadsheet application. Details for manual formatting can be found at http://crapome.org/?q=fileformatting. Alternatively, use the provided R script "export_CRAPomeSAINT_Input_File.R" to generate the SAINT/CRAPome input file. See README.txt in the **Supplemental Coding Files**.

7.3.3. Run an analysis to determine fold-change enrichment and SAINT (Significance Analysis of INTeractome) probability for each bait protein in the immunoprecipitation. Ensure that **'User Controls**' is selected in the drop-down menu under **FC-A, 'CRAPome controls'** or **'All Controls'** are selected in the **FC-B** drop-down and **Probability Score** is selected to generate SAINT probabilities. When the run has concluded, view the output that is available under **'Analysis Results'** along with a job ID. Download the data matrix from the **'Analysis Results'** for future plotting and data visualization.

7.4. Plot proteins as a function of FC-A (IPs vs. user controls) and SAINT probability by following the R-Scripts as provided in **Supplemental Coding Files**.

NOTE: A set of R scripts is provided for producing plots of FC-A vs. SAINT probability and iBAQ vs. $\log_2$ (protein abundance), colored by the adjusted p value range from empirical Bayes analysis of the label-free intensities. The details of the differential statistical analysis and plotting are found in README.txt and the R script "main_differential_analysis.R" in the **Supplemental Coding Files**.

7.5. Evaluate where known interacting proteins of the bait protein are ranked by FC-A and SAINT. Make a cutoff of FC-A > 3.00 and SAINT > 0.7 for single bait experiments in triplicate as a starting point.

NOTE: Selection of cutoffs for a "high-confidence" interactor and a "low-confidence" interactor must be informed by biological information.

8. Data visualization

NOTE: There are many programs that can effectively visualize proteomics data (e.g., R, Perseus, Cytoscape, STRING-DB). Analyzing the connectivity between high-confidence hits, and functional enrichment of these interactors can be a useful strategy for prioritizing hits for further validation and functional characterization.

8.1. Download Cytoscape, an open source network visualization tool at

https://cytoscape.org/download.html[19].

8.2. Prepare an input file for interaction data as a tab delimited file formatted with three columns: bait (source node), prey (target node), type of interaction (edge type). This can be done in Perseus or any spreadsheet program of your choice.

8.3. Select the **Import Table from File** icon towards the top left of the program (designated by a downwards arrow and a matrix in the icon). Cytoscape will auto-populate the interaction data into a network ready for custom formatting and design.

8.4. Select the **Style** tab on the control panel for Cytoscape and click on the squares in the **Def** column to adjust the attribute for the entire network. Select specific nodes or edges in the network and then select the square within the **Byp.** column of the style menu to bypass the default settings and adjust only selected objects. Alternatively, click on the drop-down menu at the top of the style menu to view the preset network formats.

NOTE: STRING-db protein-protein interaction data may be integrated into this network at this time either manually through the input file or through various enrichment tools available as plug-ins in Cytoscape, http://apps.cytoscape.org/[20]. A recommended cytoscape plug-in for enrichment analysis is found at http://apps.cytoscape.org/apps/cluego[21].

8.5. To increase the confidence in the dataset generated in this workflow, perform reciprocal IP-MS or IP-western experiments that target prey proteins of interest as the bait.

REPRESENTATIVE RESULTS:

The majority of protein mass identified in an IP-MS experiment consists of nonspecific proteins. Thus, one of the key challenges of an IP-MS experiment is the interpretation of which proteins are high-confidence interactors vs. nonspecific interactors. To demonstrate the crucial parameters used in the evaluation of data quality the study analyzed triplicate immunoprecipitations from 5 mg of HeLa nuclear extract utilizing a bead only control. The first internal check to ensure that an IP-MS experiment is reliable is whether the bait protein ranks as one of the highest enriched proteins identified by both fold-change over control and SAINT probability. In this case, the bait DYRK1A ranked among the top three enriched proteins over the control (**Figure 2A**,**B**)**.** In a nuclear interactome study of DYRK1A utilizing four independent antibodies, an FC-A cutoff of >3.00 and SAINT probability cutoff >0.7 provided a stringent cutoff for identification of both novel and previously validated interactors[22]. When applied to this experiment, a clear separation could be seen between the high-confidence interactors and >95% of copurified proteins identified as nonspecific (**Figure 2A**,**B**)**.** Applying both a fold change enrichment and probability threshold increases stringency by requiring a consistently high enrichment of protein IDs across biological replicates.

In addition to statistical scoring, the CRAPome analysis workflow also maps previously reported interactions onto bait-prey data[23]. While this mapping can be useful for thresholding high and low-confidence interactions, previously reported interactions can score poorly by FC-A and SAINT

probabilities, potentially indicating that many known interactions of a given bait may exist only in specific cell types, contexts, or organelles. For the example DYRK1A dataset, iREF interactor FC-A values were as low as 0.45, representing a very low enrichment over control (**Figure 2C**)**.** To avoid inflation of false positives, statistical thresholding should be performed in a manner that prioritizes stringency over reduction of false negatives. It should be noted that the detection of these interactions was independent of protein abundance (**Figure 2C**)**.** Calculated absolute copy number of each iREF interaction within HeLa cells showed no correlation to the detection levels of an interaction partner by IP-MS[24].

Cytoscape serves as an effective tool for visualizing multiple layers of interaction data[19]. In the DYRK1A immunoprecipitation experiment described here, the combined use of FC-A > 3.0 and SAINT > 0.9 reduced the list of high-confidence interactors to six proteins (**Figure 2D**)**.** However, when applying an FC-A cutoff of > 3.0 in isolation, eight additional proteins were added to the network. These additional protein interactors have high connectivity with the interactors already in the network, suggesting they are associated in similar complexes or functional roles. To this end, evidence from the STRING-DB of protein-protein interactions was integrated into this network as blue dashed lines[20]. While this single-bait, triplicate experiment provides a limited sample of the full DYRK1A interaction network, the use of additional baits, replicates, and integration of large public data sets can be used to expand the network of high-confidence interactions. The statistical cutoffs will thus be specific to each individual experiment and will need to be evaluated thoroughly.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative proteomics workflow for subcellular IP-MS. Cells are grown in either 4 L round bottom flasks or 15 cm tissue culture dishes and harvested at the same time for subcellular fractionation. Cells are fractionated into a cytosolic, nuclear, and a nuclear pellet, and immunoprecipitations are done from 1−10 mg of nuclear lysate using one or multiple antibodies recognizing the same bait. Filter aided sample prep (FASP) and offline sample cleanup are performed prior to single shot mass spectrometry. A downstream computational pipeline is used to process data into interpretable interaction data.

Figure 2: Representative data for a single-bait single-antibody IP-MS experiment. (**A**) FC-A and SAINT probability output from CRAPome analysis workflow for an optimal experiment using a single antibody for the kinase DYRK1A (n = 3). Beads-only controls were used for comparison. Red solid lines represent cutoffs set at FC-A > 3.00 and SAINT > 0.7. (**B**) MaxQuant protein abundance estimates (iBAQ) output vs. log2 ratio of protein abundance in DYRK1A IP to control, colored by the adjusted p value range from empirical Bayes analysis of the label-free intensities. (**C**) FC-A and estimated copy number of proteins listed as interacting proteins in the iRef database[23,24]. (D) Cytoscape network visualization of DYRK1A interactors. Blue nodes = FC-A > 3.00, SAINT > 0.7. Orange nodes = FC-A > 3.00. Black edges = proteins identified as interactors in IPMS experiment. Blue dashed edge = SAINT interaction between prey protein (confidence > .150).

Table 1: Buffer compositions

DISCUSSION:

The proteomics workflow outlined here provides an effective method for identifying high-confidence protein interactors for a protein of interest. This approach decreases the sample complexity through subcellular fraction and focuses on increasing the identification interaction partners through robust sample preparation, offline sample clean up, and stringent quality control of the LC-MS system. The downstream data analysis described here allows for a simple statistical evaluation of the proteins identified as copurifying with the bait. However, due to a high number of experimental variables (scale, cell line, antibody choice), each experiment requires different cutoffs and considerations regarding data visualization and enrichment.

The first design consideration in an IP-MS experiment is the selection of antibodies that will be used for copurification of the protein of interest along with its interacting partners. While the availability of commercial antibodies has expanded to cover larger portions of the human proteome over the past several decades, there are still many proteins for which reagents are limited. Furthermore, antibodies that have been validated for applications such as western blot detection may be incapable of selective enrichment of the target protein in an immunoprecipitation experiment. Prior to conducting a large-scale interaction proteomics experiment, it is suggested to complete an IP from a 90% confluent 10 cm dish, or equivalent cell number, and probe for the target protein of interest by western blotting. If more than a single antibody is available for immunoprecipitation, it is additionally suggested to select multiple antibodies recognizing epitopes within different portions of the protein. The binding of an antibody to a bait protein can occlude the necessary binding interface for putative interacting partners. Selection of a secondary epitope for the bait protein will increase the coverage of the interaction profile identified by a mass spectrometry-based experiment.

A second major consideration lies in the selection of the appropriate control for distinguishing high-confidence interactions from low-confidence or nonspecific interactions from those identified as copurifying with the bait. The most stringent control for an IP-MS experiment is to complete the immunoprecipitation from a CRISPR KO cell line of the bait. Such a control enables identification and filtering out of nonspecific proteins that bind directly to the antibody rather than the bait protein. In cases where generating a CRISPR KO cell line of each bait protein is not feasible, an IgG-bead control of the same isotype of the bait antibody can be used. In experiments employing a panel of antibodies representing multiple species, the use of a beads only control can be appropriate but will increase the rate of false positives identified as high-confidence interactors.

Selection of the cell line used in an IP-MS experiment is dependent on several key factors. Protein expression and localization are largely dependent on cell type. While RNA expression estimates can be found for most genes in many commonly used cell lines, protein expression is poorly correlated with RNA expression and must be determined experimentally[25]. Cell lines in which a bait protein is expressed in very low copy number should be avoided to circumvent problems associated with drastic increases in cell culture scale that may be required. It should be noted, however, that sample preparation can be optimized for the detection of very low abundance

proteins. The filter aided sample prep (FASP) method, while robust, can cause a more than 50% loss of peptide in a sample. The Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) is an efficient method of generating samples for mass spectrometry analysis that minimizes sample loss[26]. The increased recovery enabled by the SP3 method of sample preparation can be a useful alternative in this workflow for quantification of proteins that fall near the limit of detection.

This proteomics workflow has been applied across many nuclear baits, including kinases, E3 ubiquitin ligases, and scaffolding members of multisubunit complexes. Assuming proper validation of antibody reagents, successful execution of this workflow will result in detection of high-confidence protein nuclear interaction partners for a protein of interest.

ACKNOWLEDGMENTS:

This work was supported by a Grand Challenge grant to W.M.O. from the Linda Crnic Institute for Down syndrome and by a DARPA cooperative agreement 13-34-RTA-FP-007. We would like to thank Jesse Kurland and Kira Cozzolino for their contributions in reading and commenting on the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

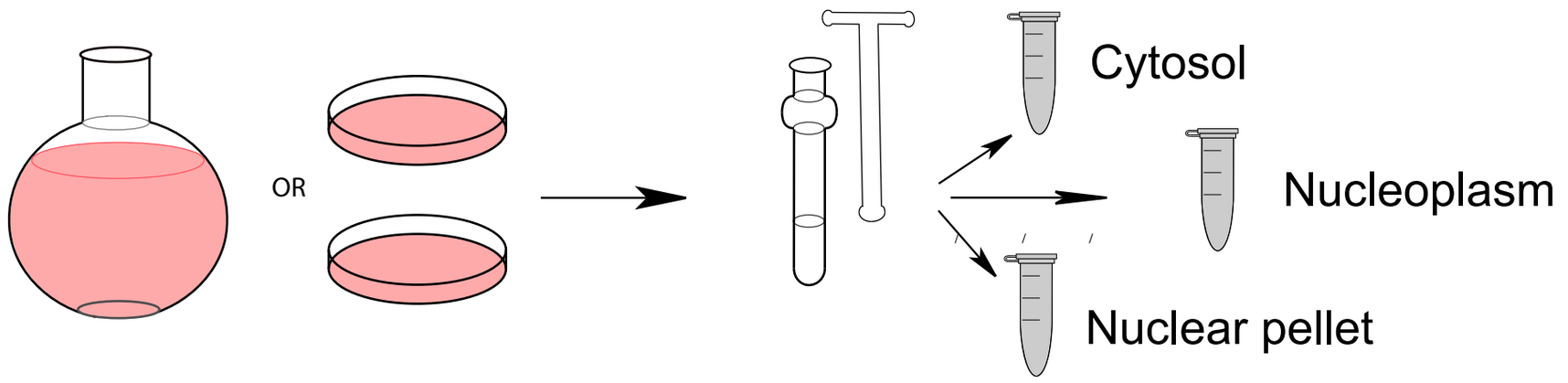
REFERENCES:

1. Varjosalo, M. et al. The protein interaction landscape of the human CMGC kinase group. *Cell Reports.* **3**, 1306–1320 (2013).
2. Kimple, M. E., Brill, A. L., Pasker, R. L. Overview of Affinity Tags for Protein Purification. *Current Protocols in Protein Science.* **73**, Unit (2013).
3. Mahmood, N., Xie, J. An endogenous 'nonspecific' protein detected by a His-tag antibody is human transcription regulator YY1. *Data in Brief.* **2**, 52 (2015).
4. Zordan, R. E., Beliveau, B. J., Trow, J. A., Craig, N. L., Cormack, B. P. Avoiding the ends: internal epitope tagging of proteins using transposon Tn7. *Genetics.* **200**, 47–58 (2015).
5. Gibson, T. J., Seiler, M., Veitia, R. A. The transience of transient overexpression. *Nature Methods.* **10**, 715–721 (2013).
6. Bronicki, L. M. et al. Ten new cases further delineate the syndromic intellectual disability phenotype caused by mutations in DYRK1A. *European Journal of Human Genetics.* **23**, 1482–1487 (2015).
7. Antonarakis, S. E. Down syndrome and the complexity of genome dosage imbalance. *Nature Reviews Genetics.* (2016).
8. Dowjat, W. K. et al. Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome. *Neuroscience Letters.* **413**, 77–81 (2007).
9. Fotaki, V. et al. Dyrk1A haploinsufficiency affects viability and causes developmental delay and abnormal brain morphology in mice. *Molecular and Cellular Biology.* **22**, 6636–6647 (2002).
10. Hämmerle, B., Elizalde, C., Tejedor, F. J. The spatio-temporal and subcellular expression of the candidate Down syndrome gene Mnb/Dyrk1A in the developing mouse brain suggests distinct sequential roles in neuronal development. *European Journal of Neuroscience.* **27**, 1061–1074 (2008).

11. Funakoshi, E. et al. Overexpression of the human MNB/DYRK1A gene induces formation of multinucleate cells through overduplication of the centrosome. *BMC Molecular and Cell Biology.* **4**, 12 (2003).
12. Yu, D., Cattoglio, C., Xue, Y., Zhou, Q. A complex between DYRK1A and DCAF7 phosphorylates the C-terminal domain of RNA polymerase II to promote myogenesis. *Nucleic Acids Research.* 1–14 (2019).
13. Towbin, H., Staehelin, T., Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America.* **76**, 4350–4 (1979).
14. Wiśniewski, J. R., Zougman, A., Nagaraj, N., Mann, M. Universal sample preparation method for proteome analysis. *Nature Methods.* **6**, 359–362 (2009).
15. Tyanova, S., Temu, T., Cox, J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nature Protocols.* **11**, 2301–2319 (2016).
16. Tyanova, S. et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods.* **13**, 731–740 (2016).
17. Mellacheruvu, D. et al. The CRAPome: A contaminant repository for affinity purification-mass spectrometry data. *Nature Methods.* **10**, 730–736 (2013).
18. Choi, H. et al. SAINT: Probabilistic scoring of affinity purificationg-mass spectrometry data. *Nature Methods* **8**, 70–73 (2011).
19. Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research.* **13**, 2498–504 (2003).
20. Szklarczyk, D. et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Research.* **45**, D362–D368 (2017).
21. Bindea, G. et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics.* **25**, 1091–1093 (2009).
22. Guard, S. E. et al. The nuclear interactome of DYRK1A reveals a functional role in DNA damage repair. *Scientific Reports.* **9**, 6539 (2019).
23. Razick, S., Magklaras, G., Donaldson, I. M. iRefIndex: A consolidated protein interaction database with provenance. *BMC Bioinformatics.* **9**, 405 (2008).
24. Kulak, N. A., Pichler, G., Paron, I., Nagaraj, N., Mann, M. Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. *Nature Methods.* **11**, 319–324 (2014).
25. Liu, Y., Beyer, A., Aebersold, R. On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell.* **165**, 535–550 (2016).
26. Hughes, C. S. et al. Ultrasensitive proteome analysis using paramagnetic bead technology. *Molecular Systems Biology.* **10**, 757 (2014).

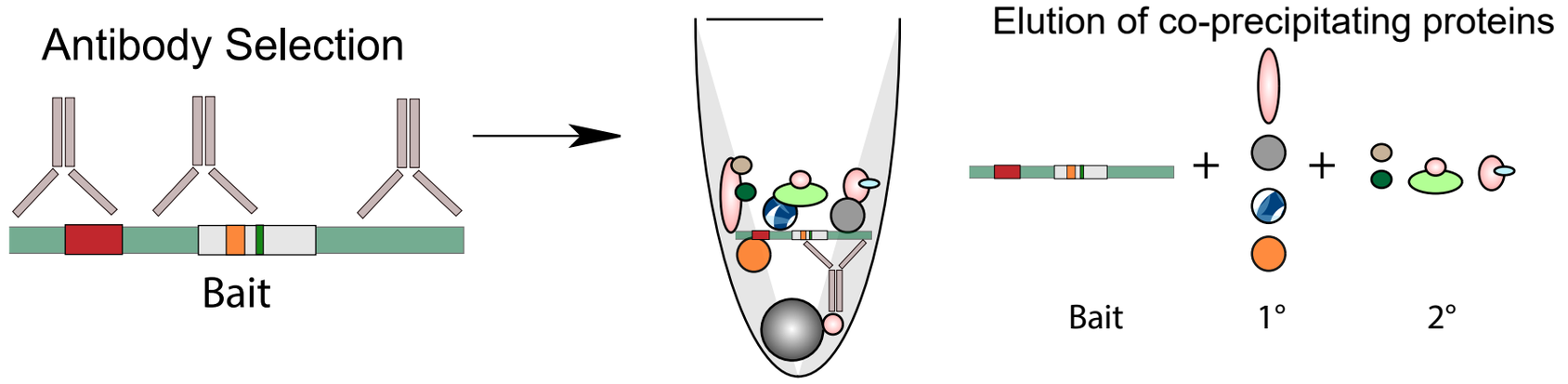
1.

Generation of Nuclear Extracts



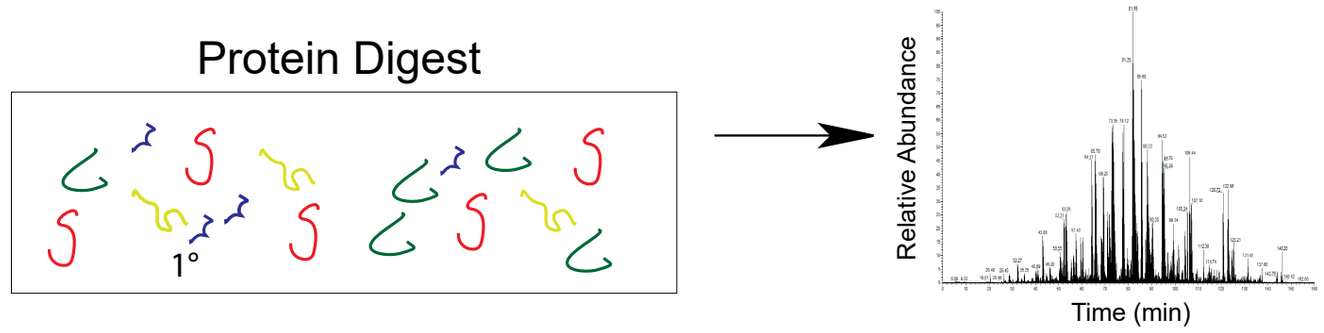
2.

Immunoprecipitation



3.

Filter Aided Sample Prep and Single-Shot Mass Spectrometry



4.

Processesing of RAW Data and Computational Analysis

Protein IDs	Gene names	Peptides	Unique peptides
A0AV96-2	RBM47	5	1
A0AVF1	TTC26	17	6
A0AVI4-2	TMEM129	1	0
A0AVT1	UBA6	58	50
A0FGR8-2	ESYT2	2	28
. . .	. . .	. . .	. . .

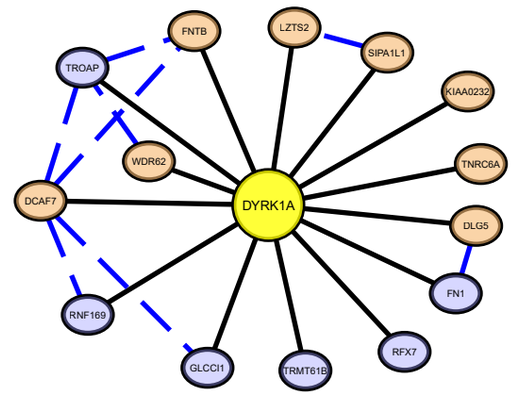
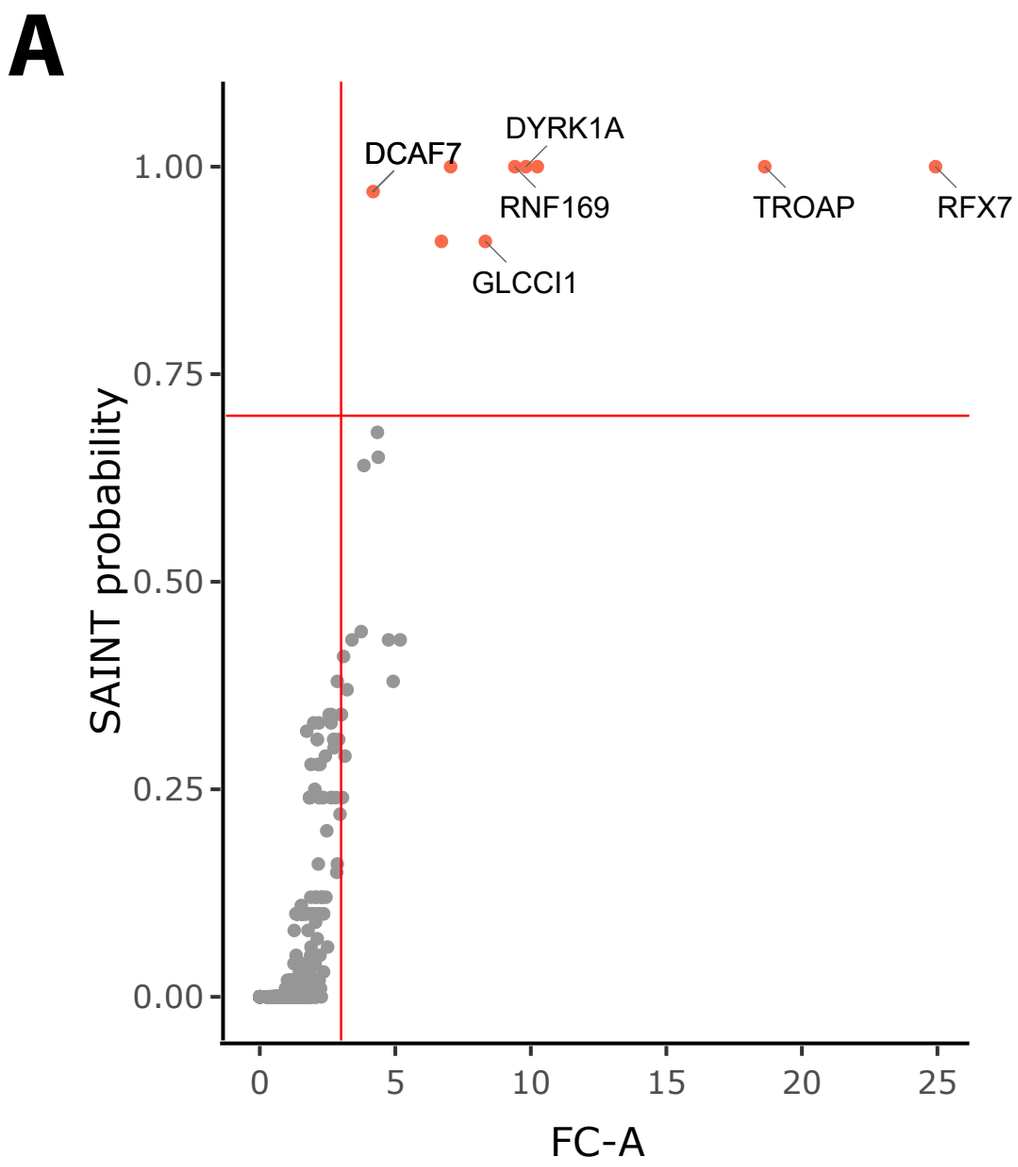
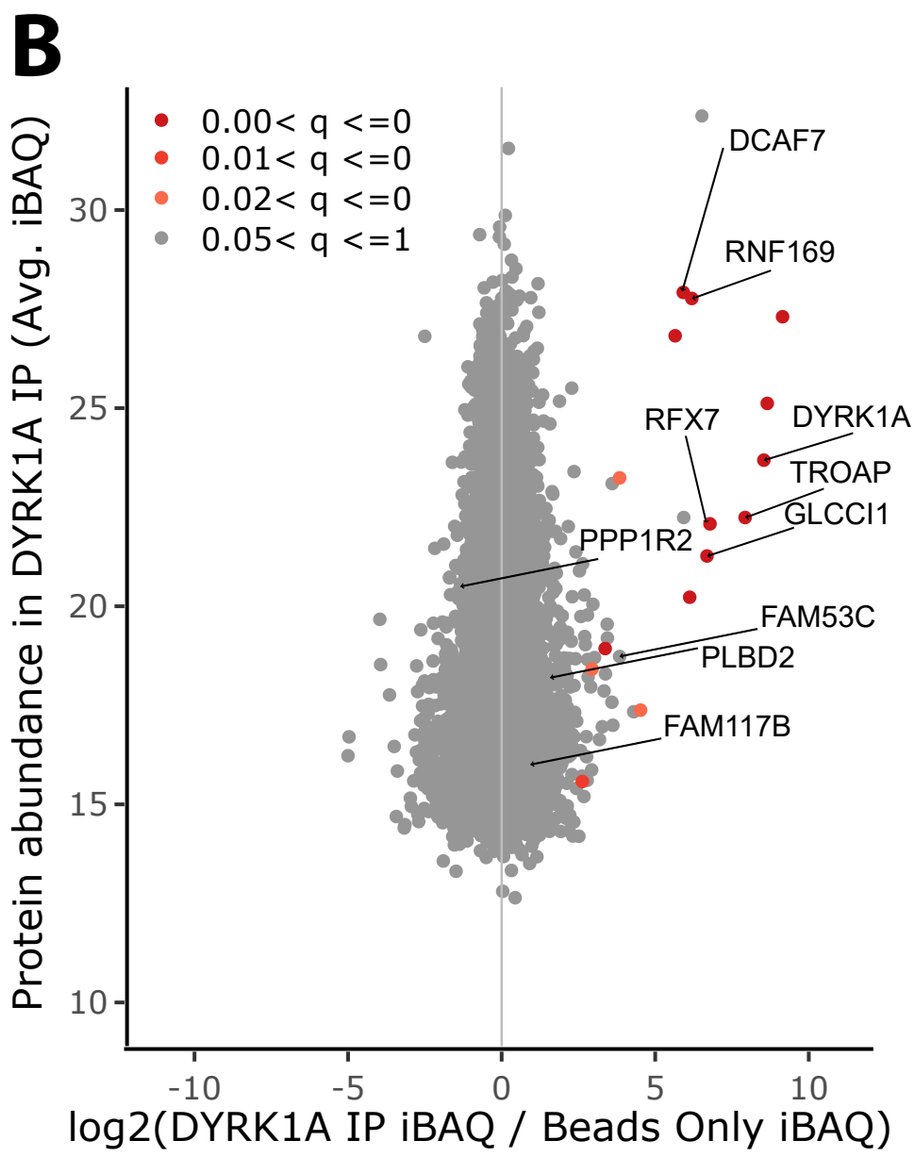
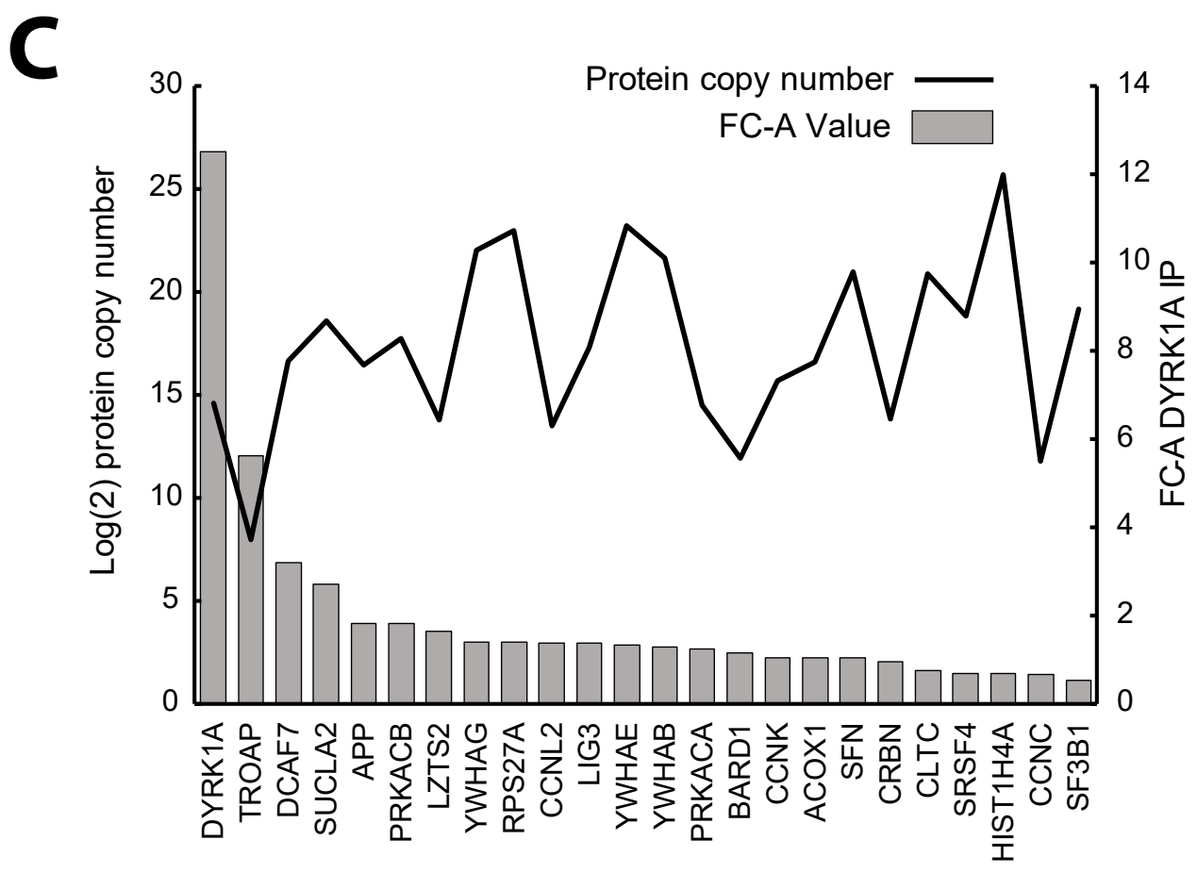


Figure 1







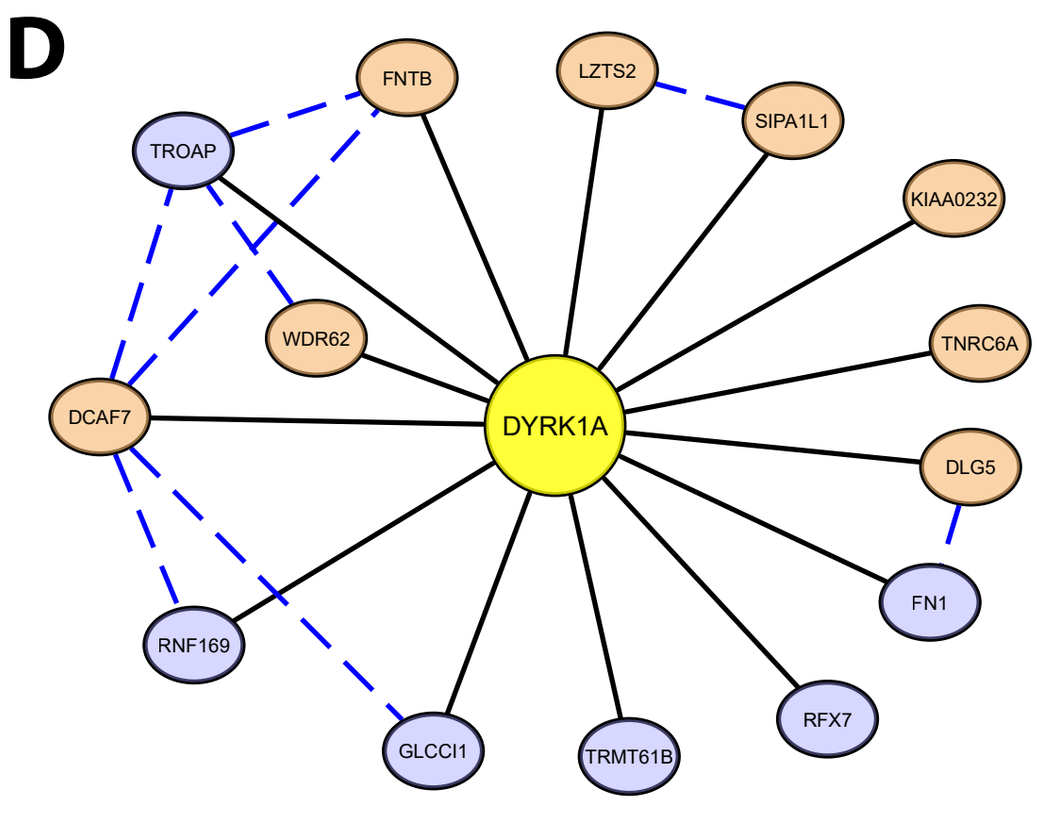


Figure 2

Table 1

Click here to access/download;Table;Buffer Composition Table.xlsx

Protease inhibitor (PI) mixture

Reagent	Final Concentration
Sodium Metabisulfite	1 mM
Benzamidine	1 mM
Dithiothreitol (DTT)	1 mM
Phenylmethanesulfonyl fluoride (PMSF)	0.25 mM

Phosphatase Inhibitor (PhI) mixture

Reagent	Final Concentration
Microcystin LR	1 µM
Sodium Orthovanadate	0.1 mM
Sodium fluoride	5 mM

Subcellular fractionation Buffers :

Buffer A pH 7.9

Reagent	Final Concentration
HEPES	10 mM
MgCl2	1.5 mM
KCl	10 mM

Buffer B pH 7.9

Reagent	Final Concentration
HEPES	20 mM
MgCl2	1.5 mM
NaCl	420 mM
Ethylenediaminetetraacetic acid (EDTA)	0.4 mM
Glycerol	25% (v/v)

Buffer C pH 7.9

Reagent	Final Concentration
HEPES	20 mM
MgCl2	2 mM
KCl	100 mM
Ethylenediaminetetraacetic acid (EDTA)	0.4 mM
Glycerol	20% (v/v)

Immunoprecipitation Buffers :

IP Buffer 1

Reagent	Final Concentration
HEPES	20 mM
KCl	150 mM
EDTA	0.1 mM
NP-40	0.1% (v/v)
Glycerol	10% (v/v)

IP Buffer 2

Reagent	Final Concentration
HEPES	20 mM
KCl	500 mM
EDTA	0.1 mM
NP-40	0.1% (v/v)
Glycerol	10% (v/v)

SDS Alkylation Buffer pH 8.5

Reagent	Final Concentration
SDS	4% (v/v)
Chloroacetamide	40 mM
TCEP	10 mM
Tris	100 mM

UA pH 8.5

Reagent	Final Concentration
Urea	8 M
Tris	0.1 M

* use HPLC grade H2O

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% Trypsin, 0.1% EDTA	Thermo Fisher Scientific	25200056	
1.5 ml low-rention microcentrifuge tubes	Fisher Scientific	02-681-320	
4-20% Mini PROTEAN TGX Precast Protein Gels	Bio-Rad	4561096	
acetone (HPLC)	Thermo Fisher Scientific	A949SK-4	
Amicon Ultra 0.5 ml 30k filter column	Millipore Sigma	UFC503096	
Benzamidine	Sigma-Aldrich	12072	
benzonase	Sigma-Aldrich	E1014	
Chloroacetamide	Sigma-Aldrich	C0267	
Dialysis tubing closure	Caroline Biological Supply Company	684239	
DTT	Sigma-Aldrich	10197777001	
EDTA	Sigma-Aldrich	EDS	
GAPDH antibody	Santa Cruz Biotechnology	Sc-47724	
Glycerol	Fisher Scientific	887845	
Glycine	Sigma-Aldrich	G8898	
HeLa QC tryptic digest	Pierce	88329	
HEPES	Fisher Scientific	AAJ1692630	
insulin	Thermo Fisher Scientific	12585014	
iodoacetamide	Sigma-Aldrich	I1149	
KONTES Dounce homogenizer 7 ml	VWR	KT885300-0007	
Large Clearance pestle 7ml	VWR	KT885301-0007	
Lysyl endopeptidase C	VWR	125-05061	
Magnesium Chloride	Sigma-Aldrich	208337	
Microcystin	enzo life sciences	ALX-350-012-C100	
Nonidet P 40 Substitute solution	Sigma-Aldrich	98379	
p84 antibody	GeneTex	GTX70220	
Phosphate Buffered Saline			
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23227	
Pierce BSA Protein Digest, MS grade	Thermo Fisher Scientific	88341	LCMS QC
Pierce C18 spin columns	Thermo Fisher Scientific	PI-89873	
Pierce Trypsin Protease, MS Grade	Thermo Fisher Scientific	90057	For mass spectrometry sample prep
PMSF	Sigma-Aldrich	P7626	
Potassium Chloride	Sigma-Aldrich	P9541	
Protein A Sepharose CL-4B	GE Healthcare Bio-Sciences	17-0780-01	
Protein G Sepharose 4 Fast Flow	GE Healthcare Bio-Sciences	17-0618-01	
SDS	Sigma-Aldrich	L3771	
Silica emitter tip	Pico TIP	FS360-20-10	
Small Clearance pestle 7ml	VWR	KT885302-0007	
Sodium Chloride	Sigma-Aldrich	S3014	
Sodium Fluoride	Sigma-Aldrich	201154	
Sodium metabisulfite	Sigma-Aldrich	31448	
Sodium orthovanadate	Sigma-Aldrich	S6508	
Spectra/ Por 8 kDa 24 mm dialysis tubing	Thomas Scientific	3787K17	
TC Dish 150, Standard	Sarstedt	83.3903	Tissue culture dish for adherent cells
TCA	Sigma-Aldrich	T9159	
TCEP	Thermo Scientific	PG82080	
TFA	Thermo Fisher Scientific	28904	
Thermo Scientific Orbitrap Fusion MS	Thermo Fisher Scientific		
Trizma Base	Sigma-Aldrich	T6066	
Urea	Thermo Fisher Scientific	29700	
Waters ACQUITY M-Class UPLC	Waters		
Waters ACQUITY UPLC M-Class Column Reversed-Phase 1.7µm Spherical Hybrid (1.7 µm, 75 µm x 250 mm)	Waters	186007484	nanoflow C18 column

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Label-Free Immunoprecipitation Mass Spectrometry Workflow for Large-scale Nuclear Interactome Profiling
Author(s):	Steven E Guard, Christopher C. Ebmeier, William M. Old

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

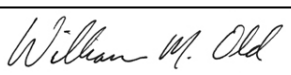
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	**William M. Old**
Department:	**Dept. of Molecular, Cellular and Developmental Biology**
Institution:	**University of Colorado Boulder**
Title:	**Assistant Professor**
Signature:	
Date:	**6/14/2019**

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

University of Colorado Boulder

Department of Molecular, Cellular and Developmental Biology
347 UCB
Boulder, Colorado 80309-0596

T 303.492.9551
F 303.492.7744
william.old@colorado.edu

26 February 2019

Dr. Vineeta Bajaj
Review Editor
JoVE

Dear Dr. Bajaj,

Enclosed please find our revised manuscript by Guard et al, entitled "Label-Free Immunoprecipitation Mass Spectrometry Workflow for Large-scale Nuclear Interactome Profiling", which we submit for reconsideration by *JoVE.*

We appreciated the useful feedback from the editors and reviewers, and the opportunity to address the referees' critiques, which we feel have improved the manuscript considerably. Included below this letter is our point by point responses to the editorial and referees' critiques, which we believe satisfactorily address the major concerns.

We hope that by addressing the previous reviews, our manuscript will be acceptable for eventual publication. We thank you for your consideration.

Sincerely,

William M. Old, Ph.D.
Assistant Professor
Dept. of MCD Biology
University of Colorado Boulder
Campus Box 347
Boulder, CO 80309-0347
Office: 303-492-9551
Lab: 303-492-9598

RESPONSES TO REFEREE COMMENTS

We thank the referees for their thoughtful comments, and have taken their feedback seriously, and address each of their concerns below.

Editorial comments:

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.**

-This comment has been addressed by editing throughout the manuscript to improve readability, and corrected spelling and grammar errors.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

-This comment has been addressed in the text.

3. The Protocol should contain only action items that direct the reader to do something.

-Individual steps throughout the protocol have been revised to read as action items. Other text or non-actionable items have been revised, removed, or moved into a note below the original step.

4. Please ensure you answer the "how" question, i.e., how is the step performed?

-This comment has been resolved where applicable. However, there are several steps where the 'how' falls outside of the scope of this protocol, specifically regarding the installation of mass spectrometry components and the specific details of the mass spectrometry elution methods, as these will be instrument/software specific and every possibility could not be covered here.

5. Software steps must be more explicitly explained ('click', 'select', etc.)

-This comment has been addressed in sections 7 and 8 of the text.

6. Step 7,8: Please use imperative tense and describe how the procedure is performed.

- These changes have been made where applicable with few exceptions:

(1) As there are many effective ways to create properly formatted input files for programs like CRAPome or Cytoscape (sections 7 and 8), we have referenced R scripts / readme instructions for doing so rather than laying out all detail within the protocol. We hope that this provides sufficient clarity without extending this workflow past the 10-page protocol limit.

7. Please move all the buffer and solution recipes to a separate table in .xlsx and upload it separately to your editorial manager account and refer the table in the text wherever applicable.

-We created a buffer and solution recipe table that is referenced throughout the text.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

-Individual steps have been simplified throughout the text, with a particularly large revision to section 7.

9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
-The protocol portion of this manuscript is less than 10 pages, and we have ensured that the highlighted portion of text is less than 2.75 pages.

10. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example Thermo's Xcalibur software package, Thermo Fisher instruments, etc.
-This comment has been addressed in the text.

11. Please ensure that all the figures are referenced in the manuscript text in the order of their numbering.
-This comment has been addressed in the text. Note that Figure 2 of the original manuscript has been removed, Figure 3 has been shifted to be new Figure 2 and an additional panel 2D has been added.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
- *All figures presented in this manuscript are original and have not appeared in a previous publication.*

13. Please do not abbreviate the journal titles in the references section.
-This comment has been addressed in the text.

14. Figure 2B: Please consider making this a separate table in .xlsx and upload all the tables individually to your editorial manager account.
- *To simplify the scope of this manuscript, Figure 2 has been removed and will no longer be discussed in the representative results sections. There are readily available reviews of liquid chromatography mass spectrometry (LCMS) system suitability and troubleshooting. We have deemed this complex subject outside of the scope of our nuclear interactome workflow.*

Reviewers' comments:
Reviewer #1:

Major Concerns:

1. The entirety of Section 6 is a major concern. It's important to ensure system suitability (especially as the authors point out for these small samples for AP-MS), but there are not enough details in this section to allow for a viewer to have success. In 6.2 -- What column? What diameter? What LC? What Emitter? In 6.4 -- What instrument? What method? Why 4500 protein identifications and not say 3000? This seems quite arbitrary as written. The choice of instrument and database for searching will have a very strong effect on the performance of the method in terms of protein identification.

-This comment has been useful in providing clarity to our workflow protocol. Section 6 has been completely rewritten to represent a more concise step-by-step protocol. Specific detail regarding column dimensions, emitter specifications and length of method have been including in the revised edition. While a Thermo Fisher Orbitrap Fusion mass spectrometer was used as the initial reference for performance, we realize that many research groups will have access to different mass spectrometers spanning a wide range of performance. Accordingly, we have changed our recommendations for protein identification and peptide identification to ranges that would provide a base level of performance required for this scale of experiment. The details of these revisions can be found within the note under step 6.4. and should be independent of instrument and database.

2. 7.1.1 -- What maxquant parameters should be used?
-Section 7 of this protocol has been rewritten to provide step by step instruction on which Maxquant parameters should be used for this protocol.

3. The use of a non-target protein or bead-only control to eliminate common "contaminants" isn't really discussed until 8.2 and should be discussed sooner.

-We strongly agree with this recommendation regarding the discussion of controls for an immunoprecipitation mass spectrometry experiment. A note has been added into the first step of the protocol regarding selection of control type and number of control replicates. This note also references the detailed examination of control conditions found in the discussion section of this manuscript.

4. Representative Results -- Lines 427-443/Figure 2a -- This section mirrors my comments on Section 6 above. Definition of poor performance as "charging" isn't always the case. A column may have gone bad, protein may have precipitated on the trap, sample could have been over/under loaded on the column, etc etc etc. It could be more complex.

-This comment has been useful in clarifying the scope of our manuscript similarly to comment #1. The section referenced in this comment has been removed from the manuscript to avoid confusion regarding evaluation of system suitability. The previous section was an oversimplified outline of several key parameters that can affect LCMS system performance. The readily available literature and troubleshooting manuals specific to each mass spectrometer serve as a better resource for instrument evaluation.

Minor Concerns:

1. **Abbreviations are used liberally, sometimes without prior definition!**
2. **4.1 -- What is meant by "Determine the V/V%"?**
3. **There are two 4.5's**
4. **4.12 -- elutions – eluates**
5. **Note below 5.2.3 -- It's the bait protein plus its putative interactors, correct?**
6. **Line 395 --> e.x --> e.g.**

-The above noted minor concerns have been clarified and resolved in the text.

Reviewer #2:

Minor Concerns:

1. The recommendation for increasing confidence in the results in line 414-424 should be included in the Quality Control section rather than Data Visualisation section.

-The text representing these lines in the original manuscript have been revised for clarification. This note now suggests a method validating protein interactions with prey proteins of interest.

2. The authors suggest Cytoscape plugins for integrating network generated by Mass Spectrometry versus String Database. It will be good if they can mention examples.

- We recommend CLUEGO as a useful plug-in for enrichment analysis, integration of public interaction evidence and clustering of nodes within your interaction data. This has been included in the note following step 8.4. alongside link to the cytoscape app store that will allow the user to find a plug-in that most specifically fits their needs.

Reviewer #3:

Major Concerns:
no major concerns.

Minor Concerns:

1. please expand abbreviations in protocol (e.g PMSF, DTT etc)
2. any advice on choice of tubes and plasticware required? other general advice that doesn't fit into the stepwise procedure but is helpful to know?

-This comment has been useful in clarifying important experimental detail. Additional notes have been added within the protocol regarding the use of low-retention plasticware, LCMS grade H_2O for sample preparation buffers, as well as important computation considerations on how to handle the data e.g. NOTE: Opening the proteingroups.txt file in Microsoft Excel will automatically convert certain gene and protein names into dates.

3. **you have 2 step 2.8s (line 147, 149)**
4. **benzonase concentration? line 201**
5. **no step 4.6**

6. **5.2.7 and 5.2.8 digestion temperature?**

--*The above noted minor concerns have been clarified and resolved in the text.*

7. **6.3 requires some expansion. Can a method really resolve all peaks? how does multiple injections ensure a system is suitable for analysis?**
   6.4 all peaks?

-Section 6 has undergone major revision to provide clarity and specificity to this portion of the protocol.

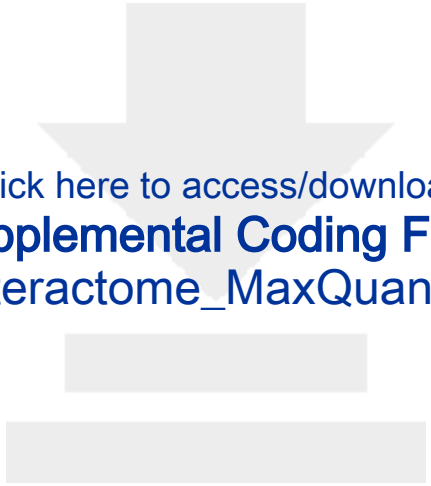
README file, start here

R script for creating SAINT/CRAPome input file

R script for statistical analysis and reproducing Figure 3

R script with plotting functions

compressed (zipped) data file of DYRK1A IP-MS data from MaxQuant and Perseus analysis



Click here to access/download

Supplemental Coding Files

DYRK1A_Interactome_MaxQuantAnalysis.zip

accessory file for R scripts

accessory file for R scripts

accessory file for R scripts