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Extract Preparation and Co-immunoprecipitation From Caenorhabditis elegans

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TITLE:

Protein Extract Preparation and Co-immunoprecipitation from *Caenorhabditis elegans*

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KEYWORDS:

C. elegans, protein extract preparation, immunoprecipitation, microRNA, ALG-1, AIN-1

SUMMARY:

This method describes a protocol for high-throughput protein extract preparation from *Caenorhabditis elegans* samples and subsequent co-immunoprecipitation.

ABSTRACT:

Co-immunoprecipitation methods are frequently used to study protein-protein interactions. Confirmation of hypothesized protein-protein interactions or identification of new ones can provide invaluable information about the function of a protein of interest. Some of the traditional methods for extract preparation frequently require labor-intensive and time-consuming techniques. Here, a modified extract preparation protocol using a bead mill homogenizer and metal beads is described as a rapid alternative to traditional protein preparation methods. This extract preparation method is compatible with downstream co-immunoprecipitation studies. As an example, the method was used to successfully co-immunoprecipitate two key *C. elegans* microRNA pathway components that interact with microRNA Argonaute ALG-1: AIN-1, a GW182 homolog, and HRPK-1. This protocol includes descriptions of animal sample collection, extract preparation, extract clarification, and protein immunoprecipitation. The described protocol can be adapted to test for interactions between any two or more endogenous, endogenously tagged, or overexpressed *C. elegans* proteins in a variety of genetic backgrounds.

INTRODUCTION:

Identifying the macromolecular interactions of a protein of interest can be key to learning more about its function. Immunoprecipitation and co-immunoprecipitation experiments can be used to identify the entire interactome of a protein through large-scale proteomic approaches[1] or to specifically test a protein's ability to coprecipitate with a hypothesized interactor. In *C. elegans*, both methods have been successfully employed to learn more about the activity of a variety of proteins, including those that closely function with microRNAs to regulate gene expression[2,3,4]. Co-immunoprecipitation experiments have the advantage of testing the protein-protein

interactions in their native cellular environment, but extract preparation can be challenging and time-consuming. Efficient lysis of the sample is necessary, but care must be taken to minimize the disruption of protein-protein interactions. Methods such as douncing[5], sonication[6], Balch homogenization[7], and zirconia beads-homogenized extracts[8,9] have been used to successfully prepare *C. elegans* total protein extracts. These methods, exception for zirconia bead homogenization, have limitations in terms of the number of samples that can be processed simultaneously. Presented is an alternative method that can be easily scaled up to allow for high-throughput, rapid protein extract preparation from *C. elegans* samples followed by co-immunoprecipitation. This extract method can be used to prepare extracts from any developmental stage of *C. elegans*. Importantly, the method can prepare up to 24 samples at a time, greatly reducing the time required for extract preparation. By contrast, for example, douncing typically allows for only one sample preparation at a time.

Described is a step-by-step procedure for animal sample collection, extract preparation, immunoprecipitation, and presentation of Western blotting data to confirm successful protein pulldown and detection of the co-immunoprecipitating protein of interest. To demonstrate the effectiveness of the protocol, two co-immunoprecipitation experiments were performed between core microRNA pathway components, 1) Argonaute ALG-1 and AIN-1, a GW182 homolog; and 2) ALG-1 and HRPK-1, a newly identified ALG-1 interactor[2]. ALG-1 and AIN-1 are core proteins that comprise the microRNA-induced silencing complex (miRISC); the interaction between these two proteins is well established[10,11]. The extract preparation protocol was effective in the ALG-1-AIN-1 co-immunoprecipitation experiment. This protocol also successfully confirmed the interaction between ALG-1 and its newly identified interactor, HRPK-1[2].

In summary, the manuscript describes a *C. elegans* extract preparation protocol that can be easily scaled up to simultaneously process 24 samples along with a co-immunoprecipitation protocol used to confirm or identify new or hypothesized interactions between proteins. The extract preparation protocol is compatible with a number of downstream experiments, including protein immunoprecipitation[2] or microRNA pulldowns[12]. Furthermore, the immunoprecipitation protocol can be adapted to test for interactions between any two or more endogenous, endogenously tagged, or overexpressed *C. elegans* proteins in a variety of genetic backgrounds.

PROTOCOL:

1. Worm sample collection

1.1. Seed mixed stage or synchronized[13] worms on NGM solid plates at the required temperature and allow the worms to grow until the desired stage. For basic *C. elegans* growth and maintenance, please see Stiernagle et al. and Porta-de-la-Riva et al.[14,13].

1.2. Collect worms in a 15 mL conical centrifuge tube by washing the worm plates with M9 buffer.

1.3. Pellet the worms by centrifuging at 400 x *g* at room temperature (RT) for 2 min and discard

the supernatant.

NOTE: The worm pellet size for extract preparation is between 100 µL and 500 µL. A 300 µL pellet of packed worms is recommended for downstream immunoprecipitation experiments and typically yields ~4.5 mg of total protein, while a 500 µL pellet will yield ~7.5 mg of total protein.

1.4. Perform additional 3–5 washes with M9 buffer (see **Table 1**) or until the supernatant is no longer cloudy.

1.5. Perform one final wash with ddH_2O.

1.6. Move the loose worm pellet to a 1.5 mL microcentrifuge tube and spin down at 400 x *g* at RT for 2 min. Discard the remaining supernatant to obtain a packed worm pellet and proceed to extract preparation.

NOTE: The protocol can be paused here. Worm pellets may be flash frozen in liquid nitrogen immediately and stored at -80 °C or in liquid nitrogen. Please note that worm pellets can only be thawed once and cannot be refrozen.

2. Extract preparation of the worm pellet

NOTE: The extract preparation should be performed on ice or at 4 °C.

2.1. If frozen, thaw worm pellet on ice.

NOTE: If the desired packed worm pellet size of 300 µL was not achieved during sample collection, multiple smaller pellets can be combined until enough material is present for further extraction.

2.2. Add an equal volume of ice-cold 2x lysis buffer (60 mM HEPES, pH = 7.4, 100 mM potassium chloride, 0.1% Triton X, 4 mM magnesium chloride, 10% glycerol, 2 mM DTT with RNase inhibitor, protease inhibitor, and phosphatase inhibitors; see **Table 1**) and vortex or pipette up and down to mix. Spin the tube(s) down to collect the mixture at the bottom of the tube.

2.3. Move the mixture into a 1.5 mL RNase-free tube containing metal beads (see **Table of Materials**) and put the sample in the bead mill homogenizer (see **Table of Materials**) at 4 °C. Ensure that the tube caps are tightened and the samples are balanced inside the homogenizer.

2.4. Homogenize the sample at the highest speed (setting 12) for 4 min.

2.5. Remove the sample from the beads and place it into a new 1.5 mL microcentrifuge tube. Alternatively, a magnet provided with the homogenizer can be used to remove the beads from the sample.

2.6. Spin down the extract at 19,000 x *g* for 20 min at 4 °C to clarify the protein extract.

2.7. Transfer the supernatant into a fresh 1.5 mL tube on ice, while avoiding carry-over of the white, cloudy precipitate that forms on top of the sample. The supernatant is now the clarified extract.

NOTE: Save 10 µL of the clarified extract to determine the total protein concentration.

2.8. Use the extract immediately for the following experiments or flash freeze the extract in liquid nitrogen and store at -80 °C.

NOTE: The protocol may be paused here. Extracts may be stored at ultralow temperature (-80 °C freezer or liquid nitrogen for ~6 months). Frozen extracts may be thawed once and cannot be refrozen.

2.9. Determine the total protein concentration of the extract using a protein concentration assay kit compatible with detergents (see **Table of Materials**) according to the manufacturer's instructions.

3. Immunoprecipitation

NOTE: All the immunoprecipitation steps for extract preparation should be performed on ice or at 4 °C. It is recommended to use 2 mg of total protein for each immunoprecipitation. However, successful immunoprecipitations with 0.8–1 mg of total protein have been performed. Always use fresh or freshly thawed protein extracts. The following protocol is outlined to perform immunoprecipitation from 2 mg of total protein, or a single immunoprecipitation experiment. The amount of beads and antibody may be increased or decreased accordingly for multiple samples or if a different amount of protein extract is used.

3.1. Place or thaw the protein extract on ice. The sample can be diluted to 10 mg/mL or 5 mg/mL with ice-cold 1x lysis buffer (see **Table 1**).

3.2. Resuspend the magnetic beads by inversion and transfer 150 µL of the 50% beads suspension into a 1.5 mL tube. Magnetize the beads on ice against a magnetic stand for 1 min or until the solution is clear. Discard the supernatant.

3.3. Remove the tube from the magnetic stand and wash the beads in 1x lysis buffer using 2 volumes (i.e., 300 µL) of bead slurry. Repeat the wash 3x.

3.4. Resuspend the beads in 150 µL of ice-cold lysis buffer.

3.5 Transfer 75 µL of the bead slurry to 2 mg of protein extract and incubate at 4 °C for 1 h with gentle agitation. Save the remaining bead suspension on ice for later use.

NOTE: This step is performed to reduce nonspecific protein binding to beads during the

immunoprecipitation step.

3.5. Place the tube with sample on the magnetic stand on ice for 1 min or until the beads are fully magnetized and the sample is clear. Transfer the supernatant to a new 1.5 mL tube; do not disturb the beads. This is the precleared protein lysate. Save 10% of the sample for Western blot analysis.

3.6. Add 20 µg of affinity purified antibody to the precleared lysate and incubate at 4 °C for 1 h with gentle agitation.

NOTE: The amount of antibody used for immunoprecipitation is specific to the antibody and protein and should be empirically determined to ensure effective immunoprecipitation of the target protein.

3.7. Add the remaining 75 µL of the prewashed beads suspension (step 3.5) to the antibody/lysate mixture and incubate for 1 h at 4 °C with gentle agitation.

3.8. Place the tube in the magnetic stand on ice for 1 min or until the beads are fully magnetized and the sample is clear. Save the supernatant for Western blot analysis (optional).

3.9. Wash the beads containing the immunoprecipitate 3x in 450 µL of wash buffer (30 mM HEPES, pH = 7.4, 100 mM potassium chloride, 0.1% Triton X, 2 mM magnesium chloride, 10% glycerol, 1 mM DTT; see **Table 1**) on ice.

NOTE: Additional washes may be performed if more stringent washing conditions are preferred.

3.10. Resuspend the bead pellet in 20 µL of 2x SDS/BME protein gel loading buffer (see **Table of Materials**) and denature by boiling at 95 °C for 5 min prior to loading onto an SDS-PAGE gel. Alternatively, denatured samples can be stored at -20 °C for several months.

NOTE: A portion of the bead immunoprecipitate can be saved for downstream RNA isolation, if desired.

4. Western blot detection of IP samples

4.1. Load the IP samples onto the SDS-PAGE gel (see **Table of Materials**). Avoid transferring the beads by placing the IP tubes on the magnetic stand for 1 min prior to aspiration of the sample.

4.2. Perform the Western blotting[15,16] and antibody staining[16] with the following modifications: dilute the ALG-1 antibody[17] 1:500 in 5% non-fat dry milk (NFDM); dilute the HRPK-1 antibody[2] 1:1,000 in 5% NFDM; and dilute the AIN-1 antibody[18] 1:10,000 in 5% NFDM. Secondary antibodies (see **Table of Materials**) were used according to the manufacturer's instructions.

4.3. Detect the bands with HRP-based chemiluminescence (see **Table of Materials**).

REPRESENTATIVE RESULTS:

This protocol (schematized in **Figure 1**) was used successfully to obtain *C. elegans* total protein extracts (**Figure 2**) effective for downstream immunoprecipitation of several proteins[2] (**Figure 3** and **Figure 4**). The presented bead mill homogenizer protocol was comparable in total protein extraction to dounce-based methods (**Figure 2**) and efficiently extracted nuclear (COL-19::GFP(NLS) (**Figure 2**) and cytoplasmic proteins (**Figure 3** and **Figure 4**). Multiple samples of various sizes were extracted simultaneously (**Figure 2**). Argonaute proteins interacted with members of the GW182 protein family, forming the miRISCs that bind to the target messenger RNAs and repress their expression[10]. **Figure 3** shows successful co-immunoprecipitation of core miRISC components ALG-1 and AIN-1, consistent with previous reports[11,17]. More recently, efforts were made to identify additional protein interactors of Argonaute ALG-1[3] in order to learn more about how microRNA biogenesis and activity might be regulated by auxiliary factors. The RNA-binding protein HRPK-1 was identified in ALG-1 immunoprecipitates[3]. This interaction was recently confirmed in a reciprocal HRPK-1 immunoprecipitation experiment[2]. The presented extract and immunoprecipitation protocols also successfully recovered ALG-1 in HRPK-1-specific co-immunoprecipitates (**Figure 4**). In addition, the ALG-1—AIN-1 interaction was tested in a variety of genetic backgrounds and HRPK-1 was shown to be unnecessary for the ALG-1/AIN-1 miRISC assembly[2] (**Figure 3**). Supplemental figures are provided to show the full membrane probed (**Supplemental Figure 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow schematic for *C. elegans* extract preparation and immunoprecipitation.

Figure 2: Western blot comparison of a nuclear localized GFP transgene, COL-19::GFP(NLS), levels in dounce prepared and homogenized samples from 250 µL and 100 µL worm pellets.

Figure 3: GW182 homolog AIN-1 co-immunoprecipitates with ALG-1. Western blotting for ALG-1 and AIN-1 proteins in ALG-1 immunoprecipitates. The ALG-1/AIN-1 co-immunoprecipitation was not affected by the absence of *hrpk-1.* Input = 10% of IP.

Figure 4: ALG-1 co-immunoprecipitates with HRPK-1. Western blotting for HRPK-1 and ALG-1 in HRPK-1 immunoprecipitates is shown. Input = 10% of IP. * indicates antibody heavy chain.

Table 1: Recipes

DISCUSSION:

C. elegans is an excellent model for studying fundamental questions in cell, molecular, and developmental biology[19]. In addition to its power as a genetic model system, *C. elegans* is amenable to biochemical approaches, including, but not limited to, protein immunoprecipitation and co-immunoprecipitation. One potential hurdle when conducting immunoprecipitation experiments is lack of antibodies specific to the proteins of interest. If no antibody is available, custom polyclonal or monoclonal antibodies can be generated. However, recent innovations in genome editing technology have allowed researchers to rapidly introduce mutations or tag

endogenous *C. elegans* genes[20,21], facilitating studies that unravel the genetic, functional, and physical interactions among the genes and the encoded proteins. Specifically, CRISPR/Cas9-mediated tagging of *C. elegans* genes at the endogenous loci has reduced the dependence of immunoprecipitation experiments on antibody availability, making co-immunoprecipitation experiments much more feasible. *C. elegans* genes can be tagged with a variety of tags ranging from fluorescent tags such as GFP or mCherry to small tags such as FLAG and HA. Antibodies recognizing these tags are readily available commercially, facilitating the studies of protein-protein interactions via immunoprecipitation approaches.

The presented protocol, outlined in **Figure 1**, can be performed for a small number of samples or scaled up, allowing for up to 24 sample preparations at a time. While the initial characterizations of protein-protein interactions via immunoprecipitation are typically done in wild type backgrounds under normal growing conditions, follow-up studies frequently necessitate testing the protein-protein interactions in a variety of genetic backgrounds or under different growth conditions. The ability to simultaneously prepare multiple extracts saves time and, importantly, ensures extract preparation consistency among the different samples. A negative control is always required, with the ideal control being a null mutation in the gene encoding for the immunoprecipitated protein of interest (See **Figure 3** and **Figure 4** for examples).

This extract protocol allows for rapid protein extract preparation from *C. elegans* samples and is comparable to zirconium bead-based homogenization[8]. Bead homogenization in general can be scaled up to multiple simultaneous sample preparations using a variety of bead mill homogenizers or similar equipment. Some more economical bead mill homogenizers may reduce the number of samples that can be processed simultaneously, however. Alternatively, the presented extract protocol is compatible with dounce-based extract preparation, which represents an economical alternative. While different bead mill homogenizers were not tested, most bead mill homogenizers are likely to be compatible with this protein extract protocol, as long as complete disruption of the *C. elegans* samples is achieved.

As presented, this extract preparation protocol is compatible with multiple downstream experiments, including protein immunoprecipitation[2] and microRNA pull-down[12] and allows for downstream collection of both protein and RNA components. It also efficiently extracts both nuclear and cytoplasmic proteins (**Figure 2**, **Figure 3**, and **Figure 4**). Similarly, the presented immunoprecipitation protocol permits RNA isolation from protein-associated immunoprecipitates. While the immunoprecipitation protocol was originally developed to identify ALG-1 protein interactors, the method can be adapted to test for interactions between any proteins of interest. In fact, the immunoprecipitation conditions used worked equally well for immunoprecipitating ALG-1 (**Figure 3**) and HRPK-1 (**Figure 4**). This protocol is an excellent starting point for immunopurification of RNA-binding proteins. It should be noted, however, that some changes in buffer composition may be required for other proteins of interest. The changes may depend on the physical and biochemical properties of the protein of interest and must be implemented on a case-by-case basis.

Once the target protein (here, ALG-1 or HRPK-1) is immunoprecipitated, Western blotting can be

used to test the co-immunoprecipitate for specific protein interactors.

Alternatively, the copurified immunoprecipitate can be subjected to mass spectrometry analysis to identify all the putative interacting proteins. Confirmed co-immunoprecipitation interactions can then be examined in a variety of genetic backgrounds or conditions to identify potential regulation of the specific interaction. For example, to determine whether *hrpk-1* plays a role in ALG-1/AIN-1 miRISC assembly, ALG-1-AIN-1 coprecipitation was assessed both in a wild type background and in the absence of HRPK-1 (**Figure 3**). *hrpk-1* testing showed it was unnecessary for ALG-1/AIN-1 interaction[2] (**Figure 3**). In addition, CRISPR/Cas9 genome editing technology can be employed to generate single point or domain deletion mutations in the proteins of interest. Retesting the ability of the generated mutants to coprecipitate with their protein interactors can reveal which domains or residues mediate the physical interaction. Such future studies can yield invaluable information about the mechanism of protein function and regulation. These approaches, combined with the power of *C. elegans* genetics, can provide important insights into the fundamental molecular processes that govern animal development and cellular function.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

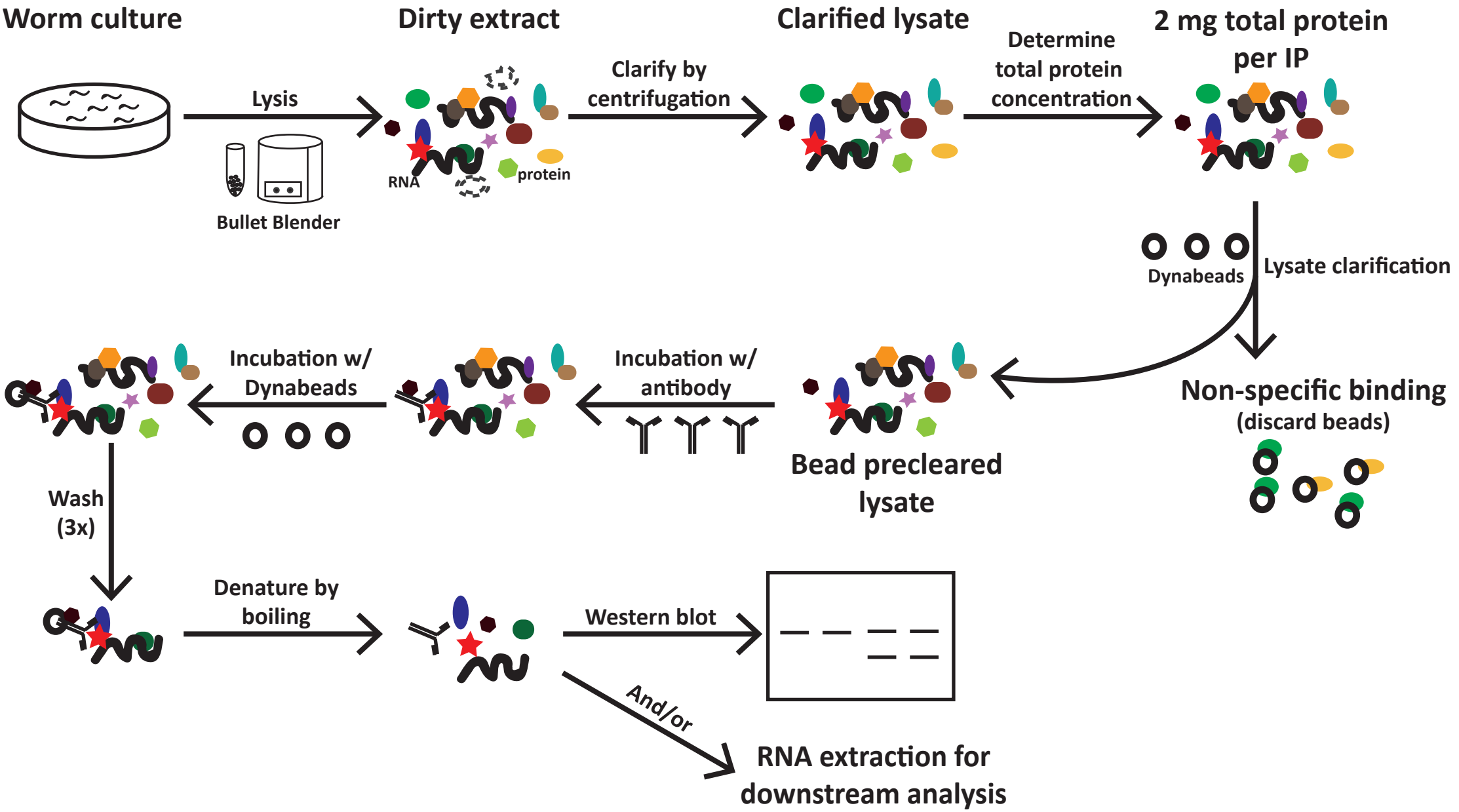

Figure 2

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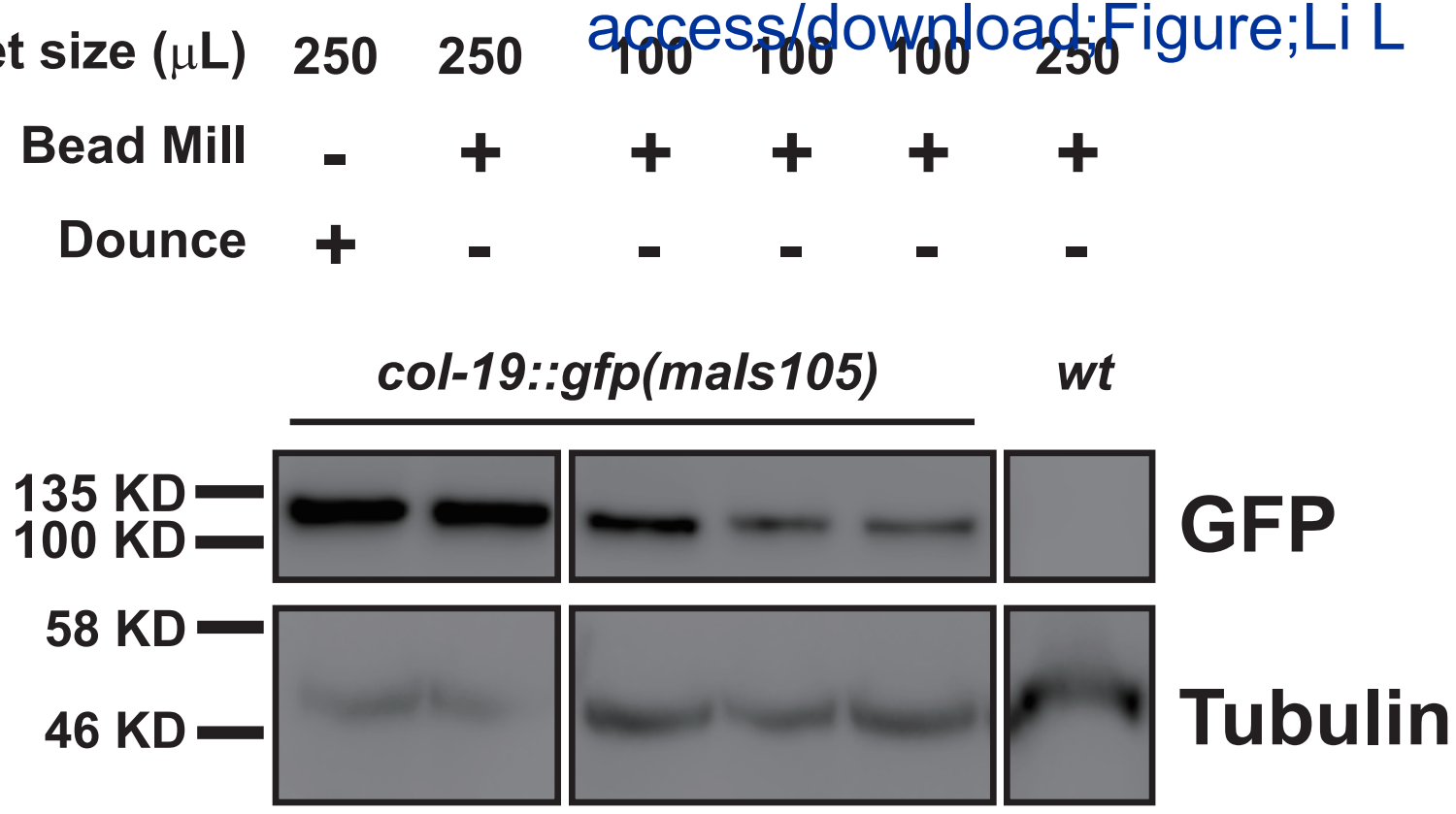

Figure 3

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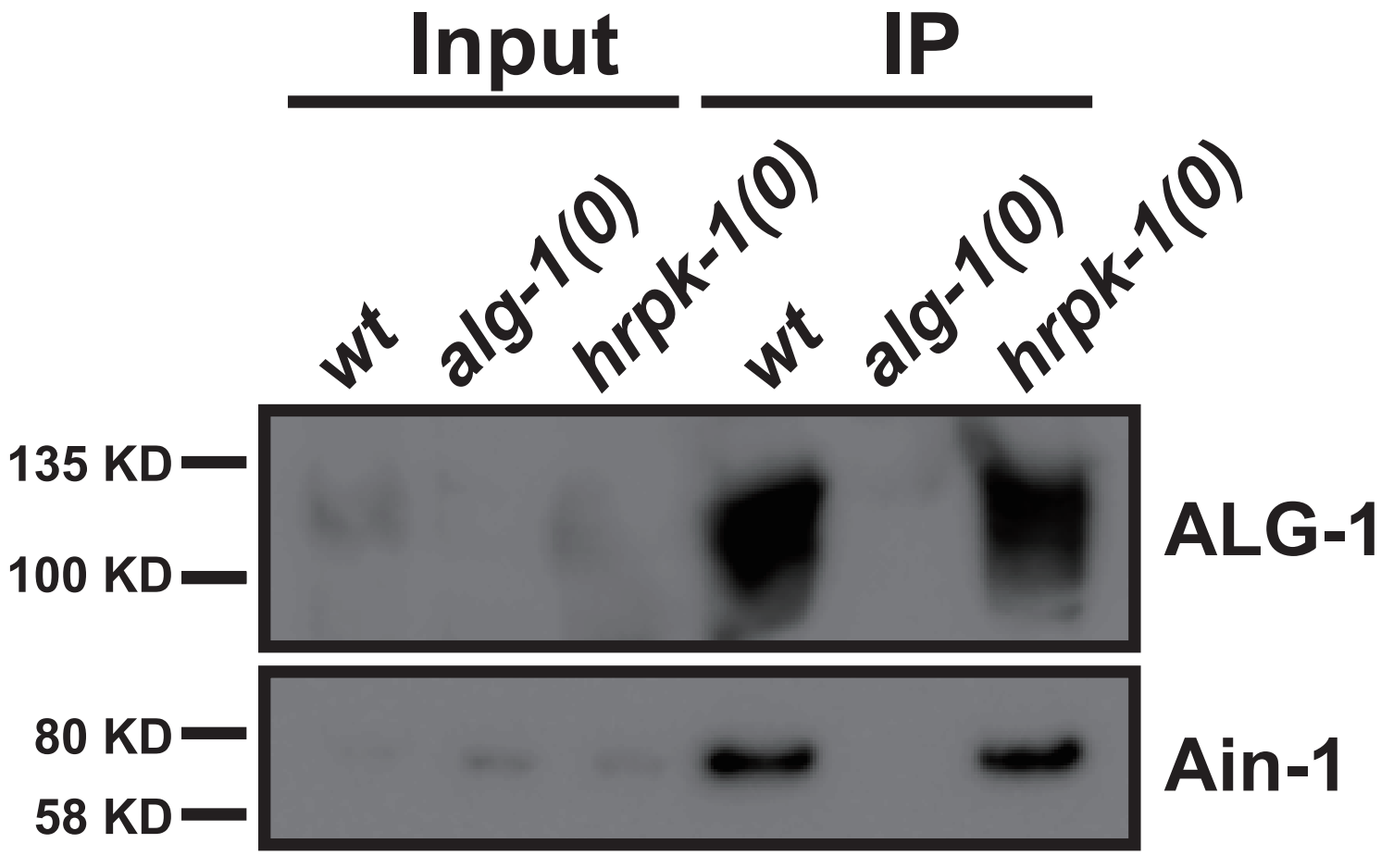

Figure 4

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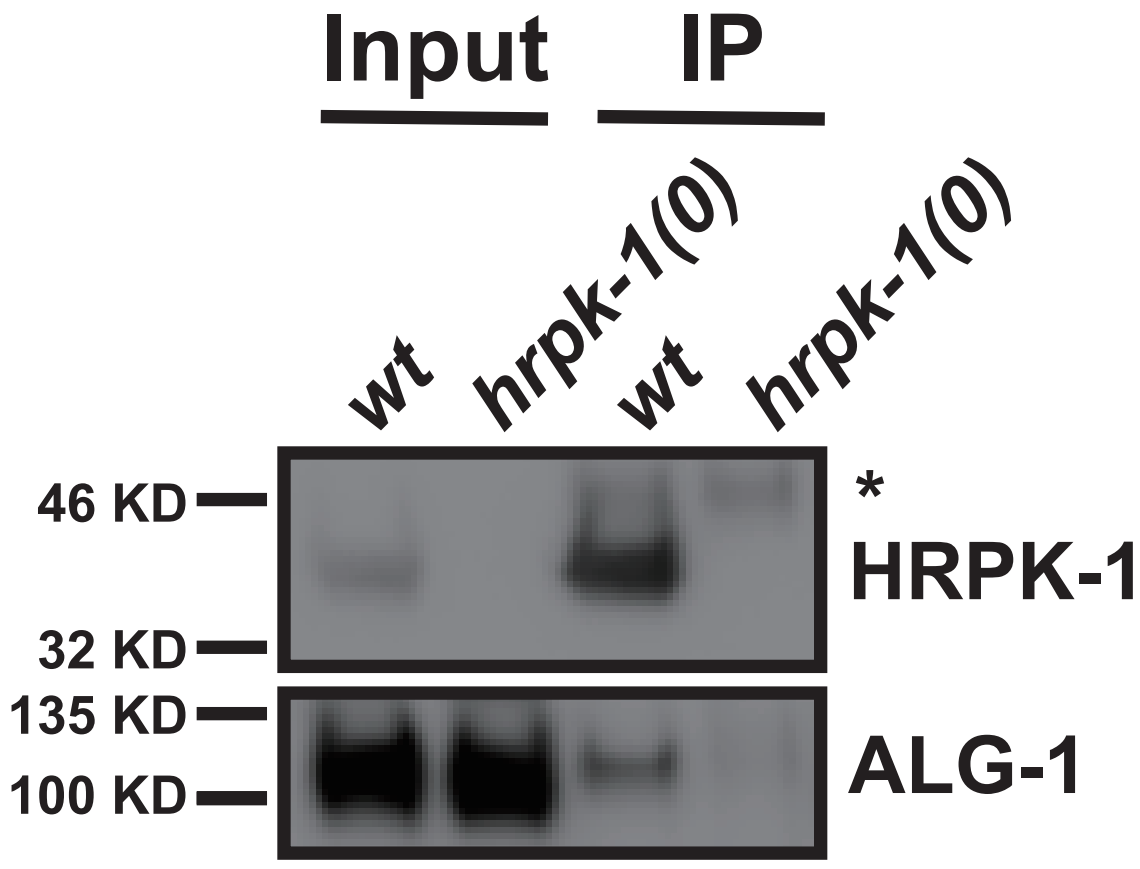

Table 1. Recipes.

M9 buffer (1 L)

KH_2PO_4	3 g
Na_2HPO_4	6 g
NaCl	5 g
1 M $MgSO_4$	1 mL
ddH_2O	up to 1 L

2x Lysis buffer (5 mL)

HEPES (pH 7.4)	200 µL
2 M KCl	250 µL
10% TritonX	100 µL
1 M $MgCl_2$	20 µL
100% glycerol	1 mL
ddH_2O	up to 5 mL

Add fresh:

1 M DTT	20 µL
EDTA-free protease inhibitor	1 tablet
phosphatase inhibitor cocktail 2	100 µL
phosphatase inhibitor cocktail 3	100 µL

1x Lysis buffer

Dilute 2x Lysis buffer with an equal volume of ddH20.

1x Wash buffer 10 mL)

HEPES (pH 7.4)	300 µL
2 M KCl	500 µL
10% TritonX	100 µL
1 M $MgCl_2$	20 µL
100% glycerol	1 mL
ddH_2O	up to 10 mL
1 M DTT	20 µL **(add fresh)**

Table of Materials

Click here to access/download;Table of Materials;Li L et al_JoVE_Table_of_Materials_revision.xlsx

Name of Material/ Equipment	Company	Catalog Number
15 mL tube	VWR	89039-664
2x Laemmli Sample Buffer	BioRed	1610737
4–20% Mini-PROTEAN TGX Precast Protein Gels	BioRed	4561096
anti-AIN-1 monoclonal antibody	custom generated	n/a
anti-ALG-1 monoclonal antibody	custom generated by PRF&L	n/a
anti-HRPK-1 monoclonal antibody	custom generated by PRF&L	n/a
Bullet Blender Storm Homogenizer	MidSci	BBY24M
DL-Dithiothreitol (DTT)	Sigma	D9779-5G
Dynabeads Protein A for Immunoprecipitation	Thermo Fisher	10002D
DynaMag-2 Magnet	Thermo Fisher	12321D
EDTA-free protease inhibitors	Roche	11836170001
GFP antibody (FL)	Santa Cruz Biotechnology	sc-8334
Glycerol	Thermo Fisher	G33-500
Goat Anti-Rabbit Secondary Antibody, HRP	BioRed	1662408
Goat anti-Rat IgG (H+L) Secondary Antibody, HRP	Thermo Fisher	31470
HEPES	Sigma	H4034-500G
LICOR WesternSure PREMIUM Chemiluminescent Substrate, 100 mL Kit	LI-COR	926-95000
Magnesium chloride hexahydrate ACS	VWR	VWRV0288-500G
Magnesium Sulfate Anhydrous	Thermo Fisher	M65-500
Microcentrifuge Tubes, 1.5 mL	VWR	20170-333
N2 wild type	CGC	
Navy RINO RNA Lysis Kit 50 pack (1.5 mL)	MidSci	NAVYR1-RNA
Phosphatase inhibitor cocktail 2	Sigma	P5726-1ML
Phosphatase inhibitor cocktail 3	Sigma	P0044-1ML
Potassium Chloride	Thermo Fisher	P217-500
Potassium phosphate monobasic	Thermo Fisher	P285-3
RC DC Protein Assay Kit I	BioRed	5000121
RNaseOUT Recombinant Ribonuclease Inhibitor	Thermo Fisher	10777019
Sodium Chloride	Thermo Fisher	S271-500
Sodium Phosphate Dibasic Anhydrous	Thermo Fisher	S374-500
TritionX-100	Sigma	X100-500ML

UY38 *hrpk-1(zen17)*	available upon request
VT1367 *col-19::gfp(maIS105)*	available upon request
VT3841 *alg-1(tm492)*	available upon request

Comments/Description
STEP 1.2
STEP 3.11
STEP 4.1
STEP 4.2, see ref. Zhang et al. 2007
STEP 4.2
STEP 4.2
STEP 2.3
Table 1
STEP 3.2
STEP 3.2
Table 1
Figure 2
Table 1
STEP 4.2
STEP 4.2
Table 1
STEP 4.3
Table 1
Table 1
STEP 1.6
STEP 2.3
Table 1
Table 1
Table 1
Table 1
STEP 2.9
Table 1
Table 1
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Table 1

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

Li et al manuscript titled "Extract Preparation and Co-immunoprecipitation from Caenorhabditis elegans" describe a simple and effective method for total protein extraction using C. elegans as a starting material. The manuscript further describes the use of antibodies to co-precipitate proteins of interest using the total protein extract. The protocol is written in a simple and easy to follow manner with many key information provided.

Clear documentation of research protocols is essential for reproducibility and provides a valuable resource for other scientist to conduct experiments more rapidly. In this context, the protocol of Li et al can be a valuable resource. However, the protocol is highly similar to bead beating approaches that have been widely used in the C. elegans community for at least over a decade. Among these bead beating of C. elegans larvae using zirconia beads is a common approach that has been detailed in multiple publications. Authors are using a specific brand bead beater / tissue lyser together with metal beads instead of zirconia beads for the lysis of the worms. In addition, all similar approaches can be used to prepare multiple extracts such as using a Precellys tissue lyser which allows processing of 24-30 samples. The emphasis on the high-throughput nature of the protocol is not unique but also is not demonstrated by the authors in this protocol.

For protein extraction protocols, it is essential to demonstrate that majority of proteins are extracted including both nuclear and cytoplasmic proteins. Authors should demonstrate that their protocol can sufficiently lyse nuclei and extract nuclear proteins. They should also compare their method with similar approaches to demonstrate that the protocol is achieving comparable levels of protein extraction.

We thank the reviewer for their thoughtful consideration of the manuscript and the feedback. We have provided our responses below each comment or concern and highlighted our response in blue text for ease of following. Revised text in the manuscript is highlighted in red.

Major Concerns:

I suggest that the authors consider doing the following;
1- show that both nuclear and cytoplasmic proteins are extracted

We appreciate the suggestion, and we have now added a Western blot of a nuclear localized GFP reporter to demonstrate extraction of nuclear proteins as Figure 1. ALG-1 and AIN-1 are cytoplasmic proteins (although ALG-1 has been previously hypothesized to be also present in the nucleus).

2- show that the extracted protein levels are comparable to other methods (to which other methods to compare can be decided by the authors as long as the data provides a certain level of comparison for the reader)

We thank the reviewer for the suggestion and now show a comparative extraction using beads vs. a dounce as Figure 1 for the above-mentioned nuclear localized GFP reporter. We note the comparable extraction levels between these two extract methods on lines 237 and 238 (Representative results).

3- include citations to similar methods (zirconia beads etc) and discuss why one or the other is better to use. As the concept of this protocol is not based on novelty but being a valuable resource, being more inclusive of similar approaches will attract wider audience.

We note the importance of this suggestion and thank the reviewer. We now mention zirconia bead extract preparations as an alternative method and the citations of manuscripts that report using zirconia beads (lines 58-60, Introduction, and lines 299-300, Discussion). In addition, we have altered our language throughout the Introduction and Discussion to emphasize that the presented protocol is just one of a number of protocols available to researchers for protein extractions.

Minor Concerns:

1- Step 1.5, it is not clear why the authors are doing a final wash with ddH2O. Wouldn't it be more suitable to do a final wash with a buffer compatible with the lysis buffer? In addition ddH2O might lead to lots of worm sticking to the side of the tubes. Have the authors explored including a low percentage detergent in this step such as %0.05 Tween-20?

We have not tried addition of a low % detergent, as we do not find issues with worms sticking to tube sides. The rational for using water is to make the pellet compatible with the 2x lysis buffer that is added to the pellet at a later step.

2- The authors should document how much total protein they obtain from 300-500ul of worm pellet for other users to compare

We have now included this information (see lines 102-104, Step 1.3 NOTE).

3- Step 2.2, do the authors spin down the tube after vortexing? Wouldn't it be better to Pipette up and down instead of vortexing and immediately transfer to the Rhino bead tube?

We have now added the spin as a follow up step for vortexing and included a suggested alternative of pipetting the sample up and down (see lines 130-131, Step 2.2).

4- Step 2.4 highest setting (setting 12) is arbitrary. Authors should contact the manufacturer to try and put a number on this value in terms of rpm or similar. The model of the machine can change in the future and setting 12 can be misleading.

We have attempted to determine the value of the setting in RPM or similar. However, the company considers this information proprietary and has not provided us with the requested information. We are continuing our inquiries and will update this information should it become available to us.

5- Step 2.6 add the reason for this step "for clarification of the protein extract"

We have now added the reason for this step as suggested (see line 143, Step 2.6).

6- "3. Immunoprecipitation" Why not show and document that the IPs work effectively with lower protein amounts?

We appreciate the reviewer's suggestion. However, successful IPs from lower total protein amounts depend on many factors, including, but not limited to, antibody quality and the abundance of the protein of interest in question. We believe it is outside the scope of this resource to demonstrate this as it is unlikely that this protocol will be used for the same antibody/same protein experiments. We have, however, added a figure panel to show that the protocol is compatible with smaller worm pellets, demonstrating that protein extractions can be conducted from smaller samples sizes (Figure 1, text line 239-240).

Similarly, can the authors back their claim that 40mg of protein is required for mass spec analysis?

This amount was suggested to us by collaborators who routinely perform mass spec analyses. 40mg is typically suggested as an amount that will allow for an excellent coverage of the recovered proteome. However, smaller total protein amounts can be used. As demonstrating the total protein mass spec requirements is outside the scope of this resource, we have removed this statement from the protocol (previously on line 172).

7- Step 3.2, what do authors mean by magnetizing beads on ice?

We have now changed the wording to hopefully clarify this step (line 178).

8- For both Figure 1 and Figure 3 original full size gel images should be included as a supplement to demonstrate if the protocol leads to clean IPs with minimal unspecific interactions. This is also important because in both figures the gel images are cut very close to the edge of protein bands.

We have now included the original full-size gel images as supplemental material (supplemental Figure 1). In addition, we have increased the cropping area in the main text Figure images.

9- Figure 2 inputs are not very convincing for a protocol paper. Is it possible the authors extracted very little protein?

Concentrations of our extracts routinely quantify anywhere from 10mg/ml to 20mg/ml, with extracts on average being 15mg/ml. The detection level is likely due to antibody reactivity and comparatively high level of IP sample (which in itself would argue against poorly extracted proteins).

10- ALG-1 bands look different in Figure 2 and 3. Both figures should also include a size marker.

We have included a size marker in all figures: please note that Figures 2 and 3 are now Figures 3 and 4.

11- Line 313, "The main advantage" over what exactly? Further in the paragraph the potential to prepare multiple extracts is again mentioned but this is not documented in this protocol.

We have removed the statement regarding “main advantage” (lines 289-290) and toned down the language to convey that this protocol is one of several available for *C. elegans* protein extractions (lines 299-312).

12- Line 323, again the emphasis on high-throughput is not documented.

We have removed “high-throughput” from our statement (lines 299-300).

If the authors can address the issues raised, I believe this can be a valuable resource for many researchers. I for one would recommend using this protocol and associated video to my team members who are going to do protein extraction and co-IP for the first time.

Reviewer #2:

Manuscript Summary:

The authors outline a streamlined protocol for preparing extracts from C. elegans larvae. Having dealt with a number of methods myself aimed at preparing lysates from developing animals, this protocol exhibits a number of features that would make this approach stand out as an excellent option of a researcher to switch to. Primary amongst these is the relatively small volumes of animals that are needed to make an abstract. Other methods including dounce homogenization require much larger volumes. The second attractive quality of this approach would be the ability to multiplex the process. As most experiments we have done aim to measure differences between wild-type animals and mutant strains, this feature is an important addition to the options currently available to researchers.

This protocol is also very clearly written with enough details in the introduction and experimental steps to guide the researcher through the process. It is essentially presentable as is and would complement the well-produced videos of the JoVE series.

We thank the reviewer for their thoughtful consideration of the manuscript and the feedback. We have provided our responses below.

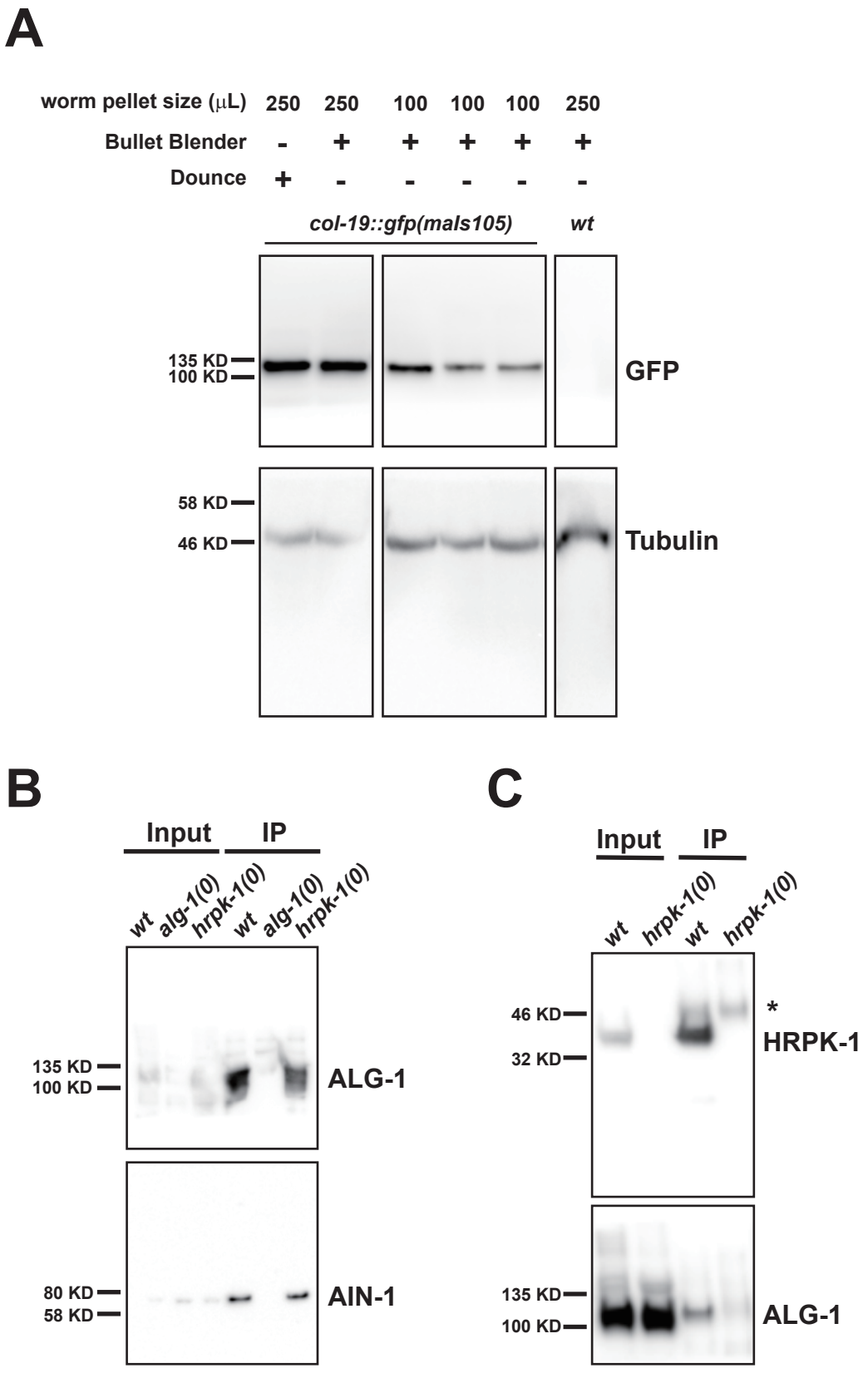
Major Concerns:
None.

Minor Concerns:
1) For portions where temperature is important, I would put that up front in the appropriate section.

We appreciate the reviewer's suggestion; we have now added the temperature in the front of both Extract preparation and Immunoprecipitation sections (see line 121, NOTE, Section 2 and line 164, NOTE A, Section 3).

2) It would be nice to give an approximation of hoe much the Bullet Blender cost in comparison to other types of tools that can be used (ie a stainless steel dounce homogenizer, etc.) this will help guide researchers to this method over other methods as each requires the purchase of special items.

We have now added that information in the manuscript's Discussion section (please see lines 299 to 312). Due to the large variety of options that are available to researchers, we have highlighted only several, but believe these provide a reasonable framework for researchers considering the costs of the necessary homogenizing equipment.



SUPPLEMENTAL FIGURE LEGEND:

Supplemental Figure 1. Full probed Western blot membranes used to generate Figures 2-4 are shown. (A) Probed membrane for Figure 2. Note that membrane was cut to allow for simultaneous probing for GFP and Tubulin, reducing the overall blot size. (B) Probed membrane for Figure 3. (C) Probed membrane for Figure 3. *denotes antibody heavy chain.