

RNA Chromatin Immunoprecipitation (RNA-ChIP) in *Caenorhabditis elegans*

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[Abstract] The RNA chromatin immunoprecipitation assay (RNA-ChIP) allows detection and quantification of RNA–protein interactions using *in vivo* cross-linking with formaldehyde followed by immunoprecipitation of the RNA–protein complexes. Here we describe the RNA–ChIP protocol that we have adapted for *Caenorhabditis elegans* (*C. elegans*) to detect interaction between the nuclear Argonaute CSR-1 (chromosome segregation and RNAi deficient) protein and its target nascent RNAs. We have used a transgenic strain expressing a recombinant long isoform of CSR-1 protein fused with N-terminal 3x FLAG epitope.

Materials and Reagents

1. *Escherichia coli* (*E. coli*) OP50 [available from the *Caenorhabditis* Genetics Center (CGC)]
2. Anti-FLAG M2 affinity gel (Sigma-Aldrich, catalog number: A2220)
3. 1-Bromo-3-chloropropane (BCP) (Sigma-Aldrich, catalog number: B9673)
4. 1 M CaCl₂ solution (Sigma-Aldrich, catalog number: 21114)
5. Distilled water (DNase/RNase-free UltraPure™) (Life Technologies, Invitrogen™, catalog number: 10977-015)
6. 10 mM dNTP mix (Thermo Fisher Scientific, catalog number: R0191)
7. EDTA (0.5 M, pH 8.0) UltraPure™ (Life Technologies, Invitrogen™, catalog number: 15575-020)
8. Ethanol 200 proof (for molecular biology) (Sigma-Aldrich, catalog number: E7023)
9. FLAG peptide (Sigma-Aldrich, catalog number: F4799)
10. Glycerol (Sigma-Aldrich, catalog number: G5516)
11. GlycoBlue™ coprecipitant (15 mg/ml) (Life Technologies, Ambion®, catalog number: AM4530)
12. 100x halt protease inhibitor cocktail (Thermo Fisher Scientific, catalog number: 78430)
13. Isopropanol (2-propanol, for molecular biology) (Sigma-Aldrich, catalog number: I9516)
14. KH₂PO₄ (Sigma-Aldrich, catalog number: P0662)
15. 8 M LiCl solution (Sigma-Aldrich, catalog number: L7026)
16. Maxima reverse transcriptase (Thermo Fisher Scientific, catalog number: EP0741)

17. 1 M MgCl₂ solution (Sigma-Aldrich, catalog number: M1028)
18. 1 M MgSO₄ solution (Sigma-Aldrich, catalog number: M3409)
19. Na₂HPO₄ (Sigma-Aldrich, catalog number: S3264)
20. 5 M NaCl solution (Life Technologies, Ambion®, catalog number: AM9759)
21. NP-40 [10% (w/v) aqueous solution] (Pierce Antibodies, catalog number: 85124)
22. Paraformaldehyde (32% solution, EM grade) (VWR International, catalog number: 15714-S)
23. Phase lock gel heavy (1.5 ml) (5PRIME, catalog number: 2302810)
24. Phenol:Chloroform:IAA (25:24:1, pH 6.6) (Life Technologies, Ambion®, catalog number: AM9730)
25. Proteinase K (~20 mg/ml) (recombinant) (PCR grade) (Thermo Fisher Scientific, catalog number: EO0491)
26. 2x QuantiFast SYBR® Green qPCR master mix (QIAGEN, catalog number: 204154)
27. Random hexamers (100 μM) (Thermo Fisher Scientific, catalog number: SO142)
28. RNase inhibitor SUPERase In™ (20 U/μl) (Life Technologies, Ambion®, catalog number: AM2694)
29. 20% SDS solution (Life Technologies, Ambion®, catalog number: AM9820)
30. 3 M sodium acetate (pH 5.5) solution (Life Technologies, Ambion®, catalog number: AM9740)
31. Sodium deoxycholate stock solution [10% (w/v) in distilled water] (Pierce Antibodies, catalog number: 89904)
32. TRI Reagent® solution (Life Technologies, Ambion®, catalog number: AM9738)
33. 1 M Tris-HCl (pH 7.5) UltraPure™ (Life Technologies, Invitrogen™, catalog number: 15567-027)
34. Triton-X 100 detergent solution (Pierce Antibodies, catalog number: 85111)
35. The sequence of primers used in Figure 1 to detect *mes-4* pre-mRNA: Forward GGATACATCAATGGAGAAATGGA (spanning exon 2 and intron 3) and Reverse ACAACTCGCGTGAATTTACTAC (spanning intron 3)
Note: DNA oligos has been synthesized by Integrated DNA Technologies (IDT).
36. 1x M9 buffer (see Recipes)
37. Nuclei extraction buffer (see Recipes)
38. RIPA buffer (see Recipes)
39. TSE 150 buffer (see Recipes)
40. TSE 500 buffer (see Recipes)
41. TSE 1,000 buffer (see Recipes)
42. LiCl buffer (see Recipes)
43. TE buffer (see Recipes)

44. Elution buffer (see Recipes)

Equipment

1. 1.5 ml Bioruptor TPX[®] polymethylpentene tubes (Diagenode, catalog number: M500-50)
2. Microfuge tubes (1.5 ml, RNase free) (Life Technologies, Invitrogen[™], catalog number: AM12400)
3. Bioruptor[®] standard UCD-200 sonicator (Diagenode, catalog number: B01010003)
4. Dura-Grind[™] stainless steel dounce tissue grinder 7 ml size (Wheaton Scientific, catalog number: 357572)
5. Real-time PCR machine (e.g. Eppendorf, Mastercycler[®] ep *realplex*⁴)
6. Refrigerated tabletop microcentrifuge
7. Labquake[™] tube rotators (Thermo Fisher Scientific, catalog number: 400220Q)
8. Vortex

Procedure

A. Fixation and nuclei extraction

1. Grow synchronized populations of worms on one large LB agar plate (100 x 15 mm) seeded with OP50 bacteria (~20,000 worms).
2. Wash worms off the plate with cold M9.
3. Centrifuge for 1 min at 1,200 x g to pellet the worms.
4. Wash 3 times with cold M9.
5. Centrifuge for 1 min at 1,200 x g to pellet the worms.
6. Fix the worms with 2% paraformaldehyde in 10 ml M9 buffer for 30 min at room temperature on rotation.
7. Centrifuge for 1 min at 1,200 x g to pellet the worms.
8. Discard the supernatant and wash one time with 10 ml cold 0.1 M Tris-HCl (pH 7.5).
9. Centrifuge for 1 min at 1,200 x g to pellet the worms.
10. Wash the worm pellet twice with 10 ml cold M9 buffers.
11. Centrifuge for 1 min at 1,200 x g to pellet the worms and discard the supernatant.
Note: At this step you can freeze the worm pellet and store at -80 °C.
12. Resuspend worm pellet in 2 ml cold Nuclei extraction buffer.
13. Transfer 2 ml of worms to a steel dounce (on ice) and stroke at least 30 times.
Note: Check under the microscope that the worms have been crushed.
14. Transfer 2 ml of lysate to two RNase free 1.5 ml microfuge tubes and centrifuge 2 min at 40 x g at 4 °C.

15. Transfer the supernatant to two new RNase free 1.5 ml microfuge tubes.
16. Centrifuge for 5 min at 1,000 x g at 4 °C to pellet the nuclear fraction.
17. Discard the supernatant and wash the nuclear pellet with 1 ml of cold nuclei extraction buffer.

Note: Combine the two pellets in one RNase free 1.5 ml microfuge tube.

18. Centrifuge for 5 min at 1,000 x g at 4 °C to pellet the nuclear fraction.
19. Repeat steps 17 and 18 twice.
20. Centrifuge for 5 min at 1,000 x g at 4 °C to pellet the nuclear fraction.
21. Resuspend the nuclear pellet in 500 µl RIPA buffer.

B. Sonication

22. Transfer the lysate to a 1.5 ml Bioruptor TPX® polymethylpentene tube.
23. Sonicate sample for 20 min at 4 °C (settings: 30 sec on, 30 sec off, level high 320 W, wave frequency 20 kHz).
24. Centrifuge for 5 min at 12,000 x g at 4 °C. Transfer the supernatant (containing chromatin) to a new RNase free 1.5 ml microfuge tube.

Note: At this step samples can be stored at -80 °C.

C. Immunoprecipitation

25. Take 1/20 fraction of the supernatant for the input RNA extraction and store on ice.
26. Use 30 µl of anti-Flag M2 affinity gel for each immunoprecipitation.
27. Equilibrate the affinity gel with 1 ml of RIPA buffer.
28. Centrifuge for 1 min at 1,000 x g at 4 °C.
29. Repeat steps 27 and 28.
30. Add supernatant from step 24 to the equilibrated affinity gel.
31. Incubate on rotation (continuous rotation at 8 rpm) for 1 h at 4 °C.
32. Centrifuge for 1 min at 1,000 x g at 4 °C.
33. Wash the affinity gel with 1 ml TSE150 buffer and incubate on rotation for 1 min at 4 °C.
34. Centrifuge for 1 min at 1,000 x g at 4 °C.
35. Discard the supernatant and wash the affinity gel 2 times with 1 ml TSE500 buffer and incubate on rotation for 1 min at 4 °C.
36. Centrifuge for 1 min at 1,000 x g at 4 °C.
37. Discard the supernatant and wash the affinity gel with 1 ml TSE1000 buffer and incubate on rotation for 5 min at 4 °C.
38. Centrifuge for 1 min at 1,000 x g at 4 °C.
39. Discard the supernatant and wash the affinity gel with 1 ml LiCl buffer and incubate on rotation for 5 min at 4 °C.

40. Centrifuge for 1 min at 1,000 x *g* at 4 °C.
41. Discard the supernatant and wash the affinity gel 2 times with 1 ml TE buffer.
42. Centrifuge for 1 min at 1,000 x *g* at 4 °C.
43. Discard the supernatant and remove as much as possible buffer using P200 pipette tips.
44. Elute the protein-RNA complexes from the affinity gel using 200 µl of elution buffer.
45. Incubate with rotation for 1 h at 4 °C.
46. Centrifuge for 1 min at 1,000 x *g* at 4 °C.
47. Transfer the supernatant to a new RNase free 1.5 ml microfuge tube.

D. Reverse crosslink

48. Take the 200 µl of supernatant from step 47 and input RNA from step 25 (to a final volume of 200 µl with elution buffer) and treat them with 1 µl (20 µg) of proteinase K for 1 h at 45 °C.

E. RNA purification

49. Add 750 µl of TRI Reagent® solution to the samples from step 48 and vortex for 30 sec.
50. Add 100 µl of BCP and vortex for 30 sec.
51. Incubate at room temperature for 10 min.
52. Centrifuge for 15 min at 12,000 x *g* at 4 °C.
53. Transfer the top aqueous phase in a new RNase free 1.5 ml microfuge tube.
54. Add 1.5 µl of GlycoBlue™ and 50 µl of 3 M sodium acetate.
55. Mix and add 1 volume of isopropanol (typically 400-500 µl).
56. Incubate at room temperature for 5 min.
57. Centrifuge for 20 min at 12,000 x *g* at 4 °C.
58. Wash the RNA pellet with 1 ml of 70% ethanol.
59. Centrifuge for 5 min at 7,500 x *g* at 4 °C.
60. Resuspend the pellet in 44 µl of RNase-free UltraPure™ distilled water.

F. DNase treatment

61. Add 5 µl of 10x TURBO DNase buffer and 1 µl of TURBO DNase to the samples from step 60.
62. Incubate 30 min at 37 °C.
63. Add 450 µl of RNase-Free UltraPure™ distilled water (total volume 500 µl).
64. Add 500 µl of Phenol: Chloroform: IAA and mix by vortex.
65. Transfer the mixture to 1.5 ml Phase Lock Gel tube and centrifuge at 12,000 x *g* for 5 min at 4 °C.
66. Transfer top aqueous phase in a new RNase free 1.5 ml microfuge tube.

67. Add 1.5 μ l of Glycoblue™ and 50 μ l of 3 M sodium acetate and mix by vortex.
68. Add 1 volume of isopropanol (typically 400-500 μ l) and mix by vortex.
69. Incubate at room temperature for 5 min.
70. Centrifuge for 20 min at 12,000 $\times g$ at 4 °C.
71. Wash the RNA pellet with 1 ml of 70% ethanol.
72. Centrifuge for 5 min at 7,500 $\times g$ at 4 °C.
73. Resuspend in 12.5 μ l of RNase-Free UltraPure™ distilled water.

G. cDNA Synthesis

74. Add to the samples from step 73, 1 μ l of random hexamer primers, 4 μ l of 5x reaction buffer, 0.5 μ l of RNase Inhibitor, 1 μ l of dNTP mix (0.5 mM final concentration), and 1 μ l of Maxima reverse transcriptase.
75. Incubate 10 min at 25 °C followed by 60 min at 37 °C.
76. Stop the reaction by heating at 70 °C for 10 min.

Note: The reverse transcription reaction can be stored at -20 °C.

H. qPCR

77. Dilute the 20 μ l sample from step 76 with 100 μ l of nuclease free water and use 5 μ l as a template in 25 μ l qPCR reaction (performed in triplicate reactions); add 12.5 μ l of 2x QuantiFast SYBR® Green mix, and primers to a final concentration of 0.6 μ M. For the amplification of nascent RNAs, the primers have to be designed in such a way that at least one PCR primer (forward or reverse) is located in an intron of a target gene and the amplicon is 70-100 bp long. The average Ct value from triplicate qPCR reactions will be considered to calculate the percentage of input. For more information about the percentage of input calculation see <http://www.lifetechnologies.com/us/en/home/life-science/epigenetics-noncoding-rna-research/chromatin-remodeling/chromatin-immunoprecipitation-chip/chip-analysis.html>.
78. For qPCR cycling conditions use the parameters suggested by QuantiFast SYBR® Green mix manufacturers.

Note: The best way to represent the data is by percentage of input (see Figure 1).

Representative data

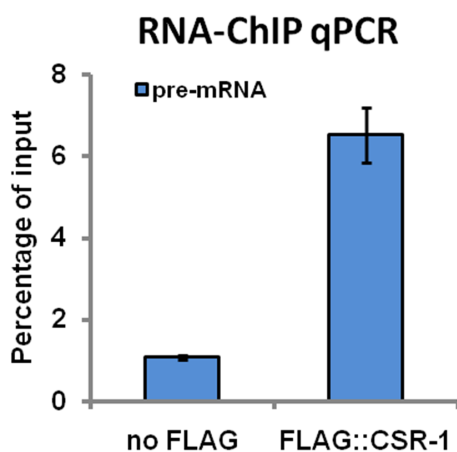


Figure 1. RNA-ChIP qPCR results obtained with an antibody against FLAG epitope and nuclear extracts from transgenic worms expressing FLAG:CSR-1 protein and control non-transgenic worms (no FLAG). The primers used in a qPCR assay have been designed to span one intron of the pre-mRNA of a known target transcript of CSR-1. The results from three independent experiments (biological replicates) are shown as percentage of input. Error bars represent the standard deviation.

Notes

The protocol has been applied to the transgenic strain expressing a recombinant CSR-1 protein fused with Flag epitope. However, it can be used with virtually any Flag-tagged nuclear proteins expressed in *C. elegans*. Moreover, if a primary antibody raised against the protein of interest is available, it can be crosslinked to Protein A/G resin and used instead of the anti-Flag M2 affinity gel. In this case, it is recommended to choose an antibody that has been tested in ChIP experiments. Because of the high background of the RNA-ChIP assay, it is highly recommended to always use a non-transgenic strain as a control in each experiment or knock out strains if primary antibody raised against the protein of interest will be used. The following protocol can also be used for cytoplasmic RNA-binding proteins. In this case, the nuclear extraction step can be omitted.

Recipes

1. 1x M9 buffer
 - 3 g KH_2PO_4
 - 6 g Na_2HPO_4
 - 17 ml 5 M NaCl
 - 1 ml 1 M MgSO_4 in 1 L of deionized water
2. Nuclei extraction buffer
 - 10 mM Tris-HCl (pH 7.5)
 - 2 mM MgCl_2
 - 3 mM CaCl_2
 - 0.5% NP-40
 - 10% glycerol
 - 100 U/ml RNase inhibitor
 - 1x protease inhibitor cocktails
3. RIPA buffer
 - 0.1% SDS
 - 0.1% deoxycholate
 - 1% Triton X-100
 - 1 mM EDTA
 - 10 mM Tris-HCl (pH 7.5)
 - 150 mM NaCl
 - 100 U/ml RNase inhibitor, protease inhibitor cocktails
4. TSE 150 buffer
 - 0.1% SDS
 - 1% Triton X-100
 - 2 mM EDTA
 - 20 mM Tris-HCl (pH 7.5)
 - 150 mM NaCl
 - 100 U/ml RNase inhibitor
5. TSE 500 buffer
 - Same as TSE 150 but with 500 mM NaCl
6. TSE 1,000 buffer
 - Same as TSE 150 but with 1 M NaCl
7. LiCl buffer
 - 250 mM LiCl
 - 1% NP40

- 1% deoxycholate
- 1 mM EDTA
- 10 mM Tris-HCl (pH 7.5)
- 100 U/ml RNase inhibitor
- 8. TE buffer
 - 10 mM Tris-HCl (pH 7.5)
 - 1 mM EDTA
- 9. Elution buffer
 - 10 mM Tris-HCl (pH 7.5)
 - 0.5% NP-40
 - 200 mM NaCl
 - 300 µg/ml FLAG peptide
 - 100 U/ml RNase inhibitor

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References

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