Cold Spring Harbor Protocols

Analysis of RNA-Protein Complexes by RNA Coimmunoprecipitation and RT-PCR Analysis from *Caenorhabditis elegans*

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Protocol

Analysis of RNA-Protein Complexes by RNA Coimmunoprecipitation and RT-PCR Analysis from *Caenorhabditis elegans*

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INTRODUCTION

RNA coimmunoprecipitation (co-IP) experiments are an extension of protein co-IP experiments in which in vivo RNA-protein complexes are investigated. This protocol describes how to perform RNA co-IPs from C. *elegans* whole-worm extracts. In principle, a protein-specific antibody is used to purify the protein of choice and its associated complex members from worm extract. This may also include RNA molecules associated with other protein components. To identify a specific mRNA molecule, all RNA molecules are first separated from the protein components after immunopurification. The mRNAs are then converted into cDNA by reverse transcription. Candidate mRNAs are detected by sensitive gene-specific amplification via polymerase chain reaction (PCR) in a semiquantitative manner. Since RNA molecules are very prone to degradation, it is crucial to avoid any kind of contamination with RNase activity in this experiment.

RELATED INFORMATION

This protocol was used to show that *gld-1* mRNA is part of the GLD-3/GLS-1/GLD-4 complex in vivo (Schmid et al. 2009). The protein co-IP procedure upon which this protocol is based is described in **Analysis of In Vivo Protein Complexes by Coimmunoprecipitation from *Caenorhabditis elegans*** (Jedamzik and Eckmann 2009).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**!**> *To prepare DEPC (diethyl pyrocarbonate)-treated solutions, add 0.1% DEPC (e.g., SERVA 18835.01) to the solution in a screw-cap bottle. Shake the bottle very hard immediately and then every 10 min for 1 h, or stir the solution with a stir bar for 1 h if detergents are included. Autoclave the solutions. Solutions that cannot be autoclaved (e.g., HEPES) should be put into a 50°C water bath overnight with the lid left loose to allow excess DEPC to evaporate.*

AMV reverse transcriptase (10 U/µL) and 5X AMV buffer (e.g., Promega)
Antibodies
Specific antibody to protein of interest
Nonspecific antibody as a negative control (preimmune serum or purified IgG, e.g., ChromPure Rabbit IgG, whole molecule, Jackson ImmunoResearch 011-000-003)

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BSA (bovine serum albumin)
<R>Buffer B70 (RNase-free)
<!>Chloroform
<!>Chloroform:isoamylalcohol (IAA) (24:1)
DNase I (RNase-free) and 10X DNase I buffer (e.g., Roche)
dNTPs, 10 mM (mix from individual dNTPs; e.g., Fermentas R0181)
Ethanol (75% [ice-cold] and 99.8%)
Gene-specific primers for nested PCR (see Step 50)
Glycogen (20 mg/mL; e.g., from oyster, SERVA 39766.02)
<!>H_2O, DEPC-treated
<!>Heparin (100 mg/mL stock) (Heparin Na-salt; e.g., SERVA 24590.01)
<!>HEPES (50 mM, pH 7.5), DEPC-treated
<R>M9 salt solution
<!>NaOAc (sodium acetate; 3 M, pH 5.2), DEPC-treated
<R>Nematode growth media (NGM) plates, 6-cm, containing starved worms
<R>Nematode growth media (NGM) plates, 10-cm and 14.5-cm
Oligo dT primer (12.5 µM) (5′-TTTTTTTTTTTTTTTV-3′)
<R>OP50 solution
<!>Phenol:chloroform:isoamyl alcohol (PCI) (25:24:1)
Protein A-agarose (e.g., Roche)

*If your specific antibody does not come from rabbit, Protein G-agarose or another type might be preferred. To pellet agarose beads, never centrifuge higher than 400*g.

Reagents for agarose gel electrophoresis
Reagents for Bradford assay
RNasin (40 U/µL; e.g., Promega RNasin Plus)
Taq polymerase and 10X PCR buffer (e.g. *Taq* DNA Polymerase from NEB M0267 S)
<!>Trizol (e.g., TRIzol, Invitrogen 15596-026)
Yeast total RNA (20 mg/mL stock; Roche 10109223001)

Equipment

Adapters for 15-mL centrifuge tubes
Centrifuge tubes (15-mL glass; e.g., Kimble 45500)
Equipment for agarose gel analysis of DNA
Falcon tubes (15-mL and 50-mL, RNase-free)
Filter (0.45 µm)
Gloves (protective; can be thick winter gloves)
High-speed centrifuge (e.g., Beckmann Coulter centrifuge Avanti J-20 or J-25)
Ice and ice water
Laminar flow hood
<!>Liquid nitrogen
Liquid-nitrogen containers (2)
Magnetic stirrer
Microcentrifuge tubes (1.5-mL, RNase-free)
Micropipette and tips (including 1-mL tips)
Mortar and pestle (prechilled)
PCR tubes (0.2-mL or 0.5-mL, RNase-free)
Pipette (glass, drawn out)
Rotor for centrifuging 15-mL glass tubes (e.g., JA25.50)
Sieve (metal)
Spatula (metal)
Spoon (metal)
Stir bar
Syringe (10-mL)

Tabletop microcentrifuge
Tabletop centrifuge for 15-mL Falcon tubes (e.g., Eppendorf centrifuge 5702)
Test tube rotation wheel for 1.5-mL tubes
Thermocycler (e.g., MJ Research DNA Engine)
Thermoshaker preset to 65°C (e.g., Eppendorf Thermomixer)
Water baths preset to 37°C and 96°C

METHOD

At all steps of the procedure, it is important to work RNase-free. Use gloves at all times, treat solutions with DEPC, bake glass equipment, use filtered tips and RNase-free plasticware, and flame metal equipment. For all chemicals, have a box/flask that is only used for RNA work. Do not share solutions with anyone.

Large-Scale Growth of *C. elegans*

This section (Steps 1-11) describes the culturing of a large number of mixed-stage worms under physiological conditions. To avoid an accumulation of inhibitory pheromones and thus a delay in worm development or an induction of starvation, a two-step plate-based amplification protocol is used. Worms are frozen in liquid nitrogen in the form of pearls to facilitate usage of smaller portions (instead of the entire worm culture) for extract preparation (Step 12).

1. Take 12 10-cm NGM plates and 30 14.5-cm NGM plates, and seed each of them with 1 mL or 2 mL of OP50 solution, respectively. Dry the plates under a laminar flow hood and store overnight at room temperature. Keep plates at 4°C for long-term storage.
2. Prepare the worms from starved-worm plates as follows:
    i. Take three 6-cm starved-worm plates and wash the worms off three times with 1 mL of M9 salt solution into 1.5-mL tubes.
    ii. Pellet the worms by centrifugation for 1 min at 400*g*.
    iii. Put the worms on ice for 2 min to settle them. Remove the supernatant.
    iv. Wash the worms three times with 1 mL of M9 salt solution.
3. Resuspend the worms in 1.2 mL of M9 salt solution. Plate 100 µL of resuspended worms on each of the 12 10-cm NGM plates under a laminar flow hood, and let the plates dry for 5 min with the lids open.
4. Let the worms grow at 20°C for 4 days until the food is almost consumed.
5. Feed the worms by adding 1 mL of OP50 solution onto the plates under the laminar flow hood, and let the plates dry with the lids open.
6. Incubate the worms at 20°C until the food is almost consumed (usually one more time overnight).
7. Treat the worms as follows:
    i. Wash the worms off the plates with M9 salt solution and collect them in 15-mL Falcon tubes.
    ii. Store the worms on ice.
    iii. Pellet the worms by centrifuging in a tabletop centrifuge at 600*g* for 2 min.
    iv. Wash the worm pellet three times with M9 salt solution.
8. Resuspend the worms in 60 mL of OP50 solution and distribute 2 mL per 14.5-cm NGM plate (30 plates in total). Dry the plates under the laminar flow hood and incubate the worms at 20°C until they reach at least the L4 stage.
    Avoid starving the animals!

9. Treat the worms as follows:
    i. Harvest the worms by washing them off the plates with M9 salt solution and collect them in 15-mL Falcon tubes on ice.
    ii. Wash the worms three times with M9 salt solution.
    iii. Wash the worms twice in buffer B70 supplemented with protease inhibitors.
10. Collect all worms in one or two 15-mL Falcon tubes (not more than 4.5 mL of settled worms per tube) and add an equal volume of buffer B70 supplemented with protease inhibitors. The yield of settled worms is ~3-6 mL and is expanded to a total volume of 6-12 mL after the addition of buffer.
    An equivalent of ~2 mL of settled worms is plenty for a typical IP experiment with two individual samples.
11. Freeze the worms as follows:
    i. Resuspend the worms well and let them drip into a container of liquid nitrogen by gently pushing them out of a 1-mL micropipette tip.
    ii. Collect the frozen worm pearl suspension with a sieve placed over an empty liquid nitrogen container.
    iii. Transfer the worm pearls to a 50-mL Falcon tube using a metal spoon. At this point, the worm pearls can be stored at –80°C.

Whole-Worm Extract Preparation

This section (Steps 12-15) describes an efficient and economical protocol for the production of cytoplasmic extract from frozen worm cultures under conditions that preserve the integrity of cellular proteins and RNA. Perform the entire procedure at 4°C unless otherwise stated.

12. Use an equivalent of 2 mL of settled worms for an IP experiment with two individual samples, or more depending on the number of IP samples in the experiment. Grind the worms to a fine powder in liquid nitrogen using a mortar and a pestle that have been precooled with liquid nitrogen. Maintain the worm homogenate as a cold paste during the entire grinding procedure by adding fresh liquid nitrogen to the mortar once it has evaporated. Grind the worms several times to produce a fine powder.
    Be careful when touching the cooled mortar. It is necessary to wear protective gloves to prevent cold burns.
13. Transfer the powder into 15-mL glass tubes with a precooled spatula and add 2 mL of buffer B70 (supplemented with fresh protease inhibitors).
    The powder produced from an equivalent of 2 mL settled worms fills approximately half the volume of a 15-mL glass tube.
14. Centrifuge as follows:
    i. Centrifuge the tubes in a high-speed centrifuge at 37,000*g* for 30 min at 4°C.
    ii. Transfer the supernatant to precooled 1.5-mL tubes.
    iii. Centrifuge again in a tabletop centrifuge at 20,000*g* for 10 min at 4°C.
15. Pass the supernatant through a 0.45-µm filter into a precooled Falcon tube using a 10-mL syringe. Immediately add 5 µL of RNasin per 1 mL of extract and proceed straight to the co-IP (Step 16). Keep some extract aside to determine the protein concentration in a Bradford assay.
    The concentrations may vary between 10 and 35 mg protein per mL extract.

RNA Co-IP

This section (Steps 16-27) describes how to immunopurify RNA-protein complexes from worm extract in two steps. First, the extract is incubated with the antibodies to allow binding of the antibody to its target protein. Second, the antibody with the associated protein-RNA complex is affinity purified using Protein A-agarose. Perform Steps 16-27 at 4°C unless otherwise stated.

16. For each IP experiment (consisting of one specific and one nonspecific antibody sample), place 60 µL of bed volume of Protein A-agarose into RNase-free 1.5-mL tubes.
17. Wash the beads three times with buffer B70 as follows:

i. Pellet the beads in a tabletop centrifuge at 400*g* for 30 sec.

ii. Remove the supernatant and add wash buffer to the beads.

iii. Mix by carefully inverting the tube at least three times.

Avoid shaking the tube to prevent foam production and fragmenting the beads.

18. Preclear 1.1 mL of fresh extract on the washed Protein A-agarose beads for 30 min on a wheel to remove all molecules that nonspecifically bind to Protein A-agarose.

19. Pellet the beads at 400*g* for 30 sec and use the supernatant for the actual IP experiment.

20. Transfer two 50-µL aliquots as input samples into two RNase-free 1.5-mL tubes.

21. Add 350 µL of Trizol to each sample (from Step 20) and incubate them for at least 10 min at 65°C and 1000 rpm in a thermoshaker. Proceed to Step 28 with the input samples after the IP has been started (Steps 22 and 23).

22. Take 500 µL of precleared extract (from Step 19); add the IP-antibody (typically ~80 µL of serum plus 3 µL of RNasin). Incubate for 1 h on a rotating wheel.

23. In the meantime, prepare new Protein A-agarose beads to be used for pulling down the antibody-protein complex from the extract.

i. For each IP sample, take 30 µL of bed volume of Protein A-agarose and wash three times with buffer B70.

ii. Block the beads by incubating them for at least 30 min on a wheel in buffer B70 containing 10 mg/mL BSA, 0.1 mg/mL yeast total RNA, and 0.1 mg/mL heparin.

iii. Wash the beads once with buffer B70.

24. Add the extract-antibody mix (Step 22) to the preblocked Protein A-agarose and incubate for 1 h on a wheel.

25. Wash the beads quickly three times in buffer B70, then wash twice in buffer B70 for 10 min on a wheel. Discard the entire wash buffer.

26. To elute the RNA from the beads, add 200 µL of Trizol and incubate for at least 10 min at 65°C in a thermoshaker at 1000 rpm.

27. Centrifuge the beads at 400*g* for 2 min at room temperature. Transfer the supernatant to a fresh tube.

Purification of Total RNA

Steps 28-38 describe how to isolate and purify total RNA from the input and IP samples. Perform the entire procedure at room temperature unless otherwise stated.

28. Add one-fifth volume chloroform (80 µL for the input samples or 40 µL for the IP samples) and shake the tube vigorously by hand for 15 sec.

29. Let the tube sit for 3 min, then centrifuge at 12,000*g* for 15 min at 4°C.

30. Transfer ~60 % of the starting volume from the upper phase to a new tube (240 µL for input and 120 µL for IP samples). Avoid the white interphase! Bring the volume up to 400 µL with DEPC-treated H_2O.

31. Add 30 µL of DEPC-treated HEPES (50 mM, pH 7.5), mix well, and centrifuge. Add 400 µL of PCI and mix well by shaking.

32. Centrifuge at 13,200*g* for 5 min.

33. Transfer the upper phase (~400 µL) to a new tube while avoiding the interphase, add 350 µL of chloroform:IAA, mix well, and centrifuge at 13,200*g* for 5 min.

34. Transfer the upper phase (~380 µL) to a new tube while avoiding the interphase, and precipitate the RNA by adding 1.5 µL of glycogen (20 mg/mL), 1/10 volume of DEPC-treated NaOAc (3 M, pH 5.2), and 2.5 volumes of ethanol. Mix the tube well and incubate at –20°C for at least 30 min.

At this point, the procedure can be interrupted and continued the next day or later.

35. Pellet the RNA by centrifugation at 16,000*g* for 15 min at 4°C.

36. Remove the supernatant with a drawn-out glass pipette and wash the pellet with 1 mL of ice-cold 75% ethanol.

37. Centrifuge the tube at 16,000*g* for 5 min at 4°C and remove the entire supernatant with a drawn-out glass pipette.

38. Air-dry the pellet until it starts to turn from white to clear, then dissolve it in 20 µL of DEPC-treated H_2O at room temperature. Keep the RNA on ice and immediately proceed to Step 39.

DNase I Treatment of the RNA

To remove genomic DNA contamination, the RNA is treated with DNase I, which is described in Steps 39 and 40. Per IP experiment, take two input samples and treat one with and one without DNase I to have a control for the DNase I treatment.

39. Assemble the following reagents in 1.5-mL tubes on ice as indicated.

	DNase I+	DNase I–
10X DNase I buffer	4 µL	4 µL
RNA	20 µL	20 µL
DEPC-treated H_2O	15 µL	16 µL
DNase I	1 µL	—

40. Mix the tubes by gently tapping the bottoms, centrifuge the contents at 5000*g* for 10 sec, and incubate for 30 min at 37°C in a water bath. Immediately proceed to Step 41.

Repurification of RNA

Prior to cDNA production (Steps 46-49), the RNA must be purified a second time (described in Steps 41-45) to prevent subsequent cDNA degradation by DNase I or inefficient reverse transcription.

41. To the DNase I-treated RNA, add 360 µL of DEPC-treated H_2O, 30 µL of DEPC-treated HEPES (50 mM, pH 7.5), and 350 µL of PCI. Mix well by shaking.

42. Centrifuge at 13,200*g* for 5 min at room temperature.

43. Transfer the upper phase to a new tube while avoiding the interphase (~400 µL), add 350 µL of chloroform, and mix well. Centrifuge at 13,200*g* for 5 min.

44. Repeat Step 43.

45. Precipitate and wash the RNA as in Steps 34 to 38. Dissolve each RNA pellet in 26 µL of DEPC-treated H_2O. Keep the dissolved RNA on ice and proceed immediately to Step 46.

Reverse Transcription of mRNAs into cDNA

The mRNA will be reverse transcribed using an oligo(dT) primer to minimize ribosomal RNA contamination (Steps 46-49). Each RNA sample is divided into an RT+ sample and an RT– sample (negative control).

46. Heat the RNA for no longer than 3 min at 96°C and immediately place it in ice water for at least 5 min.

47. Centrifuge the sample, divide it into an RT+ sample and an RT– sample, and assemble the following reactions in PCR tubes kept on ice:

Add components in the order indicated.

	RT+	RT–
5X AMV buffer	4 µL	4 µL
Oligo dT primer (12.5 µM)	1 µL	1 µL
dNTPs (10 mM)	1 µL	1 µL
DEPC-treated H_2O	—	1 µL
RNA	13 µL	13 µL
AMV Reverse Transcriptase	1 µL	—

48. Mix the reactions by gentle tapping and incubate for 60 min at 55°C. Inactivate the reactions for 20 min at 65°C in a thermocycler.

49. Centrifuge the reactions and proceed to Step 50 or store the cDNA at –20°C.

Gene-Specific PCR

During the following steps (Steps 50-54), the presence of a particular mRNA in the IP and input samples is detected in a PCR reaction with gene-specific primers. Due to the low amount of mRNA in the samples, it may be necessary to perform a two-step PCR amplification for high sensitivity and specificity. To stay in the linear range of each PCR reaction, as few cycles as possible are advised.

50. Perform a gene-specific, nested PCR to detect the presence of a particular mRNA (now cDNA) in the samples. For optimal results, design the primers so that the primary PCR product with the outer primer pair is not larger than 500 bp (spliced!) and the secondary PCR product with the inner primer pair is in the range of ~300 bp (spliced!). Try to design primers with an annealing temperature of 58°C and a GC content of 50%.

51. For the input sample, take one sample with 0.5 µL of cDNA and one sample with 1.5 µL of cDNA as template to determine whether the PCR reaction is still in the linear range. Bring the volume up to 2 µL with H_2O. For the IP samples, take 2 µL of cDNA as a template for the PCR reaction. Set up the following primary PCR reaction in a total volume of 20 µL:

Template (cDNA from Step 49)	2 µL
Primers (both 5 µM in one mix)	1 µL
dNTPs (10 mM)	0.5 µL
10X PCR buffer	2 µL
Taq polymerase (5 U/µL)	0.5 µL
H_2O	14 µL

52. Run the following PCR program:

One cycle	95°C for 2 min
10-20 cycles (optimize for each primer pair)	95°C for 50 sec
	58°C for 50 sec
	72°C for 45 sec
One cycle	72°C for 7 min
Hold	4°C

53. Set up and run the secondary PCR in the same way as the primary PCR (Steps 51 and 52), but use 0.5 µL of primary PCR reaction as a template for the PCR.

54. Analyze the complete volume of the secondary PCR reaction on a standard agarose gel.
See Troubleshooting.

TROUBLESHOOTING

Problem: There is no signal in the IP samples in the gene-specific PCR.
[Step 54]
Solution: The IP samples usually contain very little RNA (often <300 ng in total). Also, consider the following:

1. RNA is highly sensitive to any kind of degradation. To test whether degradation is occurring during the experiment, monitor the RNA quality of an input sample during the procedure. Analyze purified RNA of an equivalent of 1 µL of extract on a denaturing agarose gel, or load 1 µL of purified RNA on an Agilent Bioanalyzer chip (e.g., after Steps 15, 18, 38, and 45). Any kind of degradation should be avoided (see "There is substantial RNA degradation" below).

2. There could be low enrichment of coimmunoprecipitated RNAs as a consequence of poor antibody affinity. If this might be the problem, use a higher antibody concentration or test different antibodies that recognize the protein in question.

Problem: There is substantial RNA degradation.
Solution: Consider the following:

1. To prevent RNA degradation during the procedure, it is important to work RNase-free and fast. Do not interrupt the procedure unless the RNA has been precipitated (Steps 34 and 45) or purified.
2. Keep the RNA on ice and avoid multiple freezing/thawing steps.
3. Consider the addition of the nonspecific RNase inhibitor ribonucleoside-vanadyl complex (RVC; 200 mM; NEB S1402S) to the extract. RVC is an RNA analog and generally blocks RNases, which improves overall RNA stability. However, EDTA must be excluded in all buffers that contain RVC because EDTA disassembles the RVC. Note also that traces of RVC can inhibit reverse transcriptases.

Problem: No specificity is seen in the pull-down of mRNAs.
Solution: Consider the following:

1. Try to use high salt (1 M urea or 300 mM KAc) or heparin in the wash buffer when performing Step 25.
2. Make sure that the PCR reaction is still in the linear range at the end of the secondary PCR (the 0.5-µL and 1.5-µL input concentrations should show a threefold difference in intensity on the DNA gel). If the PCR reaction has been exhausted, differences between specific and nonspecific IP might be lost.

DISCUSSION

The method described here allows for the investigation of RNA-protein complexes and identification of mRNA targets of RNA-binding proteins in vivo. Although the method is generally applicable, it utilizes whole-worm extracts as a starting material for the co-IP procedure. Worms are protected by a tough cuticle, which makes it difficult to obtain intact extracts of high protein concentration. We have tested alternative procedures for homogenizing worms, such as sonication and the use of a French press. In our hands, grinding frozen worm pearls with a mortar and pestle gives the most reproducible results and costs the least. During sonication and French pressing, local heating of the samples was measured; this can affect the quality of the mRNA. Furthermore, these methods require larger volumes for efficient worm lysis, which either reduces the protein concentration of the extract or increases the quantity of worms needed for extract preparation. We also observed that it is more beneficial for germline protein/RNA complexes to grow the worms on solid support than in liquid culture, where the morphology of the germ line and the progeny produced are of lower quality. The use of egg plates is not recommended, because it is rather difficult to reduce the high protein and lipid background, which is a result of contaminating chicken yolk.

Good controls are crucial for a meaningful result in co-IP experiments. First of all, it is important to use a nonspecific antibody in parallel to the specific antibody as a negative control for the IP. An excellent negative control is the inclusion of an IP sample from a worm extract (e.g., from mutant animals) that does not contain the protein pulled down from the wild-type worm extract. However, make sure to adjust the wild-type and mutant extracts to the same total protein concentration prior to the pull-down reaction. Second, in the gene-specific PCR, it is important to test for an abundant mRNA that is not expected to be associated with the immunoprecipitated protein complex. Such an mRNA serves as a good specificity control, because it should either not be enriched in the specific antibody sample over the negative control or not be present in the IP samples at all.

The method described here provides a tool to examine protein-RNA complex compositions in vivo. However, it should always be kept in mind that the protein-RNA interactions identified may be mediated by other proteins. Even more importantly, it must be remembered that a positive co-IP result does not confirm that a given protein-RNA complex is a consequence of a direct physical interaction between the protein in question and the tested mRNA molecule.

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