

## **Analysis of Germline Chromatin Silencing by Double-Stranded RNA-Mediated Interference (RNAi) in *Caenorhabditis elegans***

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### **1. Introduction**

RNA interference is a simple, efficient, and highly scalable method for the analysis of gene functions in *Caenorhabditis elegans* independently of mutants availability (1,2). It is used to study individual genes, gene families, and also for genomewide screenings (3). In a RNAi experiment *C. elegans* is exposed to double-stranded RNA that corresponds to the sequence of a specific mRNA. This triggers a series of enzymatic processes, resulting in a specific degradation of this target mRNA (4–7). Consequently the animals display a phenotype that results from the depletion of the target protein. The RNAi phenotype can reach the severity of a null mutant (2). Although, generally RNAi is far more effective than antisense RNA (2,8) the effectiveness of a specific RNAi experiment depends on the target gene.

In germ cells of different organisms, the transcriptional activity is typically reduced by a variety of different molecular processes not yet completely understood (9). We used the RNAi approach to identify genes essential for the chromatin silencing in the germline of *C. elegans*, e.g., the linker histone isoform H1.1; (10).

#### **1.1. Strategies of RNAi Interference in *C. elegans***

RNAi interference in *C. elegans* is a systemic response that results from exposure to specific dsRNA, which means that the interference spreads to all cells of the individual animal, regardless of the route of administration. Addi-

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tionally, the interference is passed on from the exposed hermaphrodite animal to the next generation (F1 animals), allowing two different kinds of experiments. Exposure of F0 hermaphrodites depletes maternal, as well as zygotic, mRNAs and also the mRNA of all later stages of the F1 generation. This allows a phenotypic analysis of the F1 generation if the RNAi is not embryonic lethal and does not result in larval arrest. Because the interference occurs in all life stages, it cannot be deduced when a certain transcript is functionally required.

Alternatively, L1 larvae or later-stage animals can be exposed to dsRNA and scored directly. This being used to study embryonic lethal or larval arrest genes and also demonstrating that a certain germline phenotype originates from the postembryonic depletion of mRNA.

Four different ways of exposing *C. elegans* to dsRNA have been introduced: feeding with dsRNA expressed in bacteria (**11**), soaking of worms in dsRNA (**12,13**), dsRNA microinjection, and expression of dsRNA by transgenes in *C. elegans* (**14**). The first two methods are currently used in genomewide screening experiments (**3,13**). Microinjection of dsRNA, however, occasionally gives stronger phenotypes, even to the extent that these phenotypes occur only in microinjection experiments, but not in soaking or RNAi feeding experiments. Therefore, we recommend to start the analysis of single genes by dsRNA microinjection. Microinjection also allows the mix of multiple different dsRNA species in order to achieve a combined depletion of different mRNA species, whereas a combined dsRNA feeding is not effective. Germline silencing in *C. elegans* is a function of temperature. Higher temperatures up to 25°C favor desilencing as well as RNAi efficacy and are used in the initial depletion experiments. Further biological characterization of phenotypes then includes experiments at lower temperatures, e.g., 16°C and 20°C.

## **1.2. Effector Sequence Design**

The dsRNA sequence should not be longer than 2000 bp and no shorter than 500 bp. For larger sequences, the efficiency of T7 RNA polymerase transcription will be reduced, whereas shorter fragments generate reduced interference activity in *C. elegans*. When this is tolerable, fragments as short as 200 bp can be used. As interference originates from the degradation of the corresponding mRNA, the sequence needs to be deduced from cDNA or from genomic DNA containing mostly exons of a single gene. The specificity of the sequence can be controlled by a BLASTN analysis of the *C. elegans* genome ([http://www.sanger.ac.uk/Projects/C\\_elegans/blast\\_server.shtml](http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml)). Similar sequences with an identity of 70% or more that extend over regions longer than 100 bp potentially lead to crossreactivity of the RNAi, which is typical in gene families. By choosing appropriate cDNA fragments, the researcher can optimize

either crossreactivity or single-gene specificity. A public electronic resource is available for primer design ([http://www.sanger.ac.uk/Projects/C\\_elegans/oligos.shtml](http://www.sanger.ac.uk/Projects/C_elegans/oligos.shtml)). Public databases also contain examples of PCR primers for the amplification of any *C. elegans* gene for the purpose of RNAi interference (see **Subheading 2.6., item 4**). *C. elegans* cDNAs cloned in pBluescript II, e.g., yk85b12 encoding histone H1.1 (**10**), can be requested from the yk EST project, (<http://nematode.lab.nig.ac.jp/index.htm>) (**13**).

### **1.3. In Vitro Synthesis of dsRNA for Microinjection or Soaking**

Highly concentrated double-stranded RNA is transcribed from a linear DNA template produced as a polymerase chain reaction (PCR) product that contains T7 RNA polymerase promoters in opposite orientations on both ends (**Fig. 1**). If PCR amplification is done using genomic template DNA the 3' end of both primers should be appended with the T7 RNA polymerase promoter sequence 5'-GTAATACGACTCACTATAGGG-3'. If the fragment is already cloned between the T3 and T7 polymerase promoters of pBluescript II (Stratagene), PCR amplification can be done with a mixture of a T7 primer and the primer T7–T3, which is a T3 primer appended with the T7–RNA polymerase promoter. For fragments cloned in the RNAi feeding vector L4440 PCR amplification with the T7 primer alone is sufficient.

### **1.4. RNA Interference by Microinjection**

Any injection of dsRNA into *C. elegans* leads to a systemic interference reaction. For a maximum of interference in the next generation, a single dose of dsRNA is injected into the rachis of the syncytial gonad of L4 larvae or of young hermaphrodites (**Fig. 2A**). Alternatively, and far easier for the inexperienced operator the lumen of the intestine can also be injected. In a standard experiment, the F1 generation is scored according to germline silencing, morphology, behavior, and development. The occurrence of a RNAi phenotype, severity of this phenotype, and percentage of animals affected is a function of the time after injection. Typically, the animals from eggs laid in the first 6 h are relatively unaffected. Then, RNAi interference sets in and reaches a maximum penetrance in the F1 animals laid 16–30 h following the injection. Therefore, the injected animals are transferred every 6 h onto fresh plates in order to receive a batch of F1 animals with a maximum of phenotypic penetrance. Occasionally, RNAi penetrance can be critically low ( $\geq 5\%$ ). To enhance RNAi, efficacy the injected worms and their offspring can be fed with *Echerichia coli* HAT115 expressing the specific dsRNA. The resulting F1 animals can also receive an additional dose of injected RNAi before their adult phenotype is determined (**10**).

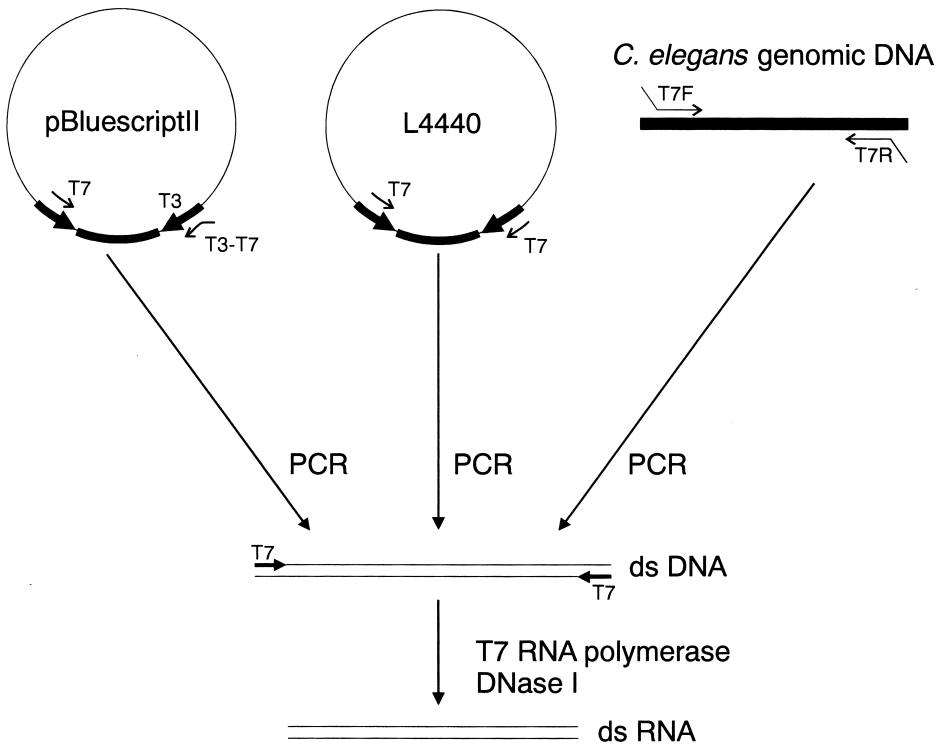


Fig. 1. In vitro synthesis of double-stranded RNA (dsRNA). *C. elegans* DNA is represented by a bold line. The fragment of interest is amplified from a feeding vector (L4440) derived plasmid with a T7 primer, from a pBluescript II clone with primers T7 and T3-T7, or from *C. elegans* genomic DNA with two specifically adapted T7 primers. Then, the PCR product is transcribed with T7 RNA polymerase. The template DNA is subsequently digested with RNase-free DNase I.

### 1.5. RNA Interference by Feeding

Systemic RNA interference can be achieved in *C. elegans* by feeding worms with transgenic *E. coli* bacteria producing specific dsRNA. The *E. coli* strain HT115 is used in combination with the expression vector L4440 (II; Fig. 1). In this system, IPTG induces T7–RNA polymerase expression and subsequent production of dsRNA, allowing continuous exposure of *C. elegans* populations to dsRNA. However, the efficiency of RNA interference achieved by feeding can be minor when compared to that of microinjection. RNA interference by feeding requires less technical skills than microinjection and can be used at far larger scales. Individual feeding clones are created by inserting genomic fragments or cDNAs into the L4440 vector. Alternatively, a full genome RNAi feeding library (approx 20,000 *E. coli* clones) has been created

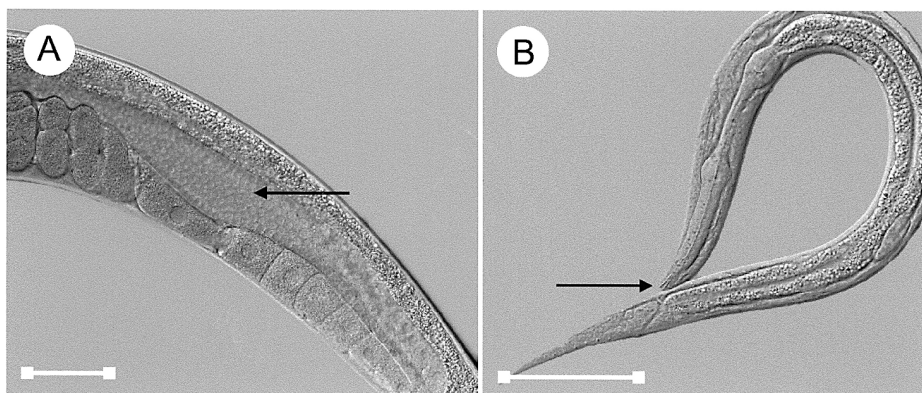


Fig. 2. Delivering dsRNA to *Caenorhabditis elegans*. (A) dsRNA is microinjected into the lumen of the syncytial gonad (the rachis) of hermaphrodites. The injection needle should be placed to where the tip of the arrow points. (B) Late L3/early L4-stage larvae are used for dsRNA feeding experiments and also for soaking. The arrow points to the mouth opening of the animal. The scale bars corresponds to 50  $\mu\text{m}$ .

by Julie Ahringer's group (The Wellcome CRC Institute, University of Cambridge, UK) and is distributed by the MRC UK HGMP Resource Center (3)(see **Subheading 2.6.;** item 7).

### 1.6. Analysis of Germline Silencing

Highly repetitive transgenes are efficiently silenced in the germline of *C. elegans*. Therefore repetitive green fluorescence protein (GFP) transgenes are used to monitor germline chromatin silencing in living *C. elegans*. Kelly and Fire (15,16) introduced *let-858::gfp* (plasmid BK48) for this purpose. We recommend using the *let-858::gfp* transgenic *C. elegans* strain PD7271 [genotype *pha-1(e2123ts) Ex 412.5/8 (ccEx7271)*] from W. G. Kelly, Emory University, Atlanta, GA. This strain needs to be maintained at 25°C in order to preserve the transgene. Alternatively, plasmid BK48 is also available in the Fire Lab 1997 Vector Kit Supplement and can be used for the production of transgenic reporter strains with standard techniques (17).

The loss of germline silencing has been implicated with severe cytological phenotypes in the germline of the *mes*-mutants: *mes-2*, *mes-4*, *mes-3*, and *mes-6* (18). Thus these *mes* mutants are useful to assess the cytological status of the germline in comparison to a RNAi phenotype of the gene under investigation. H1.1 RNAi and the *mes* mutants can be used as positive controls for a desilencing of the germline.

### 1.7. Analysis of the Depletion of the Target Protein

The easiest control of a RNA interference experiment is done in a transgenic *C. elegans* strain expressing a corresponding GFP reporter construct, which needs to contain the exons that are targeted by the RNAi. Such an expression construct is most easily generated by cloning a PCR product obtained from genomic DNA in pEGFP-N1 (Clontech). This is then used to transform *C. elegans* with standard techniques (17). GFP reporter strains, e.g., strain EC100 expressing H1.1::GFP can also be ordered from the *Caenorhabditis* Genetics Center (CGC). The fluorescence pattern in a RNAi experiment follows the time course, tissue specificity, and the intensity of the protein depletion. If an antibody specifically reacting with the target protein is available, immunofluorescence staining following standard protocols (19,20) can be used to analyze the protein depletion.

## 2. Materials

### 2.1. In Vitro Synthesis of dsRNA

1. Effector DNA sequence cloned in pBluescript II (Stratagene) or L4440.
2. Standard primers T7: 5'-GTAATACGACTCACTATAGGG-3', T7-T3:5'-CGC GCGTAATACGACTCACTATAGGGCGAATTGCCCTCACTAAAGGGA-3'.
3. Reagents: Diethylpyrocarbonate (DEPC)-treated plastic materials, gloves, Megascript T7 kit (Ambion, Cat no. 1334), dNTP, Taq-polymerase, Taq-buffer, MgCl<sub>2</sub>, water-equilibrated phenol, ethanol, RNase-free water, 96:4 chloroform-isoamylalcohol, TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0), 3 M Na-acetate, pH 5.5.

### 2.2. RNA Interference by Microinjection

1. *C. elegans let-858::gfp* reporter strain e.g., PD7271, and *E. coli* OP50 CGC. Animals need to be young freshly grown hermaphrodites that have never been starved.
2. dsRNA of the gene of interest for control experiments: irrelevant dsRNA or M9 buffer (see Subheading 2.2.; item 5).
3. Chemicals: agarose NEEO ultra-quality (Roth, Germany, cat. no. 2267.4), mineral oil (Sigma, cat. no. M-5904), 70% ethanol for sterilizing worm picks.
4. Equipment: 24 × 40 mm coverslips, worm picks (hairs from human eyebrows glued to toothpick), sterile injection needles Femtotips II (Eppendorf, cat. no. 5242 957.000), Microloader (Eppendorf, cat. no. 5242 956.003), a good binocular dissection microscope, inverted injection microscope (Zeiss Axiovert) equipped with a rotatable glide stage, differential interference contrast (DIC) optics, objective lenses with 10× and 40× magnification, mechanical micromanipulator, pressure system (the microinjection needle is connected via the output of a hand pistol to an adjustable 2000–5000 hPa pressurized air source). The

tubing connected to the pistols' outlet contains a single hole of 3-mm diameter, which can be closed with a finger when the pistol is triggered. This allows a very fast and fine-tuned microinjection of *C. elegans*.

5. Formulations: prepare M9 buffer by dissolving 3 g of  $\text{KH}_2\text{PO}_4$ , 6 g of  $\text{Na}_2\text{HPO}_4$ , 5 g of NaCl in 800 mL of  $\text{H}_2\text{O}$ , add 1 mL 1 M  $\text{MgSO}_4$ , add water to 1 L (**21**). Autoclave.
6. Preparation of seeded NGM worm plates: add 3 g of NaCl, 2.5 g of peptone, 17 g of agar, and 1 mL of a cholesterol solution (5 mg/mL in ethanol) to 975 mL of water (cholesterol is essential for the growth and development of *C. elegans*). Autoclave. After autoclaving, cool the molten agar to 55°C, and then add 1 mL of 1 M  $\text{CaCl}_2$ , 1 mL of 1 M  $\text{MgSO}_4$  and 25 mL of 1 M potassium phosphate, pH 6.0, in single steps and in exactly that order to avoid precipitation (the  $\text{CaCl}_2$ ,  $\text{MgSO}_4$ , and potassium phosphate solutions have to be autoclaved separately) before pouring the plates. Unseeded plates can be stored at 4°C for mo. Grow an overnight culture of *E. coli* OP50, dilute it with 2 vol of distilled water, and spread it onto the NGM plates (35  $\mu\text{L}$  are sufficient for a 6-cm plate). Let the bacteria grow overnight at room temperature. Seeded NGM plates can be stored at 4°C for approx 1 mo.

### 2.3. RNA Interference by Feeding

1. *C. elegans*let-858::*gfp* reporter strain e. g. PD7271 and *E. coli* OP50 (from The CGC).
2. *E. coli* strain HT115(DE3) transformed with the appropriate derivative of the RNA feeding vector L4440 (a modified version of pBluescript II with T7 promoters on both sides, which is available in the 1999 Fire lab vector kit, *see Subheading 2.6.; item 1* and *Subheading 2.6.; item 2*).
3. Chemicals: tetracycline (light sensitive; 12 mg/mL stock solution, (IPTG) isopropyl- $\beta$ -D-thiogalactopyranosid, ampicillin, carbenicillin.
4. Equipment: incubators for 16°C, 20°C, and 25°C, worm picks (*see Subheading 2.2.; item 4*).
5. Formulations: prepare NGM plates as described in **Subheading 2.2.; item 6**. IPTG and antibiotics should be added to the 55°C warm molten agar immediately before pouring the plates to avoid degradation.
6. Prepare Luria–Bertani (LB) medium by dissolving 10 g peptone, 5 g yeast extract, 5 g NaCl in 700 mL of water, and adjust pH with 2 M NaOH to 7.2. Complete to a volume of 1 L by adding water. Autoclave.

### 2.4. RNA Interference by Soaking

1. 0.2 mL PCR plastic tubes.
2. 5X M9-Mg (M9 without  $\text{Mg}^{2+}$ ): 3.4 g  $\text{Na}_2\text{HPO}_4$ , 1.5 g  $\text{KH}_2\text{PO}_4$ , 0.25 g NaCl, 0.5 g  $\text{NH}_4\text{Cl}$  per 100 mL, autoclaved.
3. 10X soaking buffer: 2.5  $\times$  M9-Mg, 30 mM spermidine (Sigma, cat. no. S2626), 0.5% gelatin; autoclaved and filtered.

## 2.5 Analysis of Germline Silencing

1. Obtain *C. elegans* strain PD7271 ([genotype *pha-1[e2123ts]* Ex 412.5/8 [*ccEx7271*]) from the CGC or from W. G. Kelly, Emory University, Atlanta, GA.
2. 4% agarose in M9 buffer, 20 mM sodium azide in H<sub>2</sub>O.
3. A fluorescence microscope capable of the observation of GFP fluorescence equipped with DIC.

## 2.6 Material Resource and Electronic Information Sources

1. Fire Lab protocol and vector/RNAi information (<http://www.ciwemb.edu/pages/firelab.html>).
2. Fire Lab FTP site (<ftp://www.ciwemb.edu/pub/FireLabInfo/>).
3. The CGC (<http://biosci.umn.edu/CGC/>).
4. WORMBASE: <http://www.wormbase.org/>.
5. Program for oligonucleotide design: [http://www.sanger.ac.uk/Projects/C\\_elegans/oligos.shtml](http://www.sanger.ac.uk/Projects/C_elegans/oligos.shtml).
6. Yuji Kohara's (yk) *C. elegans* EST project: <http://nematode.lab.nig.ac.jp/index.html>.
7. *C. elegans* RNAi libraries: <http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/Celegans.shtml> (3).

## 3. Methods

### 3.1. Synthesis of dsRNA (see Note 1)

1. Always wear gloves; use only DEPC-treated plastic materials and Rnase-free chemicals.
2. Add the following amounts of the indicated reagents in the order shown to a 0.5-mL PCR tube: 2  $\mu$ L T7 primer (100 pmol/ $\mu$ L), 0.4  $\mu$ L T7-T3 primer (100 pmol/ $\mu$ L), 4  $\mu$ L plasmid DNA (10 ng/ $\mu$ L), 4  $\mu$ L 2.5 mM dNTP, 20  $\mu$ L *Taq* buffer without MgCl<sub>2</sub>, 10  $\mu$ L 15 mM MgCl<sub>2</sub>, and 155  $\mu$ L H<sub>2</sub>O. Mix well and add 4  $\mu$ L *Taq*-polymerase (1 U/ $\mu$ L).
3. Overlay with mineral oil if your thermocycler requires it.
4. Use the following PCR protocol: once at 95°C for 5 min; once at 45°C for 20 s (required only for the T7-T3 primer); and 30 cycles at 51°C for 60 s, 72°C for 90 s, and 94°C for 60 s.
5. Check the PCR product with an analytical agarose gel, remove the mineral oil (see Note 2), and extract with 80  $\mu$ L chloroform/isoamylalcohol. Precipitate with 20  $\mu$ L 3 M Na-acetate, pH 5.5 and 400  $\mu$ L ethanol during 3 h at -20°C. Centrifuge for 30 min at maximum speed to pellet DNA. Wash the DNA pellet with 70% cold ethanol and dissolve it in 30  $\mu$ L TE.
6. Transcribe 1  $\mu$ g of the DNA with a T7 polymerase kit (Megascript T7, Ambion, cat. no. 1334) to produce the dsRNA in a single reaction. Use a reaction time of 6–12 h (see Note 3).
7. Determine the integrity of the RNA on a gel (see Note 4) and quantify it by UV



spectrophotometry.

8. Store the RNA at  $-80^{\circ}\text{C}$  (see **Note 5**).

### 3.2. RNAi by Microinjection (see **Note 6**)

1. Prepare injection pads (50–100 at once). Place drops of 0.15% agarose molten in water in the middle of a  $24 \times 40\text{-mm}$  coverslip. Dry the pads on the bench overnight (see **Notes 7–9**).
2. Load needles with 1–2 (L dsRNA using the microloader. Keep the remaining dsRNA stock on ice (see **Notes 10–12**).
3. Mount the loaded needle on the micromanipulator so that the tip of the needle is in optical axis of the microscope. Always start with  $100\times$  magnification.
4. Check the flow rate of the needle. Increase the flow rate by increasing the injection pressure up to 5000 hPa. If this is sufficient, break the tip of the needle by pushing it against the edge or corner of a coverslip. Use  $400\times$  magnification.
5. Place a drop of oil onto an agarose pad. Use a worm pick to transfer 1–2 young hermaphrodites onto the agar surface under the oil. Work under a binocular (see **Note 13**).
6. Mount the coverslip with the worms onto the microscope. Two small drops of distilled water are sufficient to adhere the coverslip to the stage. Focus onto the worm and center it together with the tip of the needle in the optical axis using  $100\times$  magnification.
7. Locate the syncytial region of one gonad arm. Search for a sausage-shaped clear area (see **Fig. 2A**).
8. Rotate the worm to allow a  $45^{\circ}$  entry angle of the needle. Then switch to  $400\times$  magnification. You need to see the germ nuclei now. Focus on the center region of the syncytial region of one gonad arm (see **Fig. 2A**).
9. Bring the tip of the needle into the focal plane.
10. Inject into the rachis.
11. You must ensure that the syncytial gonad fills with liquid.
12. Retract the microinjection needle before the gonad bursts. Remove the agarose pad from the microscope (see **Notes 14**).
13. Add  $2\text{ }\mu\text{L}$  of M9 buffer to the worm to rehydrate it.
14. Pick the injected worms onto seeded NGM plates (see **Note 15**).
15. Incubate the plates at  $25^{\circ}\text{C}$ .
16. Transfer the animals every 6–12 h onto fresh plates.
17. Screen the F1 generation for RNA interference phenotypes.

### 3.3. RNAi Feeding (see **Note 16**)

1. Clone the DNA fragment of interest between the T7 promoters of the vector L4440 and transform the construct into *E. coli* strain HT115 using ampicillin selection (see **Notes 17–19**).
2. Raise an overnight culture in LB + AT (LB with  $12.5\text{ }\mu\text{g/mL}$  tetracycline and  $100\text{ }\mu\text{g/mL}$  ampicillin) at  $37^{\circ}\text{C}$  (see **Note 20**).

3. Dilute the overnight culture 1:100 in LB + AT and grow the culture to an optical density of  $OD_{600} = 0.4\text{--}0.5$ .
4. Induce dsRNA production by adding IPTG (0.4 mM final concentration) and continue the culture at 37°C for 4 h (see **Note 21**).
5. Add a second dose of both antibiotics and of IPTG to the culture in order to double the original concentration. Incubate for another 30 min.
6. Harvest the induced cells by centrifugation and spread them onto NGM plates, which contain 1 mM IPTG, 100 µg/mL ampicillin and 12.5 µg/mL tetracycline (see **Note 22**).
7. Pick *C. elegans* larvae (**Fig. 2B**) or eggs of the reporter strain used (e.g., PD7271) onto the plates and incubate at temperatures between 15 and 25°C (see **Notes 23–25**).
8. Remove parental worms after they have laid eggs and score the phenotypes of the adult F1 generation by fluorescence microscopy of the germ nuclei. Worms can be transferred onto fresh feeding plates at any time (see **Note 26**).

Alternative method (simpler, without tetracycline, see **Note 27**):

1. Grow *E. coli* HT115 with the plasmid of interest in LB with 50 µg/mL ampicillin overnight at 37°C.
2. Spread 4 µL culture onto a 6-cm NGM plate containing 25 µg/mL carbenicillin and 1 mM IPTG.
3. Leave the plates for 5 h at room temperature.
4. Pick four M9-washed L3-stage hermaphrodites (**Fig. 2B**) of the reporter strain used (e.g., PD7271) onto the plates and incubate at temperatures between 15–25°C (see **Notes 23–25**).
5. Remove the F0 animals after they have laid 20–60 eggs.
6. Score the phenotypes of the adult F1 animals by fluorescence microscopy of the germ nuclei.

### 3.4. RNAi Soaking (see **Note 28**)

1. Collect 10–20 gravid hermaphrodites of the reporter strain used (e.g., PD7271) and wash them with M9 that contains 0.05% gelatin.
2. Cut them with two crossed injection needles in two halves and collect the emerging embryos.
3. Transfer the embryos in M9 to a 1.5-mL tube and incubate them at 25°C overnight.
4. Transfer the hatched L1 worms (see **Note 29**) to a fresh NGM plate without bacteria and let them crawl for several minutes to clean them.
5. Supplement 3.6 µL dsRNA solution with 0.4 µL 10X soaking buffer in a 200-µL PCR tube.
6. Insert 15–20 worms into the buffered RNA solution and incubate them at 20°C for 24 h (see **Note 30**).
7. Transfer the animals on to seeded NGM plates or RNA feeding plates.
8. Score the phenotypes of the adult animals.

### 3.5 Analysis of Germline Silencing

1. Produce agarose pads by dropping molten 4% agarose in M9 buffer on to a glass slide laying on a flat surface. Place two glass slides covered with two layers of transparent Scotch tape in parallel beside the first glass slide. Press a fourth glass slide in an orthogonal orientation onto the liquid agarose drop on the first slide. Wait until agarose solidifies. The resulting agarose pad has the exact thickness of two layers of Scotch tape. Release it from the top covering slide by shifting it away.
2. Add 5  $\mu$ L of 20 mM sodium azide onto the agarose pad.
3. Use a worm pick to transfer adult F1 generation animals into the sodium azide drop.
4. Cover worms with a coverslip.  
Record DIC and green fluorescent images. When desilencing occurs, the germ nuclei (**Fig. 2A**) will be green fluorescent. If desilencing is observed, additionally characterize the cytological status of the germline by identifying oocytes and developing embryos. Also count the number of germ nuclei in young hermaphrodites of a defined age.

### 4. Notes

1. In our hands, PCR fragments of this kind are superior templates for dsRNA synthesis. We prefer to use T7 promoters on both ends because T7 RNA polymerase has the highest synthesis activity.
2. Remaining mineral oil can be removed by absorption to parafilm.
3. Longer reaction times considerably increase the dsRNA yield.
4. A standard nondenaturing gel is sufficient.
5. Otherwise, it will slowly degrade.
6. Learning to microinject *C. elegans* commonly is a frustrating experience initially. Expect to practice a few times. Typically after 4–10 sessions, everyone can learn.
7. The agarose pads produced with 0.15% agarose in water will work for the cited material. Agarose from other distributors (DNA separation quality) does work as well, but the necessary concentration has to be determined experimentally. To do this, test pads have to be produced with agarose concentrations ranging from 2% to 0.07%. If the pads are too thick, the worms die too quickly from dehydration. If the pads are too thin, the worms will not be immobilized.
8. It is convenient to use thicker agarose pads (0.15% agarose) for hermaphrodites and thinner pads (0.07% agarose) for younger animals, which generate lower muscle forces, but dehydrate faster.
9. The pads can be stored at room temperature for months in the original coverslip box.
10. The needles contain an internal glass filament that will slowly transport the liquid to the tip by capillary forces without producing air bubbles. If you fill the tip of the needle directly with the microloader, you will trap air in it.
11. To release the cap of the needle, point it exactly downward and let go. This will remove the cap without breaking the needle.

12. dsRNA must be particle-free, completely dissolved, and very pure. Particularly, it may not contain traces of phenol originating from the extraction procedure. The concentration of dsRNA can be very critical. Although some proteins can be efficiently depleted with RNA concentration below 1  $\mu\text{g}/\mu\text{L}$ , other depletion experiments show phenotypes only when the RNA concentration exceeds a certain threshold. Therefore, initial dsRNA concentrations of 4–5  $\mu\text{g}/\mu\text{L}$  are used.
13. Experienced operators can handle more worms on one pad. Worms can be cleaned from adhering bacteria by transferring them onto a nonseeded NGM agar plate.
14. When the needle is retracted, no internal organs may protrude through the puncture. If this consistently happens, the needle is too thick and needs to be replaced by a thinner one.
15. A fast-working cycle is essential for animal survival. Vitality can be assessed by monitoring the locomotor activity of the animals.
16. The first feeding protocol is from Lisa Timmons (**11**), Carnegie Institution of Washington, whereas the second alternative feeding protocol is from Julie Ahringer (WellcomeTrust/Cancer Research UK Institute, University of Cambridge, UK; **3**).
17. The cloning should be performed in a standard *E. coli* cloning strain (e.g., DH5 $\alpha$ ), and the plasmid is then transferred to *E. coli* HT115(DE3). Plasmid DNA prepared from HT115(DE3) does not have the same quality as typical plasmid preparations and should be not used for further cloning steps.
18. *E. coli* HT115(DE3) has IPTG-inducible T7 polymerase activity. Competent cells can be made by using the standard methods. The strain is selectable with tetracycline. When there seems to be something wrong with the HT115(DE3) cells, test for the presence of the DE3 lysogen by PCR.
19. Julie Ahringer's feeding libraries can be obtained from the MRC UK HGMP Resource Center (see **Subheading 2.6.; item 5**).
20. Some researchers believe that the tetracycline decreases the RNAi efficacy in feeding experiments.
21. Different duration of induction time can be tested.
22. For 30 plates, a 30 mL culture is enough.
23. The ratio of worms to the bacteria is very important. Too many worms will deplete the bacteria and starve. Then, RNAi will not work.
24. *C. elegans* can be cleaned from adhering bacteria by washing worms in M9 on unseeded NGM plates or in 1.5-mL plastic tubes.
25. Initially, 25°C should be used because this enhances RNAi efficacy and germline desilencing.
26. Fresh cells work better. The feeding plates can be stored at 4°C for a maximum of 1 wk.
27. It may be necessary to test which method works best in a given environment.
28. This protocol is from Ikuma Maeda and Asako Sugimoto (**13**). We recommend soaking especially for the analysis of postembryonic development because it allows a very long exposure of L1 larvae to dsRNA. We suggest to always do this

in comparison with L1 feeding experiments, because for a given gene of interest, typically, one of the two methods works considerably better.

29. Alternatively, L1 animals may also be obtained from a clean starved plate.
30. For the analysis of the embryonic phenotype, four L4-stage larvae may be used instead.

## Acknowledgments

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