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RNA Interference in *Caenorhabditis elegans*

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1. Introduction

The introduction of double-stranded RNA (dsRNA) into *Caenorhabditis elegans* hermaphrodites results in the rapid and sequence-specific degradation of endogenous mRNAs ***(1,2)***. This RNA-mediated interference, or RNAi, effectively shuts down expression of the target gene and can phenocopy loss-of-function mutations. RNAi is also remarkably potent, requiring only substoichiometric amounts of dsRNA to elicit a response ***(1)***. Another notable aspect of RNAi in *C. elegans* is that it is systemic in that the silencing can spread between tissues throughout the adult as well as its progeny ***(1,3,4)***. Many neurons, however, are refractory to the spreading effect in wild-type backgrounds ***(5)***.

Although discovered in *C. elegans,* the use of dsRNA to silence gene expression has quickly become a widely used tool to study gene function in a number of organisms, including mammals ***(6,7)***. RNAi may have evolved from an ancient phenomenon used to regulate gene expression and combat transposable elements and viruses ***(8)***.

The ability to use RNAi to target single or multiple transcripts and the ability to apply RNAi to large-scale genomic screens further highlight the power of this technique ***(9–12)***.

2. Materials

1. Wild-type *C. elegans*.
2. NGM agar plates: 2.5 g peptone, 17 g agar, 3 g NaCl, 975 mL distilled water (dH_2O). Autoclave (30 min). Let cool to 55°C and add the following sterile solutions: 1 mL of 5 mg/mL cholesterol (stock in EtOH), 1 mL of 1 *M* $CaCl_2$, 1 mL of 1 *M* $MgSO_4$, 25 mL of 1 *M* KH_2PO_4.

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3. OP50 *Escherichia coli*.
4. Agarose, low electroendosmosis (EEO) electrophoresis grade (Fisher Scientific, Suwanee, GA).
5. Large plastic Petri dishes, 100 × 15 mm (Fisher Scientific).
6. Sterile M9 buffer: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL of 1 *M* $MgSO_4$; fill to 1 L with dH_2O.
7. 15-mL polypropylene tube.
8. 50–100–20 buffer: 50 m*M* Tris-HCl, 100 m*M* NaCl, 20 m*M* EDTA, pH 7.5.
9. 50–100–20 + Sodium dodecyl sulfate (SDS) + proteinase K: 1% SDS, 200 µg/mL proteinase K in 50–100–20 buffer.
10. Phenol : chloroform (1 : 1).
11. 3 *M* sodium acetate, pH 5.2.
12. 100% ethanol.
13. Worm pick: 36-gauge platinum wire attached to glass Pasteur pipet (Fisher Scientific).
14. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.6.
15. DNase-free RNase A.
16. Oligonucleotide primers specific to target gene.
17. Oligo $(dT)_{12–18}$ (500 µg/mL).
18. Sterile diethylpyrocarbonate (DEPC) H_2O.
19. 5X first strand buffer, supplied with enzyme (Stratagene, La Jolla, CA).
20. 0.1 *M* dithiothreitol (DTT), supplied with enzyme (Stratagene).
21. 10 m*M* dNTP stock in DEPC–dH_2O (Stratagene).
22. SuperScript II enzyme (200 U/µL) (Stratagene).
23. 0.5 *M* EDTA.
24. 2 m*M* dNTPs.
25. *Taq* polymerase.
26. Polymerase chain reaction (PCR) thermocycler and supplies.
27. PCR purification kit (Qiagen, Valencia, CA).
28. Phenol : chloroform : isoamylalcohol (25 : 24 : 1).
29. Chloroform.
30. 10 *M* NH_4Ac.
31. 70% EtOH.
32. Agarose gel electrophoresis apparatus.
33. Ultraviolet (UV) transilluminator.
34. Ethidium bromide (EtBr) (0.3 µg/mL).
35. Ultraviolet spectrophotometer.
36. Gel extraction kit (Qiagen).
37. T7 transcription kit (Ambion Megascript T7, Austin, TX or Promega, T7 Ribomax, Madison, WI).
38. Speed vacuum apparatus.
39. Borosilicate capillaries model 1B100F (World Precision Instruments, Sarasota, FL).
40. Needle puller (Sutter Instruments, Novato, CA).
41. Halocarbon oil, series 700 (Sigma, St. Louis, MO).

42. Injection pads (24 × 50 mm #1 cover slip with 2% agarose).
43. RNAi soaking buffer: 0.05% gelatin, 5.5 m*M* KH_2PO_4, 2.1 m*M* NaCl, 4.7 m*M* NH_4Cl, 3 m*M* spermidine. Add spermidine fresh before each use.
44. NGM plates + 1 *M* Isopropyl-β-D-thiogalactopyranoside (IPTG) + ampicillin poured fresh (1–3 d before use).
45. Ampicillin 100 mg/mL.
46. Luria–Bertani (LB) ampicillin agar plates (50 µg/mL).
47. LB tetracycline agar plates (12.5 µg/mL).
48. Sterile, RNase free dH_2O.
49. Siliconized 0.6 mL eppendorf tubes.
50. Siliconized 200 µL pipet tips.
51. *C. elegans* feeding library (MRC Geneservice).
52. IPTG.

3. Methods

The methods below outline (1) the preparation of genomic DNA from *C. elegans*, (2) dsRNA synthesis, and (3) RNA interference methods. Methods for handling *C. elegans* are described in **ref. *13*** and detailed methods for injection are described in **ref. *14***.

3.1. Preparation of Genomic DNA from C. elegans

1. Grow up one to two large plates of worms on NGM plates, using agarose in place of the agar.
2. Wash worms off of a recently starved plate (to avoid excess bacteria) using sterile M9 buffer, into a 15-mL polypropylene tube.
3. Pellet worms by centrifugation and carefully discard supernatant.
4. Wash worms with 10 mL of 50–100–20 buffer.
5. Pellet worms by centrifugation and carefully discard supernatant.
6. Add 2 mL of 50–100–20 + 1% SDS + 200 µg/mL proteinase K and incubate at 65°C.
7. Periodically agitate until suspension is viscous (use gentle agitation to avoid shearing DNA). This takes about 30 min.
8. Add 2 mL of phenol : chloroform and invert gently for 5 min.
9. Centrifuge to separate phenol : chloroform and aqueous phases.
10. Transfer top (aqueous) phase to a clean 15-mL tube.
11. Add 270 µL of 3 *M* sodium acetate. This results in a final concentration of 0.4 *M* sodium acetate.
12. Add 2.5 vol of 100% ethanol.
13. Wind out DNA precipitate onto the tip of a sealed glass Pasteur pipet (DNA visible as thin threads).
14. Dissolve DNA in 400 µL of TE (*see* **Note 1**).
15. Add RNase to 20 µg/mL final concentration.
16. Incubate at 37°C for 20 min.
17. DNA is sufficiently clean for use as template in polymerase chain reaction (PCR). Store at 4°C (*see* **Note 2**).

3.2. dsRNA Synthesis

3.2.1. Primer Design

Design primers to amplify approx 1 kb of mostly coding sequence (*see* **Note 3**), although 200–500 bp of target sequence often works well and may also reduce the chance of targeting other homologous transcripts. Be sure to target mostly exonic sequences and ensure that you are not targeting other homologous transcripts (*see* **Note 4**).

3.2.2. First Strand cDNA Synthesis (If Unable to Use Genomic DNA)

In those cases where only small exons separated by large introns are available, cDNA must be used to ensure you have sufficient exonic sequence to target. Before starting, make sure that all of the materials and reagents are sterilized and/or nuclease-free.

1. Mix 1 µL of oligo $(dT)_{12–18}$ (500 µg/mL), 1–5 µg of mRNA, sterile DEPC H_2O to 12 µL.
2. Heat the mixture to 70°C for 10 min, and then quickly chill on ice.
3. Centrifuge briefly to collect mixture at bottom of tube.
4. Add the following: 4 µL of 5X first strand buffer, 2 µL of 0.1 M DTT, 1 µL of mixed dNTP stock.
5. Mix the contents and centrifuge briefly to collect. Place tube at 42°C for 2 min to equilibrate temperature.
6. Add 1 µL of SuperScript II enzyme (200 U/µL) for each microgram of mRNA used in **step 1** to the mix.
7. Incubate at 42°C for 1 h.
8. Add 4 µL of 0.5 M EDTA or place tube at 70°C for 15 min in order to terminate the reaction.
9. Store cDNA at –20°C until use.

3.2.3. PCR

Amplification is performed in two steps. Designing an in vitro transcription template from a two-step PCR makes synthesizing multiple primers less expensive and may increase the yield of PCR products bearing complete T7 sites. The first step uses two 35-base pair primers, and each primer contains 15 bases of T7 sequence plus 20 bases of sequence from the gene of interest (*see* **Note 5**).

Example:

	partial T7	gene of interest
Forward primer: 5′	CGACTCACTATAGGG	CGATGAGGGCCTATTTATTC 3′
Reverse primer: 5′	CGACTCACTATAGGG	GAGAAAGTACACGATATAGC 3′

The first PCR product is cleaned using the Qiagen PCR Purification kit (as per manufacturer's instructions) and eluted in 33 µL final volume. One-tenth of

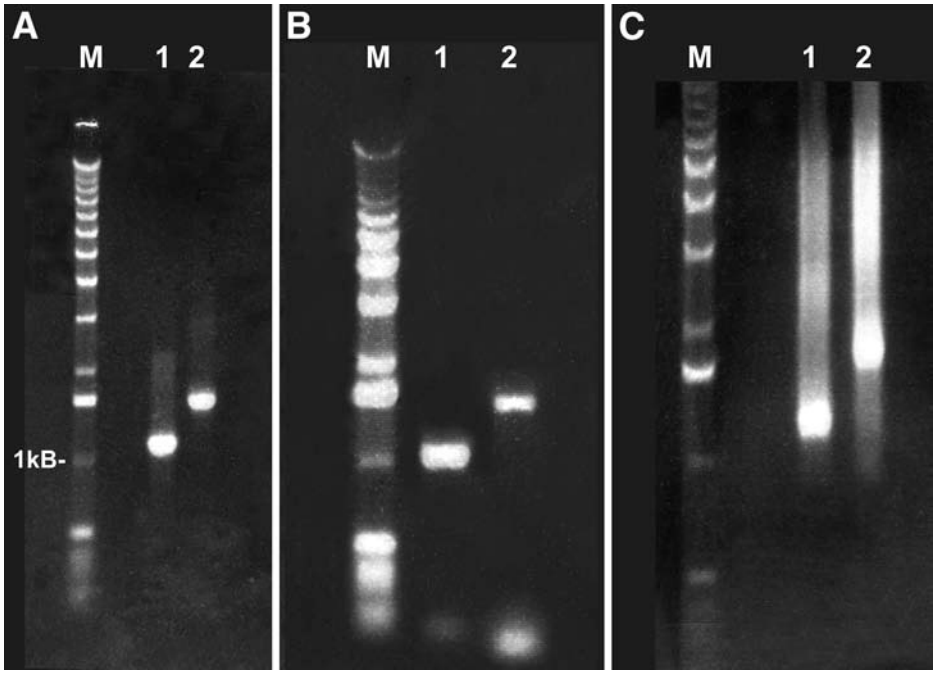

Fig. 1. **(A)** *mlc-4* (1) and *nmy-2* (2) PCR product amplified from genomic DNA with primers having partial T7 sequences. **(B)** *mlc-4* and *nmy-2* second-round PCR products that have been gel-purified using complete T7 primers and restriction sites. **(C)** Amplified and purified *mlc-4* and *nmy-2* dsRNAs. M is the DNA marker in each panel.

the recovered product is analyzed on an agarose gel (*see* **Fig. 1A**) and visualized using EtBr and UV illumination.

The second PCR uses the same primer on each end—a primer containing the full T7 polymerase promoter plus restriction sites for use with cloning: *Eco*RI, *Xba*I, and *Hind*III (*see* **Note 6**):

EXHT7: 5′ ATAGAATTCTCTAGAAGCTTAATACGACTCACTATAGGG 3′

One-tenth of the recovered product is analyzed on an agarose gel (*see* **Fig. 1B**) and visualized using EtBr and UV illumination.

3.2.4. In Vitro Transcription

The second PCR product is then gel purified (Qiagen Gel Extraction kit, as per manufacturer's instructions) and 1–2 μg of PCR product are used as the template for transcription. Commercially available in vitro transcription kits such as Ambion's Megascript-T7 kit or Promega's T7 Ribomax kit can be used to obtain high yields of dsRNA (*see* **Note 7**).

3.2.5. RNA Recovery

Pass the RNA through a Qiagen PCR purification column (treating the RNA as if it were a PCR product) to purify the RNA. RNA-specific columns can be used instead, although these are more expensive. Alternatively, dsRNA can be purified using phenol–chloroform extraction followed by ethanol precipitation (*see* **Subheading 3.2.7.**). Assess dsRNA integrity on an agarose gel (*see* **Fig. 1C**) and determine concentration using a UV spectrophotometer.

3.2.6. Storage

Mix the resulting solution with 2 vol 100% EtOH and store at –80°C. Before use, the EtOH should be evaporated in a speed-vaccum apparatus and the dsRNA pellet can be resuspended in sterile distilled water at your desired concentration.

3.2.7. Phenol–Chloroform Extraction and Ethanol Precipitation

Instead of using expensive columns (*see* **Subheading 3.2.5.**) you can purify your dsRNA using phenol–chloroform extraction followed by ethanol precipitation.

1. Add an equal volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1) to the in vitro transcription reaction.
2. Mix (vortex) the contents of the tube until an emulsion forms.
3. Centrifuge the mixture at 11,750*g* for 15 s in a microcentrifuge at room temperature.
4. Transfer the upper (aqueous) phase containing dsRNA to a fresh tube. Discard the interface and the lower (organic) phase.
5. Add an equal volume of chloroform and repeat extraction steps (**steps 2–4**).
6. Add 1/3 vol of 10 *M* NH_4Ac.
7. Add 2.5 vol of 100% EtOH and put at –20°C for 30 min. The dsRNA can be stored at this step at –20°C if desired.
8. Spin down for 10 min at 11,750*g* in a microcentrifuge at 4°C to pellet dsRNA.
9. Wash pellet with 70% EtOH.
10. Air-dry pellet.
11. Resuspend in 100 µL of TE, pH 7.6.
12. dsRNA should be stored at –20°C in 1–2 vol of 100% EtOH.

3.3. Microinjection

The dsRNA for microinjection can be injected anywhere in the body cavity or gonad of the *C. elegans* hermaphrodite ***(1)***. Additional details can be found in **ref. *14*.**

3.3.1. Preparing and Storing Needles

1. Pull capillaries to a finely tapered tip using a needle puller.
2. Use a Petri dish containing a strip of modeling clay to hold the needles. If kept dust-free, needles can be stored indefinitely.

3.3.2. Injection Pads

1. A drop of 2% agarose (in dH_2O) is flattened between two 24 × 50-mm #1 cover slips.
2. After hardening, remove the top cover slip and let the pad dry in open air (Alternatively, pads can be baked at 60°C for 1 h). If letting the pads air-dry, they should be made at least 1 d prior to use. Pads made either way can be stored for several weeks at room temperature.
3. Inject dsRNA solution into hermaphrodite gut or gonad.

3.3.3. Loading and Breaking the Needle

1. Place a 0.5 µL drop of the dsRNA solution on the back (blunt) end of the needle; the needle contains an inner glass filament that will wick the dsRNA solution to the tapered end.
2. Load needle onto an injection microscope. Use the edges of the agarose injection pad to gently break the needle. This step here takes practice to ensure that the needle does not have too large an opening, which would tend to damage the worms during injection (*see* **Note 8**).

3.3.4. Mounting and Injecting Worms

1. Place a drop of Halocarbon oil on the pad.
2. Using a worm pick, transfer young adult hermaphrodites to the oil drop on the pad (*see* **Note 9**).
3. Pat the animal down on the agarose pad with your pick until it is firmly stuck to the pad. Minimize the time that the worms spend drying out on the pad during injection to increase the viability of the worms.

3.3.5. Recovery

Put the pad under the dissecting scope and place a drop of M9 buffer on the oil drop containing your worms. Your worms will rehydrate and begin to swim. Transfer worms to a fresh NGM plate.

3.4. Feeding

RNA interference can be performed in *C. elegans* by feeding RNAse III-resistant bacteria expressing dsRNA to *C. elegans* ***(5)***. A genomic feeding library has been constructed ***(15)*** and is available commercially. The library consists of genomic fragments cloned into an IPTG-inducible T7 polymerase vector (the PCR product made in **Subheading 3.2.3.** can also be cloned into this feeding vector). The vectors are transformed into the RNAse III-resistant bacterial strain.

1. Streak out –80°C stock feeding strain that contains the desired construct onto LB tetracycline plates (tetracycline will select for feeding competent bacteria).
2. Pick a bacterial colony from streaked plates with a sterile toothpick (using sterile technique) and place it in approx 2 mL of LB/amp (ampicillin will select for the cloned gene of interest).

3. Shake overnight at 37°C.
4. Inoculate 2 mL fresh LB plus ampicillin with 20 µL of the overnight culture. Grow at 37°C until the optical density is between 0.35 and 0.40 (this usually takes about 3 h).
5. Seed NGM + IPTG + ampicillin plates with actively growing culture and allow to dry at room temperature (this will happen anytime between 3 h to overnight) (*see* **Note 10**).
6. Add worms to dsRNA-producing bacteria and allow worms to feed. Transfer worms every 24 h to new feeding plates (*see* **Note 11**).

3.5. Soaking

RNA interference can be performed in *C. elegans* by soaking worms in dsRNA ***(16)***; this method has been used in large-scale screens ***(12)***.

1. Pick four young adults onto a fresh unseeded NGM plate to clean off bacteria (*see* **Note 12**).
2. Wash worms with a drop of M9 and allow them to crawl away.
3. Add the four worms to 2 µL of RNAi soaking buffer in 0.6-mL siliconized Eppendorf tubes (final dsRNA concentration should be at 1 mg/mL).
4. Place at 20°C for 24 h.
5. Transfer worms to a fresh, seeded plate using siliconized pipet tips.
6. Wash Eppendorf tube with 100 µL of M9 to ensure transfer of all of the soaked worms.
7. Transfer to new plates daily and score progeny.

4. Notes

1. Swirl the tip of the glass Pasteur pipet containing the DNA in a microfuge tube containing 400 µL of TE.
2. Do not freeze genomic sample, as thaw cycles can shear genomic DNA. Multiple genomic DNA preparations can be used as templates when a product does not amplify from a single preparation.
3. Visit www.wormbase.org for gene structure information.
4. Visit http://www.sanger.ac.uk/cgi-bin/blast/submitblast/c_elegans for BLAST tools needed to search for and avoid dsRNAs that target other homologous transcripts.
5. When designing primers, be sure to avoid creating primers that contain primer dimers. Also, primers should not have a high GC content (>50%) and be sure that your primers are also free from strong secondary structure.
6. These DNA templates can be easily cloned into the feeding construct (L4440) discussed in **Subheading 3.4.**
7. Purchasing rNTPs, T7 polymerase, DNase, and RNase inhibitors separately and then purifying the dsRNA using phenol : chloroform followed by EtOH precipitation also works well (*see* **Subheading 3.2.7.**).
8. You can also dip needles in hydrofluoric acid to open needle tips.
9. Be sure to limit the amount of bacteria transferred, as excess bacteria can make it difficult for the worms to adhere to the pad.

10. Be sure that all media for plates are cooled to 50°C before adding IPTG, ampicillin, or tetracycline.
11. You can also place young larvae on plates or have worms lay eggs onto the feeding plates for a couple of hours, followed by removal of the mothers.
12. This protocol can be scaled up as necessary.

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