RNA interference

RNAi plates: NGM plates containing 1 mM IPTG and seeded with HT115 RNase III-deficient (note Tet-resistant transposon) *E. coli* baterial strains containing a *C. elegans* genomic DNA sequence flanked by IPTG-inducible T7 promoters (can be obtained from Ahringer / MRC geneservice RNAi by feeding library; contact C. elegans lab or http://www.geneservice.co.uk. Alternatively, can subclone your C. elegans sequence of interest into AMP-resistant pL4440 RNAi vector.

RNAi target	Predicted phenotype
L4440 vector	None
pop-1	embryonic lethal
par-6	embryonic lethal
unc-22	Unc
dpy-6	Dpy

Protocol for feeding bacteria expressing dsRNA and scoring phenotypes of treated worms:

Day 1 (morning)
- Plate #1: Place two L4's from per C. elegans strain onto RNAi plates (can use OP50 glue bacteria, which does not interfere with RNAi). Perform all experiments in duplicate to gauge reproducibility.

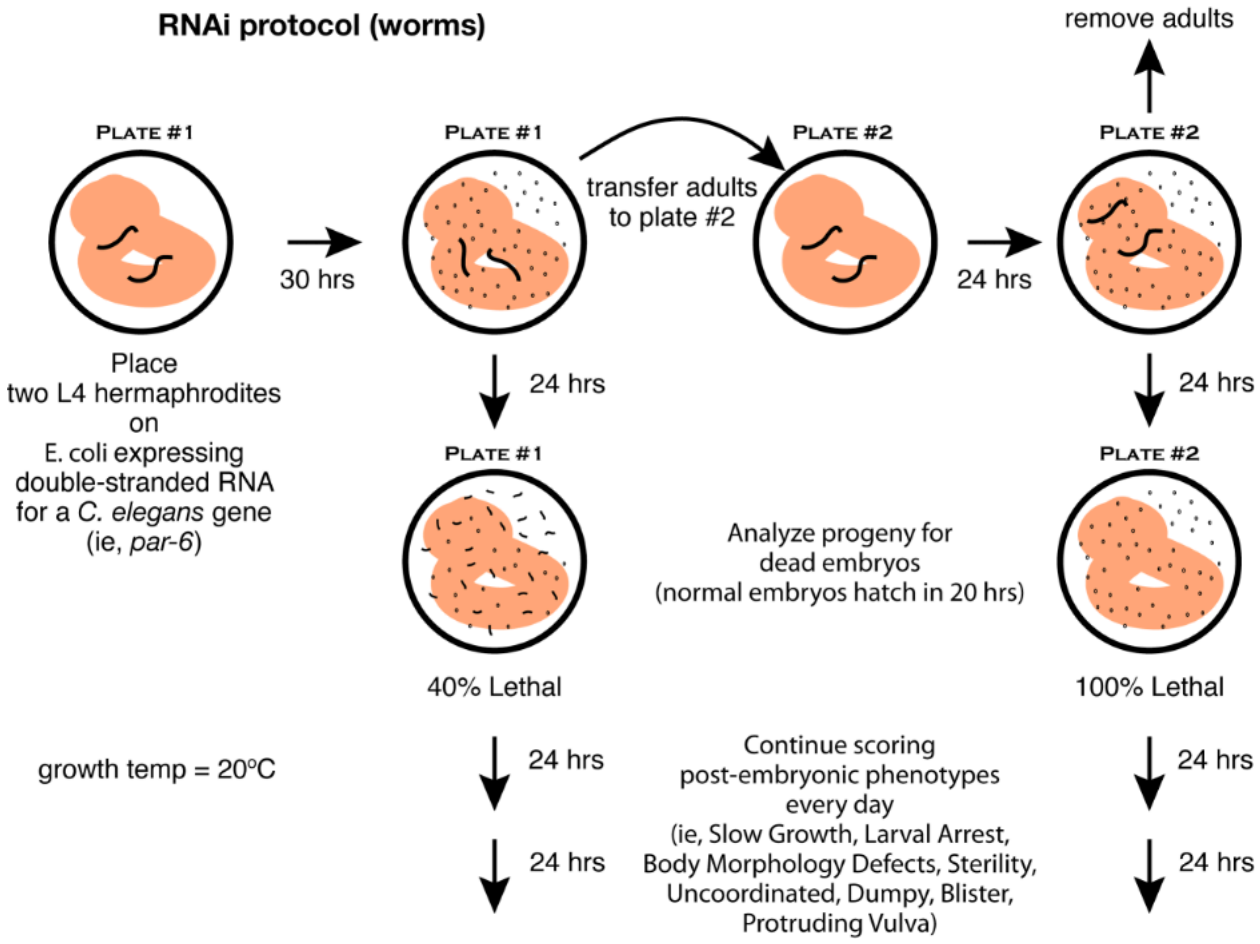
Day 2 (afternoon)
- After ~30 hours transfer adults from Plate #1 to Plate #2 (obviously, transfer to same strain of RNAi bacteria).
- Note: if an adult has burrowed, gentle shaking for 5 to 15 minutes can encourage it to surface

Day 3
- score Plate #1 for embryonic lethality (unhatched embryos) once 20 hours have passed (all normal embryos will hatch by this time)
- remove adults from Plate #2 (to waste plate)

Day 4
- score Plate #1 for larval and adult phenotypes
- score Plate #2 for embryonic lethality

Day 5
- score Plate #1 for adult phenotypes (if unstarved)
- score Plate #2 for larval and adult phenotypes

Day 6
- score Plate #2 for adult phenotypes (if unstarved)



Alternate RNAi protocol: **dsRNA injection**

RNAi by injection can result in more potent/penetrant phenotypes than RNAi by feeding.

. dsRNA Synthesis

1. ***PCR to generate a DNA template***
2. ***Transcription reactions to generate the two RNA strands***
3. ***Anneal the two strands to generate the dsRNA***
4. ***Determine the concentration of the dsRNA***
5. ***Feeding RNAi***

The first step in performing RNAi of your gene by injection or by soaking is to generate a double-stranded RNA. We recommend for a new gene that you try and generate more than one dsRNA.

1. *PCR to generate a DNA template*

Design PCR primers to amplify a region of your gene. For simplicity, we usually do this by PCR using genomic DNA as a template, although cDNA templates can also be used if convenient or if your gene has large introns that make it difficult to identify a sufficiently-large contiguous region of coding sequence. RNAs containing between 500 and 1000 bps of coding sequence are ideal—although as little as 200 bps of coding is often sufficient. Add the promoter for the T3 polymerase (AATTAACCCTCACTAAAGG) to the 5' end of one of your primers and the promoter for the T7 polymerase (TAATACGACTCACTATAGG) to the 5' end of the reverse primer.

You will be provided with genomic DNA template and primers to amplify a portion of the SAS-6 genomic region.

1. Set up a PCR reaction to generate a DNA template for the T3 and T7 transcription reaction as outlined below.

<u>PCR conditions:</u>

10X PCR buffer		10 µl
Oligos (20 µM each)		10 µl total (5 µl of each oligo)
Template		1 µl
Nucleotides	10 mM	2 µl
Taq polymerase		1.0 µl

ddH2O 76 µl

PCR conditions:

92°C	1 min
92°C	20sec
58°C	40sec
72°C	1.5 min
x34 cycles	
72°C	7 min
4°C	hold.

Run 2 µl of each reaction on a TBE gel.

2. Use the Qiagen PCR clean up kit to clean your reactions (see kit instructions). Elute with 50 µl of elution buffer or water.

2. T3 and T7 Transcription reactions to generate the two RNA strands

Note: When working with RNA—wear gloves and use RNase-free solutions.

1. Using the Ambion kits. Assemble T3 and T7 transcription reactions.

At room temperature add the following components in the order shown into an eppendorf tube from a bag dedicated for RNA work.

-20 µl rNTPs (we mix the 4 NTP solutions together when we receive the kits--each is initially 75 mM)

-5 µl 10X T3 or T7 buffer (if there is a precipitate vortex/warm to bring it back into solution before using)

-20 µl cleaned DNA template (from step II).

-5 µl T3 or T7 enzyme mix

Final volume 50 ul

Gently pipet the reaction up and down to mix. Incubate at 37°C for 3-5 hours.

2. Remove template DNA by adding 2.5 µl of DNase to each reaction (provided in the Ambion kit), incubate at 37°C for 15 minutes. Take 2 µl samples of each

transcription reaction for a gel. To each add 2 µl of RNase free water and 2 µl of 6X dye (we use 6X DNA loading dye from Sigma and keep a dedicated vial for RNA work).

3. Clean the two transcription reactions separately using the Qiagen RNeasy kit (*see instructions*). Elute each with 50 µl of RNase free water. To obtain more concentrated RNA, repeat this elution by pipetting the RNA back onto the column and eluting a second time. Take 2 µl samples of each for a gel. To this add 2 µl of RNase free water and 2 µl of 6X dye.

3. Anneal the two strands to generate the dsRNA

1. Mix 40 µl from the T3 reaction with 40 µl from the T7 reaction and 40 µl of 3X injection buffer (total 120 µl). Incubate at 68C for 10 minutes and then at 37 for 30 minutes to anneal. Take a 2 µl sample for a gel. To this add 2 µl of RNase free water and 2 µl of 6X dye.

2. Run a native 1% agarose gel in TBE to examine the RNAs.

Note: Clean the gel boxes, combs and cylinders you will use to prepare the TBE with RNase ZAP before using. It is also useful to have a container of agarose dedicated to RNA gels. Wear gloves when handling the bottle and remove agarose from the bottle by shaking it out— avoid putting spatulas into the container.

We prepare RNase free water by treatment with DEPC (mix 1 ml of DEPC with 1 liter of water—mix well and incubate at 37°C overnight—then autoclave). To make 10X TBE for RNA gels we add a packet of 10X TBE powder (Sigma) to the DEPC treated water.

Load the samples on a gel in the following order:
1. T3 strand before cleaning
2. T3 strand after cleaning
3. T7 strand before cleaning
4. T7 strand after cleaning
5. annealed dsRNA

Note: If the RNA will be used for soaking, you can mix the T3 and T7 strands with 3X soaking buffer (see recipe in APPENDIX) in a 1:1:1 ratio for the annealing step. RNA in injection buffer will not work for soaking.

4. Determine the concentration of the RNAs

Dilute 50 fold by adding 2 µl of the annealed RNA with 98 µl of RNase free ddH2O/

Read the OD 260/280 in the spectrophotometer.

OD 260=1 corresponds to a concentration of 50 µg/ml.

Concentration in µg/ml = OD (of 50 fold dilution) * 2500

We aliquot the dsRNA (typically 2 µl aliquots for injection or 5 µl aliquots for soaking and store at -80.

Protocol notes:

(1) Fidelity of the PCR is not critical so use Taq to get as much product as possible.

(2) Other kits or precipitation can be used for RNA and DNA cleanup.

(3) Samples of the single stranded RNAs can also be taken before and after cleaning—to assess whether the RNA is lost at this step, which occasionally does happen.

(4) For smaller scale RNA production—the two strands can be mixed prior to cleaning. In this case, half scale transcription reactions are performed.

(5) Quality control: You should see good bands of equivalent intensity in the T3 and T7 lanes indicating that both strands were efficiently produced. In the annealed lane you should see an up-shifted band corresponding to the double-stranded species.

(6) Trouble shooting:

A. No or weak T3 or T7 strand:

a) One of the buffers for the transcription may have gone off or has precipitated. Warm buffer solutions to bring back into solution—get new buffer

b) PCR reaction is too weak—not enough DNA template

c) Sometimes there can be a problem in the T3/T7 promoter part of the primer, so that although you obtain a good PCR product you don't get good synthesis in one direction or another. If RNA synthesis consistently fails for a specific primer set, re-order them.

B. No upshift following annealing—if the template is very small an upshift can be difficult to detect. Very large templates (1.5—2 kb) often don't anneal that uniformly and you will see a higher molecular weight smear. These RNAs usually work—so you should test whether the RNA works in this case (if the target inhibition is known to result in a detectable phenotype). RNAs between 400 bp and 1 kb are typically very well behaved.