

Methods for Delivery of Double-Stranded RNA into *Caenorhabditis elegans*

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Summary

The nematode *Caenorhabditis elegans* is often employed in investigations of diverse aspects of biology, including behavior, development, basic cellular processes, and disease states. The ability to utilize double-stranded RNA (dsRNA) to inhibit specific gene function in this organism has dramatically increased its value for these kinds of studies and has provided more flexibility in experimental design that include procedures. Here, we have collected a set of protocols from the *C. elegans* community for propagation of *C. elegans*, techniques for dsRNA preparation, four basic methods for delivery of dsRNA to *C. elegans* (injection, soaking, feeding, and in vivo delivery), and we suggest schemes that should facilitate detection of specific gene silencing.

Key Words: Double-stranded RNA; single-stranded RNA; RNA silencing; feeding; soaking; injection; HT115; HT115(DE3); transgene; transcription; RNAi.

1. Introduction

Caenorhabditis elegans is a small (1 mm long), nonparasitic nematode easily managed in the laboratory. The worm is cultured in Petri dishes using bacteria as a food source; has a short life-span (2 to 3 wk); and is transparent, with all 959 somatic cells visible under a microscope. The completed DNA sequence and the ability to generate mutants have made this an attractive system for analysis of gene function, and RNA silencing technology (RNA interference [RNAi]) has opened up amazing possibilities for genetic manipulations. RNAi in *C. elegans* affords particular advantages: First, long double-stranded RNAs (dsRNA) can be utilized as trigger molecules, and these are synthesized easily and inexpensively by in vitro transcription. Second, *C. elegans* apparently does not exhibit nonsequence-specific responses, such as the interferon/

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protein kinase R responses, that are prevalent in mammalian systems. Third, RNA silencing molecules are readily imported into the cells of most tissues. Fourth, several methods are available for delivering dsRNA to *C. elegans*, as individuals or a population. In addition, RNAi has proven particularly valuable in uncovering roles for genes in the developing germline—roles that may not be observable in genetic mutants owing to maternal deposition of gene product (**1**).

RNAi in *C. elegans* entails manufacture of dsRNA, delivery of dsRNA to worms, and extensive phenotypic analysis of affected animals. We present four protocols that should allow efficient delivery of dsRNA molecules to the animal: *injection* involves preparing dsRNA by in vitro transcription followed by injection of the dsRNA into animals; *soaking* is a forced ingestion or absorption of dsRNA by suspension of animals in concentrated dsRNA solutions; *feeding* relies on ingestion of bacteria engineered to express dsRNA; and *in vivo delivery* is accomplished by creating DNA constructs designed to express dsRNA from *C. elegans* promoters and transforming such constructs into worms to generate transgenic lines.

1.1. Considerations for the mRNA Target

Many genes, when mutated, elicit no obvious phenotypes in the laboratory setting; thus, the corresponding RNAi experiments may not result in a phenotype. Additionally, some sequences are more efficiently targeted for RNA silencing than others. The intrinsic stability of an mRNA, its accessibility to the RNAi machinery, and additional factors may determine susceptibility. In some instances, the preexisting protein, as well as the mRNA, must be diminished before a phenotype is observed. A comparative analysis of dsRNA-treated animals vs mock-treated animals by *in situ* hybridization or protein immunolocalization ensures proper execution of the protocol. Another indication that the gene of interest is not amenable to RNA silencing can be revealed by a failure to reduce green fluorescent protein (GFP) fluorescence in a transgenic animal expressing a *gene of interest::gfp* fusion following treatment with dsRNA corresponding to the gene target. Some genes expressed in the nervous system, in the pharynx, and in males can prove to be difficult targets for silencing (**2**), but the use of animals defective for *rrf-3* has enabled some of these limitations to be overcome (**3**). *rrf-3* encodes a protein with some homology to RNA-dependent RNA polymerases (**4**) and is one of several genes that function to suppress RNA-silencing mechanisms.

1.2. Considerations for Choosing an Appropriate Region from the Gene of Interest for dsRNA Trigger

It is not necessary, nor is it always advisable, to use the entire coding region of a gene as a template for dsRNA synthesis. The particular sequence chosen

for use as a dsRNA trigger can have important consequences because distinct sequences derived from the same gene may elicit dissimilar RNAi results. When selecting a DNA sequence for use as a template, one should consider the following:

1. *Region*: dsRNA trigger sequences should be derived from exons and not introns or promoter regions. Since many *C. elegans* genes are interspersed with small introns, it may be necessary to obtain a cDNA clone or to generate one using reverse transcriptase followed by DNA amplification (RT-PCR) in order to obtain a contiguous stretch of exonic sequence sufficient to trigger robust RNAi. cDNAs are available from several sources, most notably from the expressed sequence tag database of Yuji Kohara: <http://nematode.lab.njg.ac.jp/dbest/keysrch.html>.
2. *Length*: Longer dsRNAs may be more effective in eliciting RNAi since they are fragmented into a greater number of effector siRNA molecules than shorter dsRNAs. However, when longer sense and antisense strands are synthesized in separate tubes, efficient annealing of the two strands may prove difficult. Two hundred to 300 bp of sequence is often sufficient for RNAi, and in most cases, strands of this length anneal fairly readily.
3. *Specificity*: It is possible to target multiple RNAs for destruction using only one dsRNA trigger. If this is not the intent, BLAST or more sophisticated algorithms should be used under low-stringency search conditions to compare the chosen sequence to both *C. elegans* genomic DNA and expressed sequences. It may prove advisable to avoid any region of the trigger sequence that has more than 15–20 bp of perfect homology to a nontarget sequence.

1.3. Considerations for Choosing Delivery Method

It can be difficult to predict the best method for silencing a given gene target. Each of the delivery methods has particular advantages and limitations, and when the null phenotype is not known, all methods should be tried. The relative dosage of dsRNA may account for some limitations. For injection and soaking, a wide range of dsRNA concentrations can be utilized; however, the dosage of dsRNA delivered by soaking, as well as feeding, relies on an adequate ingestion or absorption by the animal. Feeding also relies on bacterial accumulation of dsRNA. The feeding protocol can be less effective for silencing in older embryos and L1 larvae because eggshell deposition prevents uptake of maternally deposited dsRNA, and animals are thus shielded from exposure to dsRNA until they feed again upon hatching. Conversely, the feeding protocol can elicit phenotypes that are not generally observed in injected animals, possibly owing to long-term or continuous exposure of worms to dsRNA. For in vivo delivery of dsRNAs from transgenes, the timing and location of silenced cells is determined by the transcriptional properties of the transgene as well as the ability of RNA-silencing signals to spread from the tissue of origin.

1.4. Considerations for Experimental Reproducibility

The penetrance and expressivity of RNAi phenotypes can vary between experiments. Growth temperature, developmental stage at the time of dsRNA delivery, or the presence of contaminating microorganisms can contribute to efficacy, and the rate of protein clearance may be affected by outside variables that may be manipulable. To fully assess an RNAi phenotype, it is advisable to administer dsRNA to animals of the same developmental stage, clone treated animals on individual culture plates, group plates into sets, and place sets under different growth conditions. Each animal and its progeny should be monitored for phenotype(s).

The methods we have provided include detailed descriptions of techniques that can be considered worm-specific or method-of-delivery-specific, or that have demonstrated advantages to the task at hand. Since space is limited, we have not provided protocols for many basic cloning and molecular techniques—especially those that might be gene specific (such as PCR conditions)—because these are available elsewhere (5). RNAi results can vary not only among laboratories but also among experiments performed by the same investigator; thus, it is important to repeat experiments and use different delivery methods for the same gene target. False negative results abound, especially from the large genomewide screens, and phenotypes that require more detailed analysis to decipher are often missed. Finally, remember that although RNAi can reliably produce a null phenocopy for some genes, it is by no means a substitute for genetic mutants.

2. Materials

2.1. *C. elegans* Husbandry

Wild-type *C. elegans* (N2 is a commonly used wild-type strain) and mutant worm stocks, as well as *Escherichia coli* OP50 bacteria, are available from the Caenorhabditis Genetics Center (CGC), a central repository for strains under contract from the NIH National Center for Research Resources (<http://biosci.umn.edu/CGC/CGChomepage.htm>). (A streptomycin-resistant OP50 strain is also available and can be useful for minimizing unwanted bacterial contamination when streptomycin is included in the culture plates.) A common means of rearing worms is to culture them on nematode growth medium (NGM) plates seeded with OP50 bacteria. OP50 is a uracil auxotroph with a slower growth rate than wild-type *E. coli* (6) and is not resistant to antibiotics. More complete guides for worm husbandry that include freezing and thawing protocols (for long-term storage) and decontamination protocols are available elsewhere (7). Protocols for bacterial maintenance and so on are also available (5).

2.1.1. Bacterial Culture

1. Erlenmeyer flask (2 L), Bacto-tryptone, Bacto-yeast extract, NaCl, agar, Teflon-coated stir bar, aluminum foil, stir plate, appropriate antibiotics.
2. *E. coli* OP50 strain.

2.1.2. Preparation of NGM Plates

1. Erlenmeyer flask (2 L), Bacto-tryptone, agar, NaCl, 10 mg/mL of cholesterol in ethanol, 2 M Tris base (sterilize by autoclaving), 3.5 M Tris-HCl (sterilize by autoclaving).
2. Sterile Petri plates (60 mm diameter).

2.1.3. Maintenance of *C. elegans* Stocks

1. Dissecting stereomicroscope with transmitted light source ($\times 1$ to $\times 65$ magnification).
2. Thin metal spatula.
3. Worm pick: This can be fashioned from a 1- to 2-in piece of 30- to 32-gage platinum wire mounted into a bacteriological loop holder or onto a glass Pasteur pipet (by melting the tip of the pipet around the base of the wire). The tip of the wire can be hammered flat and shaped into a tapered point by cutting with scissors. Alternatively, the tip can be shaped into a hook or loop using dissection forceps.
4. Refrigerated incubator(s).

2.2. Generation of DNA Templates Used in dsRNA Production

The dsRNA trigger used in soaking or injection protocols is synthesized using a DNA template that consists of a bacteriophage promoter sequence properly oriented with respect to the gene of interest. PCR products, as well as plasmids, can be used as templates for in vitro transcription reactions. In vivo delivery of dsRNA requires a DNA template with a *C. elegans* promoter to drive expression of dsRNA. dsRNA delivery by bacterial feeding requires either a plasmid with two T7 promoter sites in inverted configuration flanking the gene of interest, or a plasmid with a single T7 promoter site preceding two copies of the gene of interest, in inverted repeat configuration. The protocols in this chapter outline some required elements for transcription from plasmid templates, strategies for transcription from PCR templates, points to consider when choosing the target region for the gene of interest, and assembly of the final DNA construct to be used as a template for in vitro transcription.

2.2.1. Preparation of Plasmid DNA Templates for dsRNA Production

Plasmid vectors are available from several suppliers of molecular biology products. These should contain bacteriophage promoters flanking a restriction bank. Transcription from these promoters proceeds toward the restriction

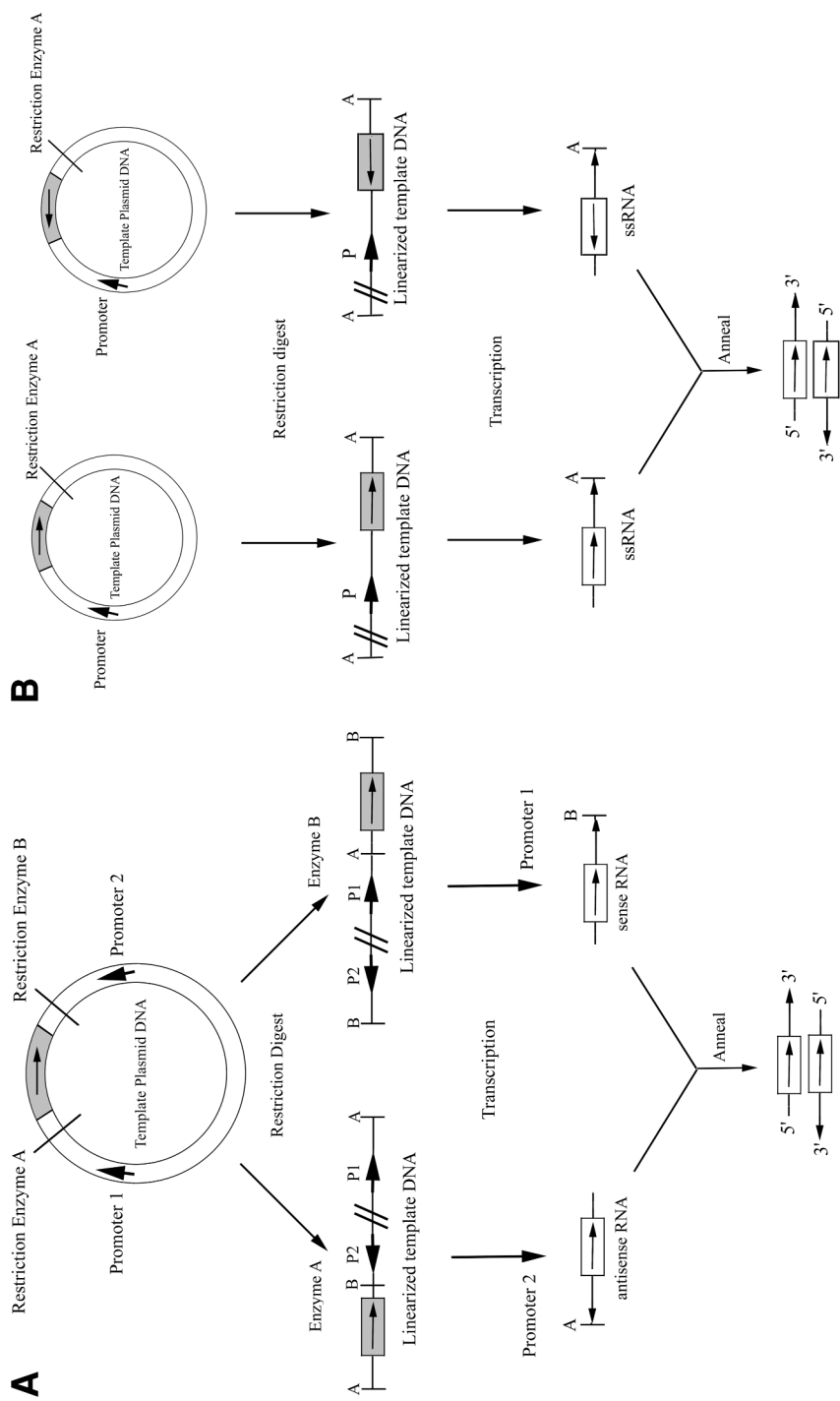
bank—where the target gene sequence (RNAi trigger) will be inserted (**Figs. 1–3**; gray boxes represent the target gene sequence). The promoter sequences generally employed in these vectors are derived from a defined promoter region from a gene in T7, T3, or SP6 bacteriophage; however, the precise promoter sequence may not be identical in all vectors. The vector may have a combination of two distinct promoters (T3 and T7, or T7 and SP6; *see Fig. 1A*), or two identical promoters in inverted repeat orientation flanking a restriction bank (double T7; *see Fig. 2*), or a single promoter (generally T7; *see Figs. 1B and 3*). A double-T7 plasmid (L4440) is available from the Caenorhabditis Genetics Center (<http://biosci.umn.edu/CGC/CGChomepage.htm>).

1. Plasmid vector, plasmid with DNA corresponding to target gene, restriction enzymes, supplies for agarose gel electrophoresis and DNA analysis.
2. DNA affinity purification kit, phenol/chloroform/isoamyl alcohol (25:24:1), 100% ethanol, 3 M sodium acetate (pH 5.2), 70% ethanol.

2.2.2. Preparation of PCR-Amplified Templates for dsRNA Production

1. N2 worms cultured on OP50-seeded plates with a thin layer of 2% agarose overlay, 15-mL conical centrifuge tubes, sterile dH₂O; Pasteur pipets, clinical centrifuge, benchtop microfuge, vortexer.

Fig. 1. (*see facing page*) Plasmid configurations for in vitro dsRNA synthesis. **(A)** Dual-promoter plasmids. The represented plasmid contains two oppositely positioned bacteriophage promoters (bold arrows) and a section of the gene of interest (gray box) pasted between restriction sites A and B. Linearized plasmid can be used to transcribe sense and antisense RNA strands (white boxes)—two separate RNA transcription reactions are required for this. An antisense strand is generated by linearizing the plasmid DNA at the position of enzyme A and using the RNA polymerase corresponding to promoter 2. A sense strand can be generated in a separate tube by linearizing the plasmid with enzyme B and using the RNA polymerase corresponding to promoter 1. Annealing of sense and antisense strands produces a dsRNA trigger for injection and soaking. **(B)** Single-promoter plasmids. A typical single-promoter plasmid with one copy of the *C. elegans* gene of interest (gray boxes) pasted behind a bacteriophage promoter (bold arrow is shown). This configuration yields both sense and antisense strands (white boxes) provided that two versions of the plasmid—with the target gene in both orientations—are available. The two plasmids can be linearized with the same restriction enzyme (A), but in separate tubes so that each reaction can be monitored for completeness. The in vitro transcription reactions can be performed in separate tubes that are then mixed for annealing into dsRNA, or by combining both linearized plasmids (if both plasmids have the same promoter). Efficient annealing of strands is generally best when the strands are cotranscribed; however, it is sometimes desirable to have ssRNA preps as controls.



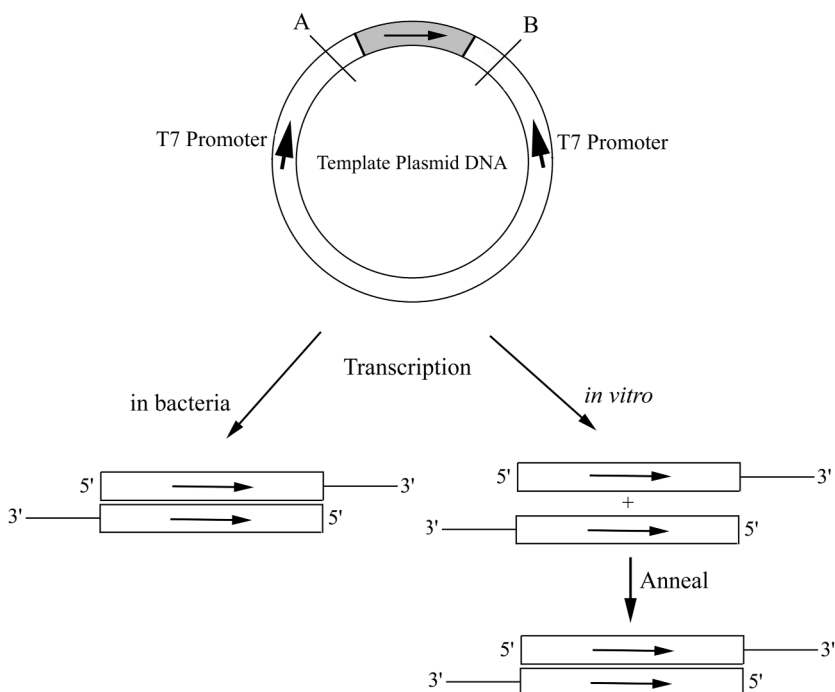


Fig. 2. Double-promoter configurations for *in vitro* dsRNA synthesis or for dsRNA feeding experiments. Plasmids contain two identical, inverted promoters (e.g., two T7 promoters, bold arrows) flanking a DNA insert (gray box). When utilized in the bacterial feeding method (left), T7 RNA polymerase is manufactured by the bacteria and utilizes the T7 promoter to synthesize dsRNA by cotranscription. This plasmid may also be used to generate dsRNA *in vitro* (right) (see **Subheadings 2.3.** and **3.3.**; also see **Fig. 1A**). Two versions of linearized plasmid should be generated using restriction enzymes A and B in separate tubes. Since the same promoter is present at each end of the template, the linearized plasmids can be combined and one *in vitro* transcription reaction can be performed. Efficient annealing of strands is generally best when strands are cotranscribed.

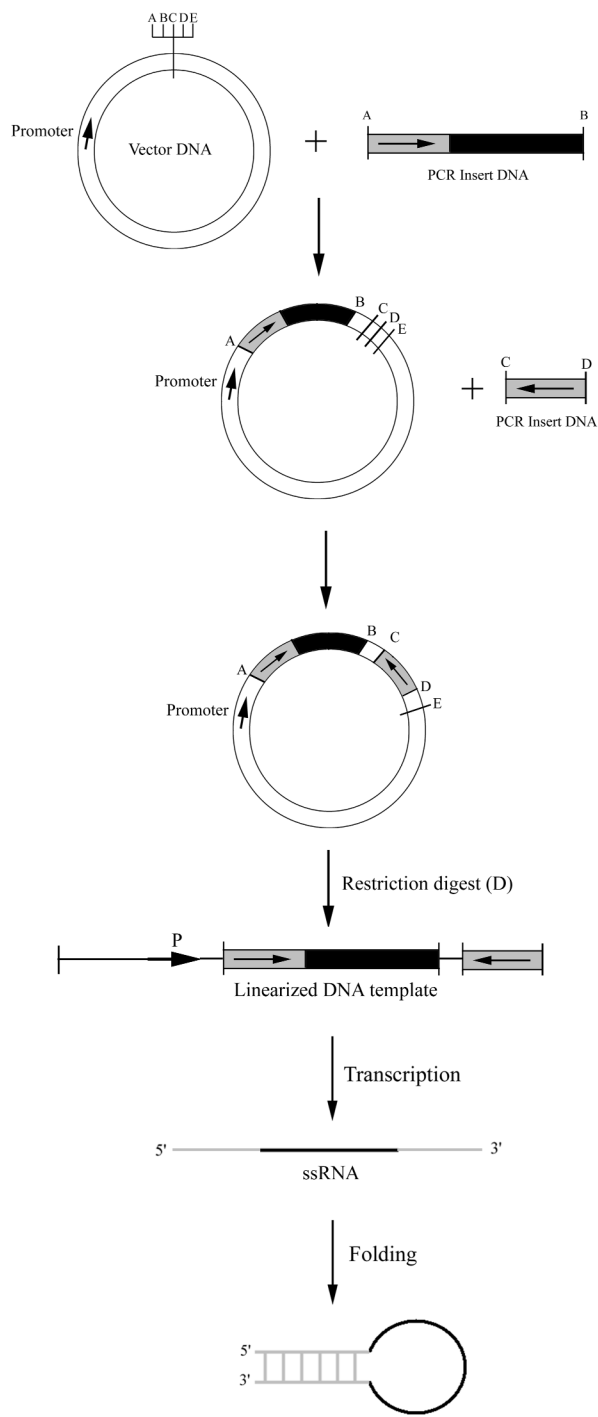
2. NTE: 100 mM NaCl; 50 mM Tris; 20 mM EDTA, proteinase K, 10% sodium dodecyl sulfate (SDS).
3. Water baths, incubators, or PCR machine for various incubations.
4. Phenol/chloroform (1:1), 100% ethanol, 3 M sodium acetate (pH 5.2), 70% ethanol, DNase-free RNase A.
5. Trizol reagent (cat. no. 15596-026; Life Technologies, Gaithersburg, MD), chloroform, RNA affinity purification kit, RNase-free DNase I.
6. Ultraviolet (UV) spectrophotometer and supplies.
7. Materials for reverse transcription reactions (commercial kits are available).

8. PCR primers with promoter sites at 5' ends, thermostable DNA polymerase and reagents for PCR amplification, PCR machine and supplies.
9. Agarose gel electrophoresis supplies for DNA analysis, DNA affinity purification kit.
10. Gloves.
11. PCR primers: Each primer should have a bacteriophage promoter sequence at its 5' end followed by sequences corresponding to the targeted gene. The melting temperature of the primer should be calculated based on the *C. elegans*-derived sequences alone because the promoter sequences will not hybridize to *C. elegans* DNA. This melting temperature should be higher than standard annealing conditions in PCR reactions. (Web-based sources such as <http://alces.med.umn.edu/rawtm.html> can aid in calculating the T_m for each primer.) The same promoter site can be included in both primers to generate a double-promoter construct. This strategy has the advantage that dsRNA can be synthesized in one tube. Two separate in vitro transcription reactions may be required if two different promoter sites are incorporated into the PCR primers.

2.3. Production of dsRNA by In Vitro Transcription

dsRNA of sufficient quantities (several micrograms or more) for most soaking and injection experiments can be obtained relatively easily using in vitro transcription protocols. These reactions employ a simple bacteriophage RNA polymerase and a DNA template with promoter sequences corresponding to the RNA polymerase. The RNA polymerase binds to the promoter and synthesizes a copy of single-stranded RNA (ssRNA) in a directional and strand-specific manner (relative to the DNA template) that is specified by the orientation of the promoter sequence. PCR products as well as plasmids can be used as templates. In vitro transcription kits are available from several commercial sources. These kits are supplied with detailed instructions, and since other protocols are readily available (5), we provide here only a general outline for performing these reactions. In vitro transcription involves preparation of the template DNA (purification and restriction digestion); synthesis of RNA; annealing of sense and antisense strands; and, finally, analysis of the ssRNA and dsRNA products.

1. Purified template DNA (plasmid or PCR product; see **Figs. 1, 3, and 4**), restriction enzymes, agarose gel electrophoresis apparatus, UV transilluminator.
2. 0.5X TAE: 20 mM Tris-acetate, 0.5 mM EDTA, 0.3 µg/mL of ethidium bromide [EtBr]; filtered; 1% agarose gel for RNA analysis (gel should be made and run in 0.5X TAE).
3. DNA affinity purification kit, phenol/chloroform/isoamyl alcohol (25:24:1).
4. 100% ethanol.
5. 3 M sodium acetate (pH 5.2).
6. 70% ethanol.



7. Reagents for transcription reactions: 200 mM dithiothreitol (DTT), ribonucleotide solution (GTP, ATP, UTP, and CTP, each at 5 mM), nuclease-free H₂O, 10X transcription buffer (standard buffer: 400 mM Tris, pH 7.5; 100 mM NaCl; 60 mM MgCl₂; 20 mM spermidine [5]), RNase inhibitor, bacteriophage RNA polymerases (e.g., T7, T3, and SP6). Alternatively, a commercially available in vitro transcription kit can be used.
8. TE: 25 mM Tris-HCl; 10 mM EDTA, pH 8.0; 3X injection buffer (20 mM phosphate buffer, pH 7.5; 3 mM potassium citrate, pH 7.5; 2% PEG 6000).
9. Incubators, water baths, or PCR machine for various incubation temperatures.
10. Gloves.

2.4. Delivery of dsRNA into *C. elegans* by Injection

RNAi by injection is the “classic” method for dsRNA delivery (8), and it is effective (dilute solutions of dsRNA can induce RNAi) and multipurpose (several distinct mRNAs can be targeted simultaneously using an injection mix comprising multiple dsRNAs) (9,10). (However, the RNAi machinery can be saturated—several groups have had difficulty targeting more than three separate genes at once.) Injection can also result in transmission of RNA-silencing signals to progeny, resulting in a larger subject population (8,11). With a workable injection system, even novice injectors can achieve RNAi successfully, because targeting the delivery needle to a specific tissue is not required for phenocopy production. Injected dsRNA can elicit systemic RNA silencing in the injected animal and its progeny even when the dsRNA is delivered to a body cavity (8). Injection allows some flexibility regarding the study of gene function at particular stages. For example, it is possible to analyze gene function in later-developing tissue for genes that are essential earlier in development. In these instances, dsRNA may be injected into young larvae (L1/L2 larvae), allowing the requirements for gene function to be examined at a later stage (e.g., L4 larvae or adults).

Fig. 3. (*see opposite page*) Plasmid used as template for transcribing hairpin dsRNAs in vitro. A plasmid containing the gene of interest configured as an inverted repeat (gray boxes) with a stuffer fragment (black boxes) flanked by a single promoter (bold arrows) can be generated in two steps. First, a fragment of the coding region corresponding to the gene of interest is inserted behind the promoter using restriction sites A and B. A second DNA fragment is then inserted behind the first using sites C and D. (Sites C and D can be created by appropriately designed PCR primers.) This suggested design for an inverted repeat makes use of sequences from the gene of interest as the stuffer region (black boxes). The purified plasmid is linearized at site D or E, and runoff transcription is performed using RNA polymerase. This plasmid configuration can be used in the feeding protocol if a bacteriophage T7 promoter is present.

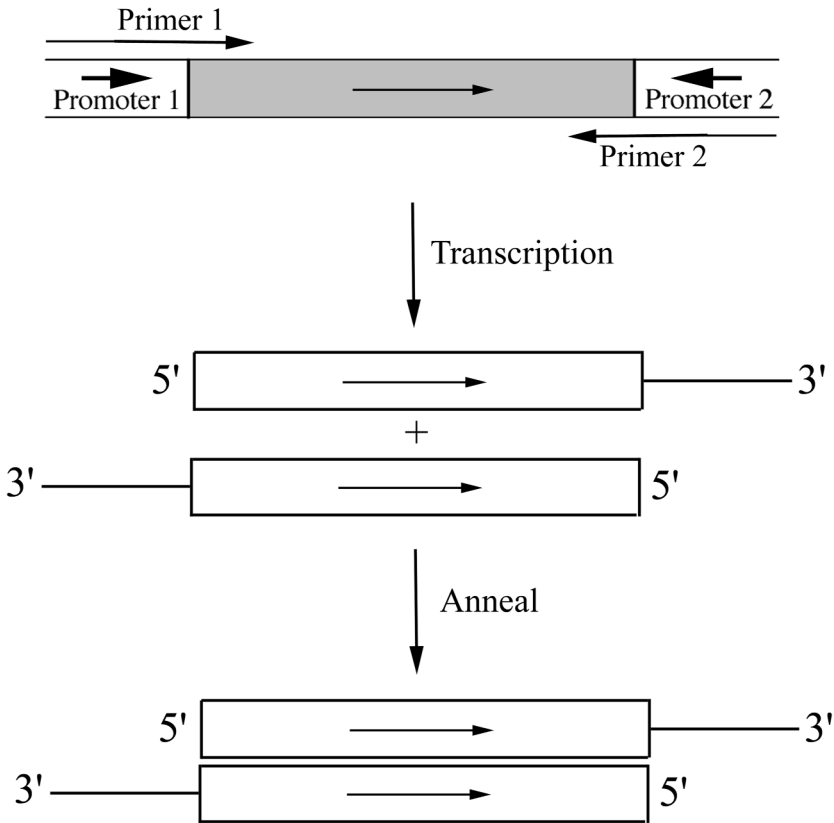


Fig. 4. PCR-amplified template DNA for RNA soaking and injection experiments. PCR product contains a DNA insert (gray box) flanked by two promoters. The promoter sites can be incorporated into the primer sequence, as indicated. The PCR fragment can be used directly (after cleanup) in an in vitro transcription reaction. A runoff transcript will be produced with either RNA polymerase. If the two promoters are identical, only one in vitro transcription reaction is required to generate dsRNA.

Delivery of dsRNA to *C. elegans* involves preparing a dsRNA injection mix, injection needles, and an “injection pad” of agarose; loading the mix into an injection needle; injecting the dsRNA into an animal; recovering the animal; and assessing any phenotype(s). More complete descriptions of microinjection are available (12,13). References for DNA injections (14,15) may provide additional information.

1. dsRNA (0.1–3 $\mu\text{g}/\mu\text{L}$) prepared by in vitro transcription (see **Subheadings 2.3.** and **3.3.**) or other means.

2. Microinjection equipment: inverted microscope with attached micromanipulator, needle puller, Pasteur pipets, forceps, glass slides, standard borosilicate thin-wall filamented capillary tubes with outer diameter/inner diameter within the range of 1.0/0.58–1.5/0.84 mm. The microinjection needles will be made from the capillary tubes and thus the choice in size will depend on the design of the needle holder.
3. Freshly prepared 2% agarose solution in water, 60°C water bath or incubator, 37°C incubator, microfuge.
4. Large Petri dishes or other covered containers for holding needles.
5. Clay fashioned into a pencil shape and inserted into the needle container. Pulled needles can be mounted horizontally into the clay, preventing accidental breakage of tips. A similar setup can be used to hold needles loaded with dsRNA solution; a small paper towel moistened with water should be included to prevent dehydration of the injection solution.
6. Binocular dissection microscope with transmitted light source, mineral oil (heavy white oil; viscosity at 100°C: 340–360), M9 medium (for 1 L, 3 g of KH_2PO_4 , 6 g of Na_2HPO_4 , 5 g of NaCl, 1 mL of 1 M MgSO_4), recovery buffer (M9 media + 4% glucose).
7. OP50-seeded NGM plates (see **Subheadings 2.1.2.** and **3.1.2.**).

2.5. Delivery of dsRNA by Soaking

An RNAi phenotype can be induced in *C. elegans* by soaking the worms in a concentrated solution of dsRNA made by in vitro transcription (**16,17**).

1. dsRNA (0.2–5 $\mu\text{g}/\mu\text{L}$), 1.5-mL microfuge tubes, sterile dH_2O and M9 medium (see **Subheading 2.4.**), appropriate strain of *C. elegans*.
2. 15°C Incubator, additional incubators at appropriate temperatures.
3. OP50-seeded NGM plates (see **Subheadings 2.1.2.** and **3.1.2.**), mineral oil.
4. Appropriate microscope for phenotypic analysis.

2.6. Delivery of dsRNA by Feeding Worms dsRNA-Expressing Bacteria

The laboratory food source for *C. elegans* is bacteria, and bacterial strains have been established that can transcribe specific RNAs from engineered plasmids. Sufficient quantities of dsRNA accumulate within the bacterial cell such that these strains can be used as a food source for *C. elegans* and can also induce RNAi (**2**). The feeding protocol involves generating a plasmid with the gene of interest following a T7 bacteriophage promoter (production of these plasmids is described in **Subheadings 2.2.1.** and **3.2.1.**; see **Figs. 2** and **3**), transforming an HT115(DE3) bacterial strain with such a plasmid, plating transformed cells on NGM plates under induction conditions, applying worms to plates, and monitoring phenotype(s).

1. DNA plasmid containing the gene of interest inserted between two T7 promoter sites (**Fig. 2**). A common double-T7 plasmid (Amp^r) is L4440, available from the CGC (<http://biosci.umn.edu/CGC/CGChomepage.htm>). Alternatively, DNA plas-

mid containing a single T7 promoter site followed by the gene of interest in inverted repeat orientation can be used (**Fig. 3**).

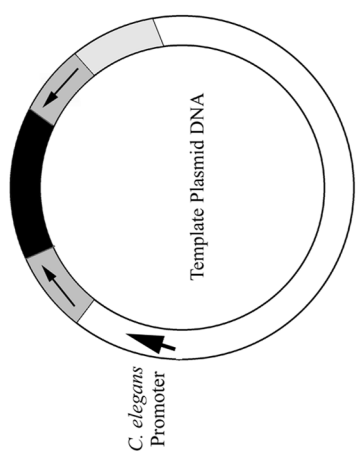
2. HT115(DE3) bacterial strain (**2**)—a tetracycline (Tet)-resistant, RNaseIII (–) strain available from the CGC; LB broth and agar plates; 12.5 mg/mL of Tet; 50–100 mg/mL of ampicillin (Amp); 37°C shaking incubator.
3. Cold 50 mM CaCl₂, filter sterilized; clinical centrifuge housed in a cold room or a refrigerated centrifuge; 15- and 50-mL sterile polypropylene centrifuge tubes.
4. 80% Glycerol, autoclaved; isopropyl-β-D-thiogalactopyranoside (IPTG): stock solution = 4 mM (1000X).
5. Plates (60 × 15 mm) containing NGM agar supplemented with 12.5 μg/mL of Tet, the antibiotic appropriate for plasmid selection (e.g., 50–100 μg/mL of Amp), and 0.4 mM IPTG; appropriate strain of *C. elegans*.

2.7. Production of dsRNA by In Vivo Transcription

Transcription of specific dsRNA within worm cells can be achieved by generating worm strains transformed with DNA constructs designed to express dsRNA. First, a plasmid is configured with a worm promoter and the gene of interest. The plasmid is then injected into the germline of a suitable worm strain. The DNA enters the nuclei of the syncytial germline, where the plasmids recombine/ligate into a “minichromosomelike” structure that is repetitive in nature and is maintained in cells as an extrachromosomal array (**15**). Multiple plasmids from the same injection mix are often incorporated into the array, and most arrays are marked with a dominant selectable marker that is derived from a plasmid in the injection mix. It is possible to observe RNAi when a transgene array is formed from a mix of two different plasmids with the same promoter: one plasmid capable of expressing the sense strand of an RNAi trigger and a second plasmid expressing the antisense strand (**18**). Alternatively, a single worm promoter can be placed in front of two copies of the gene of interest arranged as inverted repeats (**Fig. 5**).

1. Plasmid containing inverted DNA repeats (gray boxes in **Fig. 3**) and a stuffer fragment (black boxes in **Fig. 3**) flanked by one promoter and various restriction endonuclease sites (see **Subheadings 2.2.1.** and **3.2.1.2.**).
2. Plasmid containing a *C. elegans* promoter and 3' untranslated region (UTR).

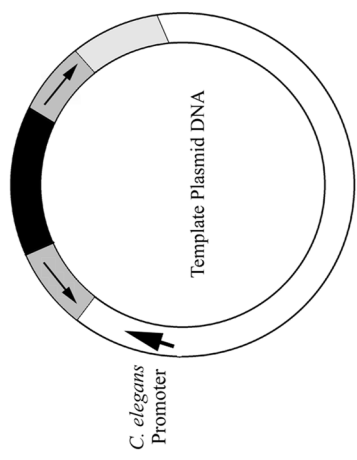
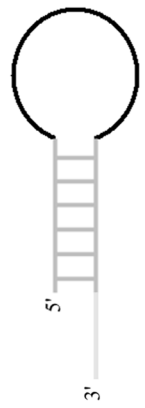
Fig. 5. (see facing page) Plasmid configuration for in vivo transcription of RNA hairpins. A hairpin-generating plasmid that contains inverted DNA repeats (gray boxes) and a stuffer fragment (black boxes) as in **Fig. 3** can be used for expression in *C. elegans* cells. A *C. elegans* promoter (bold arrows) should be inserted in front of the inverted repeat and a 3' UTR (light gray) from a stably expressing gene should be inserted at the end of the inverted repeat. No differences in effectiveness have been reported to date between the configurations of inverted repeats depicted here. Such plasmids are used to generate transgenic worms that transcribe dsRNA in vivo.



Transcription



Folding



Transcription



Folding



3. Microinjection equipment and associated supplies (*see Subheading 2.4.*).
4. Injection mix composed of transformation marker plasmid (e.g., *rol-6* [22]) and dsRNA-expressing plasmid (*see Subheading 3.2.1.2.*).

3. Methods

3.1. *C. elegans* Husbandry

3.1.1. Bacterial Culture

1. Luria-Bertani (LB) medium, pH 7.5: Dissolve in a 2-L Erlenmeyer flask: 10 g/L of Bactotryptone, 5 g/L of Bacto yeast extract, 5 g/L of NaCl. Dispense into 100-mL bottles. Sterilize by autoclaving.
2. LB plates: Follow instructions for LB medium (do not dispense) and add 15 g/L of agar before autoclaving. Carefully drop a Teflon-coated stir bar into the flask, cover the flask with aluminum foil, and autoclave. (Agar will not dissolve unless heated. Use of the stir bar will speed cooling of the solution when removed from the autoclave and will prevent solidification at the bottom of the flask.) Stir the autoclaved solution until the temperature lowers to ~60°C. Add antibiotics as necessary. Pour into sterile 100-mm Petri dishes and allow to solidify.
3. Using sterile technique, streak the starter OP50 bacterial culture onto sterile LB agar plates and incubate, inverted, overnight at 37°C. The plate can be stored for routine use for several months at 4°C when sealed with Parafilm to prevent desiccation.
4. Inoculate a bottle of LB with a single colony from the OP50 culture plate. Incubate overnight at 37°C.
5. Store the OP50 liquid stock at 4°C. The stock can be used for several months to seed NGM plates, barring contamination (*see Subheadings 2.1.2. and 3.1.2.*).

3.1.2. Preparation of NGM Plates

1. Add the following to a 2-L Erlenmeyer flask: 1 mL of 2 M Tris base, 1 mL of 3.5 M Tris-Cl, 0.5 mL of 10 mg/mL cholesterol, 3 g of tryptone, 2 g of NaCl, 17 g of agar. Bring the volume to 1 L with dH₂O and carefully drop a Teflon-coated stir bar into the flask. Cover the flask with aluminum foil and autoclave.
2. Stir the autoclaved solution on a stir plate until the temperature lowers to ~60°C. Dispense into 60 × 15 mm Petri dishes under sterile conditions (*see Note 1*).
3. Allow time (overnight) for the plates to evaporate excess moisture. The plates can then be stored at 4°C or at room temperature in airtight containers.
4. Using a sterile technique, apply the OP50 bacterial culture dropwise onto the surface of the plates (~100 µL). Tilt the plates to spread out the lawn, but do not seed with so much bacteria that the lawn reaches the edge of the plate because worms may crawl up the plastic sides and dehydrate.
5. Allow the bacterial lawn to grow overnight at room temperature. Seeded plates can be stored in an airtight container for 2 to 3 wk at room temperature or at 15°C.

3.1.3. Maintenance of *C. elegans* Stocks

Worms can be transferred from plates lacking food (starved plates) to freshly seeded plates using a worm pick. The platinum pick is first flamed for sterility

before each attempt at transfer—this also prevents cross-contamination of stocks. Mounting the worms onto the pick is achieved using a gentle swiping motion to lift the worm off the plate. The animals are removed from the pick using a reverse motion into the bacterial lawn of the fresh plate. Bacteria can facilitate adherence of the worm onto the pick, acting as a sticky surface bridge between the pick and the worm. A successful transfer will gouge neither the worm nor the surface of the agar plate. Worms tend to burrow into the agar using these imperfections, and monitoring and collection of burrowed worms can prove difficult. Novice worm pickers may require a few days of practice to master the art of transfer.

Worms can also be transferred to a fresh plate by transferring a “chunk” of agar. This method quickly moves many worms to a fresh plate and is particularly useful when plates are starved, when the genotype of each worm is identical, or when a mating is not required to maintain the stock. To chunk worms, a metal spatula is flame-sterilized, then used to cut a cube of agar from the old plate. The spatula is then used as a spoon or shovel to scoop the worm-laden cube onto a new plate, preferably just adjacent to, but not directly on, the bacterial lawn.

C. elegans is generally reared at temperatures between 15 and 25°C (typically 20°C). Higher temperatures produce a faster growth rate: worms grow twice as fast at 25°C than at 16°C. Transferring worms every 1 to 2 d ensures a good supply of worms at every developmental stage.

3.2. Generation of DNA Templates Used in dsRNA Production

Subheading 3.2.1. describes methods that can be utilized when the DNA sequence corresponding to the dsRNA trigger is in hand (e.g., a cDNA). **Subheading 3.2.2.** describes methods to generate a trigger DNA by PCR or RT-PCR if the appropriate sequence is not readily available.

3.2.1. Assembly of Plasmid DNA Templates for dsRNA Production

3.2.1.1. ASSEMBLY OF DOUBLE- AND SINGLE-PROMOTER PLASMIDS HARBORING TRIGGER DNA

The template sequence (the *C. elegans* gene of interest) is inserted using restriction enzymes corresponding to sites in the restriction bank and standard cloning techniques (see **Figs. 1** and **2**; also see **Note 2**). For double-promoter templates, the final construct will contain a DNA insert (gray boxes in **Figs. 1A** and **2**) flanked by two promoters with some restriction endonuclease sites remaining. For single-promoter templates, the final construct will contain a DNA insert (gray boxes in **Fig. 1B**) flanked at one end by a promoter. For dsRNA production from single-promoter plasmids, it will be necessary to con-

struct two plasmids that differ with respect to gene orientation (**Fig. 1B**). dsRNA transcribed in vitro from these templates can be used for injection (*see Subheadings 2.4. and 3.4.*) and soaking (*see Subheadings 2.5. and 3.5.*) experiments; double-T7 plasmids can be utilized in the bacterial feeding protocol (*see Subheadings 2.6. and 3.6.*).

3.2.1.2. ASSEMBLY OF SINGLE-PROMOTER PLASMIDS WITH INVERTED-REPEAT CONFIGURATION OF TRIGGER DNA

The final construct (**Fig. 3**) will contain two oppositely oriented copies of the same DNA segment (gray boxes in **Fig. 3**) flanking a stuffer fragment (black box in **Fig. 3**). The promoter will be located at one end of the inverted repeat sequence, while the other end should contain at least one restriction endonuclease site (**Fig. 3**). A promoter sequence for RNA polymerases T7, T3, or SP6 can be utilized.

Use standard cloning techniques to insert a *C. elegans* coding region into the restriction bank of the plasmid. Choose a set of enzymes that leaves at least two restriction sites at one end of the restriction bank. If the DNA segment is cloned into a singly cut vector, it will be necessary to determine the orientation of the insert before proceeding.

Figure 3 depicts a cloning strategy in which the left-hand end of the inverted repeat is the first to be cloned into the vector. (A mirror image cloning strategy is also possible where the right portion of the inverted repeat is cloned into the vector first.) For the second fragment to be oriented properly, it may be necessary to synthesize PCR primers with restriction sites on the 5' ends that correspond to insertion sites in the vector (**Fig. 3**, sites C and D). It is often convenient and advantageous to generate the stuffer fragment from sequences derived from the target gene itself; this eliminates a cloning step and alleviates concern that a nonrelated stuffer fragment might elicit RNAi for another gene. This is accomplished when one of the sections is longer than the other (black boxes in **Fig. 3**). For the repeat to be properly maintained by bacterial cells, the stuffer should consist of at least 100 bp. Cloning of DNA with inverted repeats can be challenging, as they are often not faithfully maintained in bacteria. Transformation of ligations into a bacterial strain that is defective in multiple recombination mechanisms (such as SURE cells from Stratagene) can improve the cloning success rate.

Once the construct has been generated, a large, clean batch of DNA should be prepared. A variety of DNA purification kits that utilize affinity resins are available for this purpose. The final purified construct can be digested with a restriction enzyme at the end of the inverted repeat (not the promoter end; **Fig. 3**, site E) so that a runoff transcription product can be generated. dsRNA generated from such templates is suitable for soaking (*see Subheadings 2.5.*

and 3.5.) and injection (*see Subheadings 2.4. and 3.4.*) experiments and can be in bacterial feeding experiments if a T7 promoter site is present (*see Subheadings 2.6. and 3.6.*).

3.2.2. Assembly of PCR Templates for dsRNA Production

A PCR fragment can be generated (**Fig. 4**) that is composed of the coding region from the target gene of interest (gray box in **Fig. 4**) flanked by two promoters. The PCR reaction can be performed on purified genomic DNA (*see Subheading 3.2.2.1.*), directly on DNA released from worms (*see Subheading 3.2.2.2.*), or on cDNA synthesized from purified worm RNA (*see Subheading 3.2.2.3.*) The latter protocol is preferred when the target sequence is broken up into small exons; an RT-PCR fragment allows a more contiguous stretch of homology between trigger dsRNA and target mRNA. Sequences corresponding to T7, T3, and SP6 bacteriophage promoters are small enough that they can be incorporated into a PCR primer (**5,19**). However, SP6 RNA polymerase does not efficiently transcribe RNA from a PCR-amplified DNA template (**20**); therefore, primers with T7 or T3 promoter sequences are preferred.

3.2.2.1. PREPARATION OF N2 WORM GENOMIC DNA

1. Pick (not chunk) N2 worms onto 10 NGM/OP50 plates (100 × 15 mm) with a thin layer of 2% agarose overlayed and grow until worms are gravid. (The agarose prevents worms from digging into the plates and provides a barrier to the agar, which might contain impurities that could prevent subsequent molecular procedures.)
2. Collect worms from all stock plates into a 15-mL conical centrifuge tube by dislodging the worms from the plates with sterile-filtered dH₂O and pooling washes into the 15-mL tube using a Pasteur pipet.
3. Centrifuge the worms at low speed in a clinical centrifuge for 10–20 s to pellet the worms.
4. Remove the dH₂O by aspiration, and gently wash the worms with an additional 2 mL of dH₂O.
5. Repellet the worms at low speed in a clinical centrifuge for 10–20 s and aspirate the dH₂O.
6. Freeze the worm pellet at –80°C for at least 1 h (this helps crack open the worms).
7. Thaw the pellet on ice and then add 2 mL of NTE, 20 µg of proteinase K, and 100 µL of 10% SDS. Mix.
8. Incubate at 65°C for 2 h.
9. Transfer the worm mixture to microfuge tubes (500 µL/tube) and add an equal volume of phenol/chloroform, preequilibrated to room temperature.
10. Vortex the mixture well, and then centrifuge for 5 min in a room temperature microfuge at maximum speed.
11. Transfer the top aqueous phase to a new microfuge tube and repeat the phenol/chloroform extraction an additional two times (do not transfer more than 400 µL of aqueous solution to each new tube after the final extraction).

12. Add 1 mL of ice-cold 100% ethanol to each tube of 400 μ L and mix gently by inverting.
13. Centrifuge for 30 min at 4°C in a microfuge at maximum speed to pellet the DNA.
14. Carefully remove the ethanol and gently add 500 μ L of 70% ethanol to the pellet.
15. Centrifuge for 5 min at 4°C in a microfuge at maximum speed.
16. Carefully remove the ethanol and dry the pellet at room temperature (approx 1 h).
17. Dissolve the DNA in 400 μ L of sterile dH₂O.
18. Add 8 μ g of RNase A and incubate for 30 min at 37°C.
19. Phenol/chloroform extract the DNA three times (transfer 400 μ L of the aqueous DNA solution to each fresh tube after the final extraction).
20. Add 1/10 vol of 3 M sodium acetate, pH 5.2, and 1 mL of ice-cold 100% ethanol to each tube. Mix gently by inverting the tubes.
21. Centrifuge for 30 min at 4°C in a microfuge at maximum speed to pellet the DNA.
22. Carefully remove the ethanol and add 1 mL of ice-cold 70% ethanol, being careful not to dislodge the pellet.
23. Centrifuge for 5 min at 4°C in a microfuge at maximum speed and carefully remove the ethanol.
24. Air-dry the DNA pellet at room temperature (do not completely dry the pellet because this can cause the DNA to fragment).
25. Resuspend the DNA in 200 μ L of sterile dH₂O. Estimate the yield on a 0.7% agarose gel by comparing the EtBr staining intensity with that of a known concentration of marker DNA.
26. Use 0.1–1 μ g of this genomic DNA in a 50- μ L PCR reaction. To generate a PCR fragment for use in an in vitro transcription reaction (**Fig. 4**), use hybrid primers with bacteriophage promoter sequence at the 5' end (T7, T3, or Sp6) and target gene sequence at the 3' end (*see Note 3*) (23).

3.2.2.2. SINGLE WORM PCR OF TARGET SEQUENCE

1. In a PCR tube, add 9 μ L of sterile dH₂O, 1 μ L of 10X PCR polymerase buffer (with 1.0–2.5 mM Mg⁺²; 1.5 mM Mg⁺² is standard), and one to three gravid N2 worms (10- μ L total volume per tube).
2. Place at –80°C for at least 20 min (this helps crack open the worms).
3. Remove the tubes from the freezer and thaw on ice.
4. Add 0.5 μ L (~5–10 μ g) of proteinase K.
5. Incubate at 65°C for 1 h, then 95°C for 15 min. Proteinase K will help lyse the worm cuticle and also degrade proteins, especially DNases. A 95°C inactivation step is required so that the DNA polymerase will not be degraded when added later during the amplification step.
6. Add the remaining PCR reaction components (more buffer [with Mg⁺²], dH₂O, dNTPs, primers, and thermostable polymerase) to generate a PCR fragment for use in an in vitro transcription reaction (**Fig. 4**). Some parameters, such as Mg⁺² concentration and cycle conditions, will be sequence- or primer-specific. A number of sources are available for advice on these matters (5).

3.2.2.3. TOTAL RNA PREPARATION FROM N2 WORMS AND RT-PCR AMPLIFICATION OF TRIGGER DNA

1. Grow N2 worms on a minimum of 20 NGM/OP50 plates (60 × 15 mm) until gravid.
2. Collect the worms from all stock plates into one 15-mL conical tube using sterile-filtered dH₂O and a Pasteur pipet.
3. Centrifuge the worms at low speed in a clinical centrifuge for 10–20 s to pellet the worms.
4. Remove the dH₂O by aspiration and wash the worms with an additional 2 mL of dH₂O.
5. Repellet the worms at low speed in a clinical centrifuge for 10–20 s and aspirate the dH₂O.
6. Resuspend the worm pellet in the residual dH₂O and transfer to a microfuge tube.
7. Pellet the worms one final time by centrifuging for 10–20 s in a microfuge at low speed. Remove the remaining dH₂O.
8. For every 50 µL of worms, add 400 µL of Trizol Reagent (Life Technologies) (*see Notes 4 and 5*).
9. Shake the mixture by hand frequently over a 10-min incubation period at room temperature.
10. Add 80 µL of chloroform, mix by inverting for 15 s, and incubate for 2 to 3 min at room temperature.
11. Centrifuge the sample at 8000 rpm for 15 min at 4°C in a microfuge.
12. Transfer the top aqueous layer to a fresh microfuge tube.
13. Purify the RNA by an affinity method to concentrate; commercial kits are available.
14. Add 10 U of RNase-free DNase I, 25 µL of 10X DNase buffer, and sterile dH₂O to purified RNA (final reaction volume of 250 µL) and incubate at 37°C for 20 min to remove the DNA.
15. Add an equal volume of phenol/chloroform, mix well, centrifuge at 4°C for 5 min at maximum speed in a microfuge, and transfer the top aqueous phase to a new microfuge tube.
16. Add 1/10 vol of 3 M sodium acetate, pH 5.2, and 1 mL of ice-cold 100% ethanol to the tube and mix by gently inverting.
17. Centrifuge for 30 min at 4°C in a microfuge at maximum speed to pellet the RNA.
18. Carefully remove the ethanol and add 1 mL of ice-cold 70% ethanol, being careful not to dislodge the pellet.
19. Centrifuge for 5 min at 4°C in a microfuge at maximum speed. Carefully remove the ethanol.
20. Air-dry the RNA pellet at room temperature (approx 1 h). Do not dry completely—the RNA pellet can be difficult to resuspend.
21. Resuspend the RNA in 50 µL or less of sterile dH₂O.
22. Determine the concentration of the purified RNA by spectrophotometric measurement. Generally, 5 µg of total RNA is utilized for a reverse transcription reac-

tion (see **Note 6**). A number of commercially available kits are available for performing these reactions. The reverse transcription product is used as a template for PCR (see **Note 7**). The amplified DNA can be ethanol precipitated or purified using a number of commercially available kits. The purified DNA can then be subcloned into a plasmid or used directly as a template for in vitro transcription (see **Subheadings 2.3.** and **3.3.**).

3.3. Production of dsRNA by In Vitro Transcription

The methods in **Subheading 3.2.** provide guidelines to generating plasmid DNA or PCR-amplified DNA that can be used as a template for dsRNA production. The following protocols describe how dsRNA is produced from such templates. The resulting dsRNA can be delivered to worms by injection or soaking, as described in **Subheadings 3.4.** and **3.5.** **Subheading 3.3.1.** describes the preparation necessary for the template DNA, **Subheading 3.3.2.** describes the in vitro transcription reaction, and **Subheading 3.3.3.** describes how to anneal sense and antisense strands to generate dsRNA.

3.3.1. Preparation of Template DNA

3.3.1.1. dsRNA PREPARATION FROM A PLASMID TEMPLATE

Plasmid DNA should be of high quality and can be purified by an affinity method that minimizes salt concentrations (a number of commercial kits are available).

1. Linearize the template DNA (5–10 µg) with the appropriate restriction enzymes (see **Figs. 1–3**). For dual-promoter plasmids, two digestions in separate tubes will be required: one tube will be used to generate the sense strand and the other for the antisense strand. For double promoter (e.g., double T7) plasmids, the two digested plasmids can be combined into one tube and transcribed simultaneously using one RNA polymerase. For single-promoter plasmids harboring an inverted repeat DNA sequence, only one site (at the end of the inverted repeat) should be cut. Enzymes that leave a 5' overhang or blunt end should be chosen since RNA polymerases may initiate transcription from a 3' overhang.
2. Analyze a small aliquot of the digestions by agarose gel electrophoresis to confirm that the plasmid DNA was completely linearized.
3. Clean up the digestions to remove enzymes and salts by purifying over a DNA affinity column (commercial kits are available) or by utilizing phenol/chloroform extraction and ethanol precipitation.

3.3.1.2. dsRNA PREPARATION FROM A PCR TEMPLATE

DNA generated by PCR (**Fig. 4**) can be used directly as a template in in vitro transcription reactions following a cleanup step. Purification of the DNA

from reaction components can be easily accomplished by using commercial kits or by phenol/chloroform extraction followed by ethanol precipitation.

3.3.2. *In Vitro* Transcription (see **Notes 8–10**)

1. “Homemade mixes”: Mix the following reaction components in the order listed to prevent precipitation of DNA with the spermidine in the buffer (equilibrate components to room temperature unless otherwise noted):
 - a. 0.5–1 μg of linearized template DNA or PCR fragment.
 - b. 1 μL of 200 mM DTT.
 - c. 2 μL of 5 mM NTPs.
 - d. H_2O to 16 μL .
 - e. 2 μL of 10X transcription buffer.
 - f. 24 U of RNase inhibitor (stored on ice).
 - g. 15–20 U of RNA polymerase (stored on ice).The total reaction volume is 20 μL .
2. Commercially available kits: Mix the reaction components and template DNA per the manufacturer’s instructions.
3. Incubate the reaction for 1–4 h at 37°C, or per the manufacturer’s instructions.

3.3.3. Annealing RNA Strands (see **Note 11**)

When sense and antisense strands are synthesized separately, it is necessary to perform an annealing step. An annealing step may also optimize the yield of fully double-stranded RNA when the strands are synthesized in the same tube. If loss of ssRNA is a concern or if the transcription product is a hairpin, **steps 2a–e** can be omitted.

1. Remove a 0.5- μL sample from each single-stranded reaction for analysis by agarose gel electrophoresis (**Fig. 6**; see **Notes 12** and **13**). Proceed to **step 2** if clean ssRNA preparations are required; otherwise proceed to **step 3**.
2. The following protocol, adapted from Andrew Fire, results in dsRNA, as well as clean preparations of sense and antisense RNA for use as controls:
 - a. Bring the volume of single-stranded reactions to 400 μL by adding 40 μL of 3 M NaOAc (pH 5.2) and 360 μL of H_2O .
 - b. Add 200 μL of phenol/chloroform (1 : 1), mix by inverting, and centrifuge at maximum speed for 5 min in a microfuge at 4°C.
 - c. Transfer the upper aqueous phase to a new tube, add 200 μL of chloroform, mix by inverting, and centrifuge as in **step 2b**.
 - d. Transfer the upper aqueous phase to a new tube, add 1 mL of ethanol, mix by inverting, and centrifuge for 20 min at maximum speed in a microfuge at 4°C.
 - e. Dry the ssRNA pellets at room temperature and resuspend in 10 μL of TE.
 - f. Add 2 U of RNase-free DNase I, 2 μL of 10X DNase buffer, and sterile dH_2O to each ssRNA sample (20- μL final reaction volume) and incubate at 37°C for 15 min. (This step is important when the ssRNA will be injected but is not required for soaking experiments.)

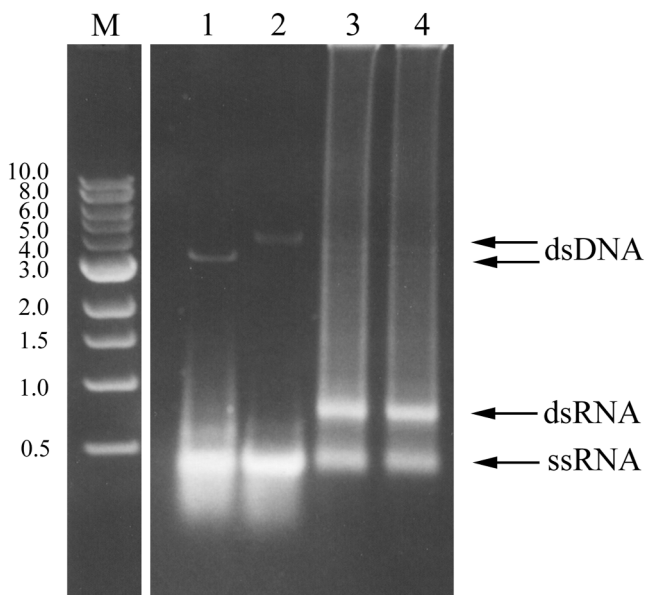


Fig. 6. Agarose gel analysis of ssRNA and dsRNA synthesized by in vitro transcription. A plasmid with a configuration similar to **Fig. 1A** was used for in vitro transcription to generate dsRNA corresponding to the *C. elegans unc-22* gene. T7 and T3 polymerases were used to generate the single strands in separate transcription reactions that were incubated for 4 h at 37°C to produce sense and antisense RNA. The reactions were then mixed and incubated an additional 2 h at 37°C. Further annealing of the strands was accomplished by incubating the RNA mixture at 75°C for 5 min, then cooling to room temperature for 30 min. M, DNA marker; lane 1, sense RNA; lane 2, antisense-RNA; lane 3, mixed ssRNA; lane 4, mixed and annealed ssRNA. Arrows indicate dsDNA template, dsRNA, and ssRNA. Note the shift in size between the ssRNA and the dsRNA and that this protocol resulted in partial annealing of strands (see **Note 14**).

- g. Phenol/chloroform extract, chloroform extract, and ethanol precipitate as described in **steps 2a–d**. Dry the ssRNA pellets at room temperature and resuspend in 10–50 μ L of TE.
- h. Reserve the necessary amount of clean ssRNA for use as controls and analyze yield by agarose gel electrophoresis (**Fig. 6**; see **Notes 12** and **13**). Combine the remaining volume of the single-stranded samples into one tube and anneal by incubating at 68°C for 10 min, then 37°C for 30 min in 1X injection buffer.
3. Alternatively, to maximize the yield of dsRNA (and when clean ssRNA is not required), combine the transcription reactions. Then try one or more of the following methods followed by phenol/chloroform extraction and ethanol precipitation to achieve complete annealing:

- a. Continue incubating the reactions at 37°C for an additional 2 h, heat to 75°C for 5 min, and slowly cool to room temperature over a 30-min period.
- b. Heat to 75°C for 5 min and slowly cool to room temperature.
- c. Heat to 65°C for 15 min, then 37°C for 30 min.
- d. Heat to 65°C for 15 min, decrease to 37°C at a rate of $-1^{\circ}\text{C}/\text{min}$, incubate at 37°C for an additional 30 min, then decrease to room temperature at $-1^{\circ}\text{C}/\text{min}$.

3.4. Delivery of dsRNA into *C. elegans* by Injection

3.4.1. Preparation of Injection Pads (see Note 15)

1. Using a Pasteur pipet (preheated in an oven or heated by repeated pipetting of water maintained at 60°C), place a drop of 1 to 2% agarose at 60°C onto a glass slide or cover slip that is suitable for your microinjection system.
2. Quickly place another glass slide on top of the agarose drop, orienting the top slide perpendicular to the bottom slide.
3. Allow a few minutes for the agarose to solidify, and then peel apart the slides. A quick sliding motion generally accomplishes this; some practice may be required. A square of agarose will be left on the surface of one of the slides. Trim the square with a razor to remove bulges at the edges that might interfere with needle movement later.
4. Allow the slides to dry overnight at room temperature or dry in a 60°C oven for 1 h.

3.4.2. Preparation of Injection Needles

Several needles may be necessary for each injection mix, because some may become plugged during injection. Most clogged needles are useless and must be disposed. Several different needle-pulling devices are available from Sutter, Narishige, and other companies that will shape a needle for injection.

3.4.3. Loading Injection Needles With dsRNA Solutions

Several methods can be used to load the needles with the solutions. With filamented needles, the bottom of the needle can be sterilized by brief passage through a flame. Then do the following:

1. Warm the needles in a 37°C incubator for 10 min.
2. First, centrifuge the dsRNA solution briefly in a microfuge to remove sediment that might clog the injection needle. Then, insert the bottom end of the prewarmed needle into dsRNA-containing solution using forceps to hold the needle. As the air inside the needle cools, the liquid will rise by capillary action. (Using your fingers to hold the needle prevents efficient cooling.)
3. When some liquid has risen into the injection needle, remove the needle from the solution and gently lodge, tip down, into the clay in the hydrated needle chamber (do not let the tip touch the bottom of the chamber). The liquid should continue to move while the needle is stored in the chamber. Once the liquid has reached the

tip, the needle is ready for injecting; liquid movement can be monitored using a dissection microscope and generally takes 5–20 min.

4. Mount the loaded needle into the needle holder of a microscope set up for micro-injection and focus on the tip of the needle under low power. Place a slide with an agarose pad topped with mineral oil onto the microscope, lower the needle into the oil, focus on the tip, and check the flow rate. It may be necessary to break the tip of the needle to get the liquid to flow out. This can be accomplished by using the tip to pick at imperfections on the pad while the needle is under pressure. Replace this slide with one mounted with worms for injections.

3.4.4. Mounting *C. elegans* onto Injection Pads and Injecting (see **Note 16**)

1. Place a drop of mineral oil onto an injection pad (see **Note 17**).
2. Transfer well-maintained worms from an OP50 bacterial lawn onto an unseeded plate (this allows the worms to shed bacteria from their cuticles).
3. Using a platinum worm pick fashioned into a point (see **Subheading 2.1.3.**), gently transfer worms from the unseeded plate onto the agarose pad. Monitor transfer under a dissection microscope. Here it is important to avoid a swiping motion of the pick. When a part of the worm has touched the agarose surface, it should immediately adhere, and any further motion of the pick will rip the worm apart. The rest of the worm is brought to the pad as the worm struggles to free itself. For novice injectors, limit one to five worms per pad.
4. Mount the slide onto the injection microscope already loaded with a working needle.
5. Inject the worms with liquid. Injection into the body cavity or gut is sufficient to elicit an RNAi response. However, if the phenotype is to be monitored in the progeny of injected animals, a greater number of affected progeny may result by targeting the gonad (**Fig. 7**).

3.4.5. Recovery of Injected Animals

1. Place a large drop of recovery buffer or M9 medium over the injected animals. The animals should be released from the pad immediately. Allow 15 min for recovery.
2. Place a drop of M9 and a drop of mineral oil onto an unseeded area of an OP50-seeded plate. Remove the worms from the agarose pad using a 200- μ L pipetman set at 20 μ L (higher volume settings will provide more pipet tip surface area for the worms to stick).
3. Place the worms into the M9 drop on the NGM plate—use the same pipet tip to transfer all worms. Worms should be counted before and after transfer. If worms have stuck to the pipet tip, they can be dislodged by pipetting mineral oil from the plate slowly up and down the pipet tip, expelling the mineral oil onto the plate, followed by pipetting similarly with more M9. The mineral oil will release the worms from the plastic tip surface, and the M9 will help wash the worms and mineral oil from the tip.
4. Allow the worms to recover on the plates overnight.

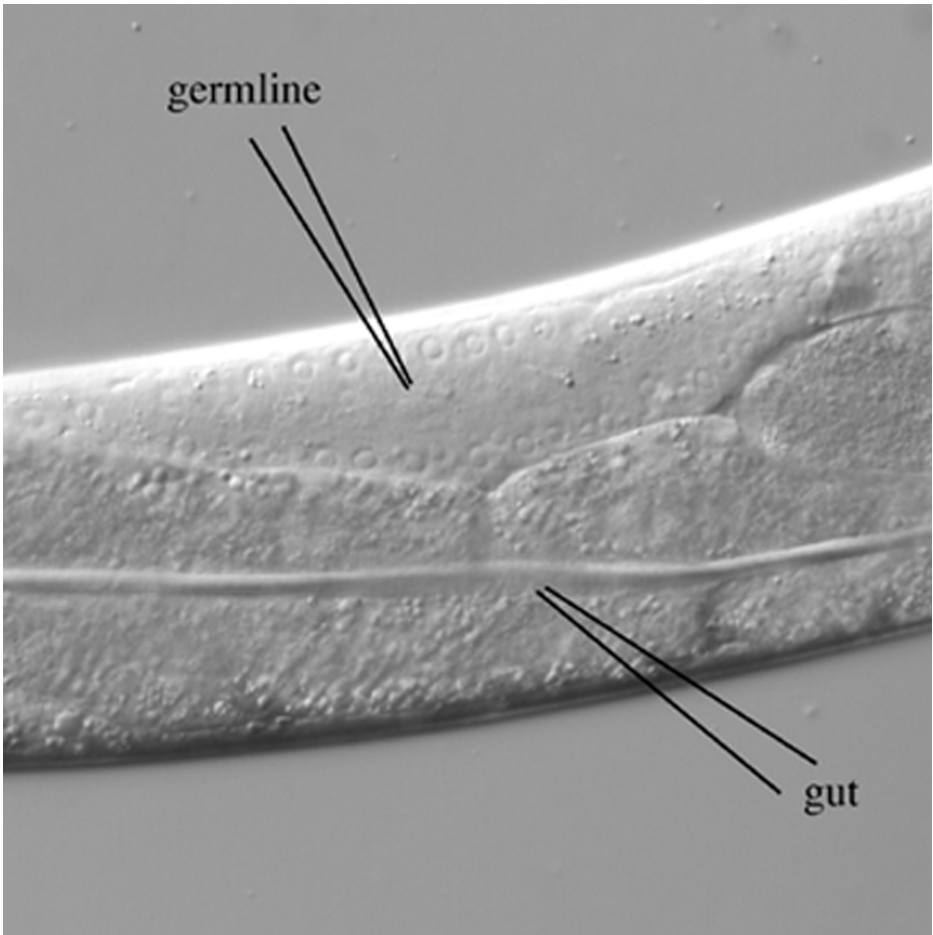


Fig. 7. Image of adult hermaphrodite highlighting the gut and gonad ($\times 40$). Lines represent an injection needle. dsRNA may be injected into the gut, body cavity, or germline of the animal, DNA must be injected into the gonad for germline transformation. The gut cells have a darker, grainy appearance, are much larger than other cells of the animal; and the central lumen is often visible. The gonad consists of two U-shaped structures that are enclosed in basement membrane and is generally observed as a clear region of the animal. At one end lies a region of mitotically dividing nuclei. The mitotic gonad is a syncytium of nuclei that are arranged similar to the “kernels on a corn cob.” In this image, outermost gonadal nuclei are in focus and are colinear with the central point of the lumen where DNA is injected.

3.4.6. Analysis of RNAi Phenotypes

1. Transfer individual worms from the recovery plate onto separate OP50-seeded NGM plates.
2. Transfer each injected worm onto a fresh plate on a daily (or more frequent) basis. Label the plates so that all progeny of an injected individual can be monitored as a group.
3. Monitor each batch of progeny for phenotypes. The first batch of progeny may contain unaffected animals, because, they may have been too developmentally progressed at the time of dsRNA delivery. Later batches of progeny may not be affected because the dsRNA may have become degraded or may become limiting.
4. It is wise to perform several sets of injections, culturing each set of worms post-injection under different temperature or other growth conditions. Compare the phenotypic distributions among the sets of progeny and among sets of injections.

3.5. Delivery of dsRNA by Soaking

1. Set up varying dilutions of dsRNA (0.2–5 $\mu\text{g}/\mu\text{L}$) in 1.5-mL microfuge tubes (*see Note 18*) in a 5- μL volume (minimum). Dilutions can be made using a 1:1 mixture of sterile M9 in water.
2. Add 10–20 worms of the appropriate strain and developmental stage to the diluted dsRNA.
3. Incubate overnight at 15°C. The length and temperature of incubation may be varied.
4. After incubation, carefully transfer the worms from the tube to a seeded NGM plate using a 200- μL pipet tip set at 20 μL . Carefully rinse the pipet tip and the tube with a small amount of sterile dH_2O and mineral oil to ensure that all worms have been transferred (*see Subheading 3.4.5.*).
5. After a few hours, transfer soaked worms individually to an OP50-seeded NGM plates.
6. Once F_1 embryos are observed, transfer the soaked worms to a fresh plate. This is done to flush out the first F_1 progeny that may have been present within the adult animal at the time of soaking, and therefore may not show a phenotype. Subsequent transfers of soaked animals on following days is also advisable because the RNAi effects do wear off and phenotypes may not be observed in later progeny.
7. Periodically monitor the soaked animals and F_1 progeny for phenotype (*see Fig. 8* for results of soaking experiments using ds *unc-22* RNA; *also see Note 19*).

3.6. Feeding *C. elegans* dsRNA-Expressing Bacteria

The feeding protocol requires a plasmid with T7 promoter sequences and DNA corresponding to the dsRNA trigger. The plasmid can be configured in a “double T7” configuration with two T7 promoter sites (plasmid L4440, available from the CGC, is one example (*see Subheading 3.2.1.1.* and **Fig. 2**) or the insert can be configured as an inverted repeat behind a single T7 promoter

Worm #	Experiment A		Experiment B		Experiment C	
	Twitching P0	Twitching F1	Twitching P0	Twitching F1	Twitching P0	Twitching F1
1	+	++	-	+++	+	-
2	+	++	-	+++	+	+
3	+	++	-	+++	+	+
4	+	++	-	+++	+	-
5	+	-	-	+++	+	-
6	-	-	-	+++	+	-
7	-	++	-	+++	+	-

Fig. 8. Experimental variability among soaking experiments. Experiments A and B: N2 worms were incubated in *unc-22* dsRNA overnight at 15°C, recovered, cloned, and kept at 20°C during phenotypic analysis of F₁ progeny. Experiment C: N2 worms were incubated in *unc-22* dsRNA overnight at 15°C, recovered, cloned, kept at 15°C for 48 h, then shifted to 20°C before phenotypic analysis of F₁ progeny. These experiments were performed using the same batch and concentration of dsRNA. Animals were scored for the corresponding loss-of-function twitching phenocopy: +, 25–50% of progeny exhibited the twitching phenotype; ++, 50–75% of progeny exhibited the twitching phenotype; +++, 75–100% of progeny exhibited the twitching phenotype. This set of experiments highlights the variability among individual experiments, and this may be influenced by factors such as the developmental stage of the soaked worm and incubation temperature after soaking (cf. experiment A with C). Although the penetrance varied, the expressivity of the phenotype was strong in all cases.

site (*see Subheading 3.2.1.2.* and **Fig. 3**). The plasmid is transformed into HT115(DE3) host cells, and dsRNA production is maintained on NGM plates supplemented with antibiotics and IPTG. Generally, feeding experiments are performed using one bacterial strain harboring one plasmid at a time (*see Note 20*). Worms are placed directly on such plates and phenotypes are monitored in the presence of food. The following protocols require Amp selection for maintenance of plasmids in the bacterial strain. If another plasmid is used that contains a different antibiotic resistance gene, replace the Amp with the appropriate antibiotic. Use sterile techniques for all the protocols.

3.6.1. Producing CaCl₂-Competent HT115(DE3) Cells

1. Inoculate 2 mL of LB+Tet (12.5 µg/mL) medium with HT115(DE3) host cells. Incubate overnight at 37°C with shaking (150–225 rpm).
2. Inoculate 0.5–1 mL of the overnight culture into 20 mL of LB+Tet (a 50-mL sterile centrifuge tube is a convenient vessel). Incubate at 37°C with shaking (150–225 rpm) until an OD₆₀₀ of 0.4–0.8 is attained (this usually requires 1–4 h).
3. Pellet the cells by centrifuging the culture tube in a clinical centrifuge for 15 min at maximum speed at 4°C.
4. Decant the medium. Resuspend the bacterial pellet by gently vortexing in the residual medium.

5. Incubate the tube on ice for 5 min. *In subsequent steps, the cells should be kept on ice.*
6. Add 10 mL of sterile, cold 50 mM CaCl₂. Swirl the tube gently to mix, and incubate on ice for 1 h.
7. Pellet the cells by centrifuging the tube at 4°C in a clinical centrifuge for 15 min at maximum speed.
8. Decant the medium. Resuspend the cells in the residual solution by gently flicking the bottom of the tube.
9. Add 0.1–0.5 mL of cold 50 mM CaCl₂, and swirl the tube gently to mix. The cells are now ready for transformation. Cells stored at 4°C may be used for 72 h with little loss of competency.

3.6.2. Transforming HT115(DE3) Cells With Plasmids

Use 50–200 µL of competent cells prepared in **Subheading 3.6.1.** for transformation of supercoiled plasmids (5).

1. Using a sterile technique, add 10–100 ng of plasmid to the cells and incubate on ice for 1 h.
2. Heat-pulse the cells in a 42°C water bath for 30 s, and then incubate the tube on ice for 2 min.
3. Add 500 µL of LB medium without antibiotics to the tube and incubate at 37°C for 1 h.
4. Plate the cells on LB-agar plates containing 12.5 µg/mL of Tet and 50–100 µg/mL of Amp. *Do not add IPTG to the plates.*

3.6.3. Freezing HT115(DE3) Expression Strains

1. Using a sterile technique, inoculate a colony of freshly transformed HT115(DE3)+plasmid into 1 mL of LB broth+Tet and 50–100 µg/mL of Amp. Incubate at 37°C with shaking (225 rpm) until an OD₆₀₀ of 0.4–0.8 is attained (this usually requires 1–4 h).
2. Add 750 µL of cells and 250 µL of sterile 80% glycerol to a labeled, sterile freezer vial and mix by gently inverting. Quick-freeze in a dry ice/ethanol bath. Place immediately in a –80°C freezer.

3.6.4. Coculture of *C. elegans* With dsRNA-Expressing Bacteria (see **Notes 21 and 22**)

1. Inoculate a 20-mL 2X YT culture containing 12.5 µg/mL of Tet and 50–100 µg/mL of Amp with a single colony of HT115(DE3)+plasmid and incubate overnight at 37°C with shaking at 225 rpm (see **Note 23**).
2. Dilute the culture more than 100-fold, and continue to grow until the culture reaches OD₆₀₀ = 0.4–0.8.
3. Add IPTG to the culture to a final concentration of 0.4 mM, and incubate with shaking (225 rpm) for an additional 1 h at 37°C. This induces transcription of T7 RNA polymerase within the cells.

4. Supplement the culture with an additional 50 µg/mL of Amp, 12.5 µg/mL of Tet, and 0.4 mM IPTG.
5. Directly seed the cells onto NGM plates containing 50–100 µg/mL of Amp, 12.5 µg/mL of Tet, and 0.4 mM IPTG. Allow the cells to incubate and the plates to dry at room temperature overnight (*see* **Note 24**).
6. Transfer the worms to plates using a worm pick to transfer individuals or a metal spatula to transfer a small agar chunk with more worms.
7. Monitor phenotypes in the transferred animals and in their progeny (*see* **Notes 25–28**).

3.7. *In Vivo* Transcription of dsRNA

1. Subclone the inverted DNA repeats (gray boxes in **Fig. 5**) and stuffer fragment (black boxes in **Fig. 5**) behind a *C. elegans* promoter and in front of a *C. elegans* 3' UTR (light gray) using standard cloning methods (**Fig. 5**) (5).
2. Refer to **Subheading 3.4.** for instructions on *C. elegans* injections (*see* **Notes 29** and **30**); the injections of dsRNA and plasmid DNA are similar (*see* **ref. 12** for more information).
3. Recover each set of animals onto NGM/OP50 plates. Clone the progeny that exhibit a phenotype corresponding to the transformation marker onto separate plates. Monitor these F₁ progeny for the presence of the marker phenotype—not all F₁s will give rise to lines. Maintain those plates producing F₂ animals with the marker phenotype as separate lines.
4. Examine each transformed line for an RNAi phenotype using different culture conditions (e.g., different temperatures).
5. Monitor the efficacy of RNA silencing by performing *in situ* RNA hybridizations or protein immunolocalization if an antibody to the target protein is available.

4. Notes

1. The plate protocols can be scaled up, and an automatic dispenser such as Wheaton Omnisense may be used to facilitate pouring. If pouring plates by hand, it may be more convenient to make the medium in an autoclavable container with a spout and handle.
2. It is possible to produce a hybrid dsRNA molecule by inserting two trigger sequences into an RNA expression plasmid. Efficient RNAi for both gene targets can be observed. If one of the sequences corresponds to *rrf-3* or other endogenous inhibitor of RNAi, the efficiency of RNAi can be enhanced.
3. Bacterial DNA is likely to be present in this genomic DNA prep.
4. Wear gloves when preparing RNA to protect against the introduction of RNases. All solutions used during an RNA preparation should be autoclaved or filter sterilized to remove RNases and other contaminants.
5. Care should be taken when using the Trizol reagent because it contains phenol and can cause burns.
6. When a contiguous coding region of sufficient length is not available (i.e., the gene is disrupted by too many introns), RT-PCR can be utilized to generate a trig-

ger molecule of adequate length (≥ 200 bp). The resulting DNA can be inserted into a plasmid (**Fig. 1**), or, alternatively, if hybrid primers composed of promoter and target gene sequences were utilized in the RT-PCR reaction, the DNA can be used directly (after cleanup) in *in vitro* transcription reactions (**Fig. 4**).

7. When performing RT-PCR reactions, a control tube lacking the RT enzyme should be included with each primer set. This control should help detect the presence of contaminating genomic DNA in the RNA preparation (no amplification product should be produced). If DNA is present, another round of DNase treatment, followed by phenol/chloroform extraction and ethanol precipitation, can be performed on the RNA sample.
8. Wear gloves whenever working with RNA or reagents used to synthesize RNA to protect against the introduction of RNases.
9. All solutions used during an RNA preparation should be autoclaved or filter sterilized to remove RNases and other contaminants.
10. To obtain the highest yield of ssRNA, it may be necessary to optimize the reaction conditions. The suggested buffer is typical, but buffers that are supplied with commercially available RNA polymerases can vary: for SP6 reactions, NaCl may be omitted and DTT lowered to 1 mM; for T3 and T7 reactions, concentrations for NaCl, MgCl₂, and DTT can range from 10 to 25, 6 to 8, and 5 to 10 mM, respectively. The amount of template DNA and incubation time can also be varied to increase the yield of RNA.
11. To obtain the highest yield of dsRNA, it may be necessary to optimize the annealing conditions. This can be influenced by factors such as length and complexity of the ssRNA transcripts. For RNAs that are difficult to anneal, try using a smaller fragment of the gene of interest as template.
12. EtBr is a mutagen and a carcinogen, and gloves should be worn during its use.
13. Wear safety glasses to protect eyes from UV light when viewing agarose gel.
14. A linearized plasmid will migrate much more slowly than the resulting dsRNA or ssRNA when resolved on an agarose gel (**Fig. 6**). The yield of dsRNA can be estimated by comparison with the fluorescence intensity of a known quantity of DNA in the marker lane. However, a PCR template used for *in vitro* transcription may be similar in size to the resulting dsRNA, possibly skewing quantitation of the dsRNA. To avoid this problem, the dsRNA preparation should be treated with DNase.
15. The agarose pad provides a sticky surface for mounting worms and prevents them from moving during injection. Worms mounted on this surface slowly dehydrate, allowing them to accept the injected fluid. The pad composition can be manipulated to suit work style (or injection speed). A faster-dehydrating pad may be preferred by experienced users while a slower-dehydrating pad may be preferred by novice injectors or by injectors using younger (smaller) animals. The rate of dehydration depends on the following:
 - a. The thickness of the pad: We have found that the weight of a glass slide is sufficient to spread out the drop of agarose. Furthermore, since glass slides have a relatively consistent weight, the resulting pads are also relatively

- consistent in thickness. (We have not found it necessary to measure the precise amount of agarose dropped onto the first slide; a large drop from a Pasteur pipet is spread completely underneath the top slide.)
- b. The temperature of the agarose: Hot agarose will spread faster and produce a thinner (slower-dehydrating) pad. Slightly thicker pads will result when using cooler agarose. The agarose solution can be maintained at 60°C in covered glass vials in a heat block for about 1 wk with good results.
 - c. The concentration of agarose: Some batch-to-batch variability in performance has been observed. We test each new bottle of agarose using concentrations within the range of 1–4% and determine the best working concentration for that bottle, which is then reserved exclusively for injections. Generally, 1% agarose works best.
16. For novice injectors, it is advisable to master mounting and recovery of worms before attempting injections. To practice, place worms onto pads (in mineral oil), wait 20 min, add recovery buffer, wait 20 min, and then transfer the worms to seeded plates. When this can be accomplished with full survival, proceed with mastering the injection. This strategy also allows the quality of the reagents to be checked.
 17. It is not a bad idea to test a new batch of mineral oil for toxic effects: mount some worms onto pads and overlay with mineral oil, allow to sit for 10 min, drop M9 medium onto the worms, and transfer to a seeded NGM plate. Assay for viability. If worms float off the agarose pads into the mineral oil, it may need to be replaced.
 18. Some proprietary buffers in commercial in vitro transcription kits may kill worms. Dilution or ethanol precipitation of in vitro transcribed dsRNA may be necessary. Worms should be soaked in various concentrations (e.g., 1X, 0.5X) of a commercial buffer in the absence of dsRNA to determine toxicity.
 19. Since it is not possible to control how much dsRNA a worm will ingest during soaking, the penetrance and expressivity of the resulting loss-of-function phenotypes may vary greatly from worm to worm, especially for dilute dsRNA solutions.
 20. “Multiplex” RNAi—in which more than one dsRNA trigger is delivered to the worm simultaneously—is possible using the feeding method. In general, bacterial strains harbor only one double-T7 plasmid, and two bacterial strains each expressing a different dsRNA can be mixed and fed to worms. However, the success rate for achieving RNAi for two targets simultaneously is very low. Better success is achieved when one bacterial strain expresses two distinct dsRNA sequences simultaneously. This is most easily accomplished by juxtaposing two DNA sequences into the double-T7 vector such that a hybrid dsRNA can form.
 21. This protocol is particularly useful when a few dsRNA-expressing bacterial strains (i.e., a few triggers) are used to study phenotypic effects on multiple strains of worms. However, another variation of this protocol may facilitate analyses of one strain of worms (wild-type) with multiple strains of dsRNA-expressing bacteria (i.e., multiple RNA triggers) (21). In this protocol, bacterial cells are grown in liquid culture to log phase or saturation in the absence of IPTG and Tet, seeded

- onto plates containing 1 mM IPTG and 75 µg/mL of carbenicillin without Tet, and allowed to induce expression of trigger RNA overnight at room temperature. This protocol reduces the need to handle many tubes of different bacterial strains but also adds the hazard and expense of higher concentrations of IPTG in the plates.
22. dsRNA can also be extracted from the bacterial cells (2). The extracted dsRNA can elicit RNAi when injected into worms.
 23. We have found that the feeding protocol works best when the HT115(DE3) cells are freshly transformed. Storage of expression strains at 4°C on LB plates often results in a loss of competency for dsRNA production. We make freezer stocks from newly transformed colonies and generally inoculate from frozen stocks. Additionally, we regenerate our frozen stocks of commonly used dsRNA-expressing bacterial strains at least every 3 mo, again from freshly transformed colonies.
 24. Freshly seeded plates or plates stored for as long as 3 wk at 15°C can produce RNAi phenotypes; however, it is always best to use freshly seeded plates.
 25. RNAi phenotypes are usually observable within 16 h to 3 d, depending on the target gene and quality of food.
 26. Plates contain sufficient quantities of bacteria to support growth of the worms for one generation; subsequent generations can be transferred to fresh plates.
 27. At no time during dsRNA administration should the animals be depleted of food because the RNAi phenotype can diminish. Best results are achieved when animals are transferred frequently onto freshly seeded plates—allowing more food for fewer F₁ animals.
 28. For long-term maintenance of animals on dsRNA-expressing food, worms from successive generations should be transferred to fresh feeding plates.
 29. Plasmid DNA *must* be injected into the gonad of the worm in order to obtain stable lines that will express the trigger RNA in vivo.
 30. The dsRNA hairpin may not elicit a visibly discernable phenotype, so a transformation marker must be injected along with the dsRNA to confirm that the worms have incorporated the injected DNA. When plasmid pRF4 is injected along with the inverted repeat plasmid, worms with pRF4-containing arrays will exhibit a “roller” phenotype. (pRF4 harbors the *rol-6* gene with a dominant mutation that produces this phenotype.) Other dominant markers, including GFP-expressing plasmids, can be used.

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