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**TITLE:**

# 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. Identification of new, or confirmation of hypothesized protein-protein interactions 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, we describe a modified extract preparation protocol using a bead mill homogenizer and metal beads as a rapid alternative to traditional protein preparation methods. Furthermore, we demonstrate that this extract preparation method is compatible with downstream co-immunoprecipitation studies. As an example, we show successful co-immunoprecipitation between key microRNA pathway components, *C. elegans* microRNA Argonaute ALG-1 and a GW182 homolog, AIN-1 and another ALG-1 interactor, HRPK-1. This protocol includes 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 for 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 co-precipitate 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 function closely 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, yet care must be taken to minimize the disruptions to protein-protein interactions. Methods such as douncing[5], sonication[6], Balch homogenization[7], and zirconia beads homogenization[8,9] have been used to successfully prepare *C. elegans* total protein extracts. These methods, with the exception of zirconia bead homogenization, have limitations in terms of the number of samples that can be processed simultaneously. Here, we present 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 development stage of *C. elegans*. Importantly, the method has the ability to prepare up to 24 samples at the same time, greatly reducing the time required for extract preparation. By contrast, for example, douncing typically only allows for one sample preparation at the time.

Overall, we describe a step by step procedure for animal sample collection, extract preparation, and immunoprecipitation and present Western blotting data that confirms the successful protein pulldown and detects the co-immunoprecipitating protein of interest. Specifically, to follow up on our extract preparation protocol we show two successful co-immunoprecipitation experiments between 1) two core microRNA pathway components, Argonaute ALG-1 and a GW182 homolog, AIN-1 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) and the interaction between these two proteins is well established[10,11]. We show that our extract preparation protocol is compatible with ALG-1-AIN-1 co-immunoprecipitation experiments. This protocol is also fully compatible with co-immunoprecipitation experiments aimed at confirming interactions between ALG-1 and newly identified interactors, such as HRPK-1[2].

In summary, we describe a *C. elegans* extract preparation protocol that can be easily scaled up to simultaneously process 24 samples. We present a co-immunoprecipitation protocol that can be used to identify new or confirm 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[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 100µL and 500 µL. A 300µL pellet of packed worms is recommended for downstream immunoprecipitation experiments and typically yields ~4.5mg of total protein, while a 500µL pellet will yield ~7.5mg of total protein.

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

1.5 Perform one final wash with dd$H_2O$.

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 as worm pellets may be flash frozen in liquid nitrogen immediately and stored at -80 $^oC$ 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: Steps 2.1 -2.8 of extract preparation should be performed on ice or at 4$^oC$.

2.1 If frozen, thaw worm pellet on ice.

NOTE: If the desired packed worm pellet size of 300µL was not obtained 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 KCl, 0.1% Triton X, 4 mM $MgCl_2$, 10% glycerol, 2 mM DTT with RNAse inhibitor, protease inhibitor and phosphatase inhibitors, see **Table 1. Recipes**) and vortex or pipet 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 place 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 Bullet Blender 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 for total protein concentration determination.

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 as extracts may be stored at the 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 the a protein concentration assay kit compatible with detergents (see **Table of Materials**) according to manufacturer's instructions.

**3. Immunoprecipitation**

NOTE A: All immunoprecipitation steps of extract preparation should be performed on ice or at 4°C.

NOTE B: 2 mg of total protein is recommended for each immunoprecipitation. However, we have performed successful immunoprecipitations with 0.8-1 mg of total protein. 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. You may increase or decrease the amount of beads and antibody accordingly for multiple samples or if you wish to work with more or less protein extract.

3.1 Place or thaw the protein extract on ice. You can dilute your sample to 10 mg/mL or 5 mg/mL with ice cold 1x lysis buffer (see **Table 1. Recipes**).

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 magnetic stand and wash beads in 1x lysis buffer using 2 volumes (300 µL) of bead slurry. Repeat for a total of three washes.

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 non-specific protein binding to beads during the immunoprecipitation step.

3.6 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 while taking care not to disturb the beads. This is your precleared protein lysate. Save 10% of your sample for Western blot analysis (this is your input).

3.7 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 antibody and protein specific and should be empirically determined to ensure effective immunoprecipitation of target protein.

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

3.9 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).

3.10 Wash the beads containing your immunoprecipitate 3 times in 450 µL of Wash buffer (30 mM HEPES, pH 7.4, 100 mM KCl, 0.1% Triton X, 2 mM $MgCl_2$, 10% glycerol, 1 mM DTT) (see **Table 1. Recipes**) on ice.

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

3.11 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 modification. ALG-1 antibody[17] was diluted 1:500 in 5% non-fat dry milk (NFDM). HRPK-1 antibody[2] was diluted 1:1000 in 5% NFDM, and AIN-1 antibody[18] was diluted 1:10000. Secondary antibodies (See **Table of Materials**) were used per manufacturer's instructions.

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

**REPRESENTATIVE RESULTS:**

This protocol (schematized in **Figure 1**) has been used successfully to obtain *C. elegans* total protein extracts (**Figure 2**) competent for downstream immunoprecipitation of several proteins[2] (**Figures 3 and 4**). The presented bead mill homogenizer protocol is comparable in total protein extraction to dounce-based methods (**Figure 2**) and can efficiently extract nuclear (COL-19::GFP(NLS), **Figure 2**) and cytoplasmic (**Figures 3 and 4**) proteins. We also show that multiple samples of various sizes can be extracted simultaneously (**Figure 2**). Argonaute proteins interact 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 a successful recapitulation of co-immunoprecipitation of core miRISC components ALG-1 and AIN-1, consistent with previous reports[11,17]. More recently, efforts have been 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. An RNA binding protein, HRPK-1, has been previously identified in ALG-1 immunoprecipitates[3]. This interaction has been recently confirmed in a reciprocal HRPK-1 immunoprecipitation experiment[2]. The presented extract and immunoprecipitation protocols allow for successful recovery of ALG-1 in HRPK-1-specific co-immunoprecipitates (**Figure 4**). In addition, we demonstrate that the ALG-1-AIN-1 interaction can be tested in a variety of genetic backgrounds and show that HRPK-1 is dispensable 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 250µL and 100µL 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 is 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.

**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 in the ability to conduct 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 proteins encoded by them. 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 can be 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).

Our 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. Alternatively, the presented extract protocol is compatible with dounce-based extract preparation, which represents an economical alternative to cost conscious researchers (current stainless steel dounce list price is $757). A current cost comparison of several homogenizers places the Bullet Blender (list price $4,425) at a lower end of other homogenizers, such as Precellys24 (list price $8,950), and MiniBead Beater (list price

$5,988). More economical bead mill homogenizers, such as BeadBug6, for example, are available (list price $1,862) but reduce the number of samples that can be processed simulteneously. As we have not tested additional bead mill homogenizers, we cannot guarantee protein extract compatibility with downstream experiments. However, we believe most bead mill homogenizers are likely to be compatible with our protein extract protocol, as long as complete disruption of the *C. elegans* samples is achieved.

As presented, our extract preparation protocol is compatible with multiple downstream experiments, including protein immunoprecipitation[2] and microRNA pulldown[12] and allows for downstream collection of both protein and RNA components. It efficiently extracts both nuclear and cytoplasmic proteins (Figures 2-4). 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 work equally well for immunoprecipitating ALG-1 (Figure 3) and HRPK-1 (Figure 4). We believe this protocol is an excellent starting point for immunopurification of RNA binding proteins. We should note, 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 have to be implemented on a case by case basis.

Once the target protein (in our examples, ALG-1 or HRPK-1) is immunoprecipitated, the co-immunoprecipitate can be subjected to Western blotting to test for specific protein interactors. Alternatively, the co-purified immunoprecipitate can be subjected to mass spectrometry analysis to identify all of 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 co-precipitation was assessed both in wild type background and in the absence of HRPK-1 (Figure 3). We found that *hrpk-1* is dispensable 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 co-precipitate 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 its 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.

**ACKNOWLEDGMENTS:**

This work was in part supported by Kansas INBRE, P20GM103418 to Li and Zinovyeva and R35GM124828 to Zinovyeva. We thank Min Han for generously sharing the anti-AIN-1 antibody. Some of the strains used in the course of this work were provided by the *Caenorhabditis* Genetics Center (CGC), funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

**DISCLOSURES:**

The authors have nothing to disclose.

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