HiScribe™ RNAi Transcription Kit

High-yield Synthesis of Double-stranded RNA for RNA Interference (RNAi) Studies

Instruction Manual

Catalog #E2000S
Store at –20°C

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Table of Contents:

Kit Components 2

Introduction
RNA Interference (RNAi) Background 4
Kit/LITMUS Vector Features 5

HiScribe RNAi Protocols
Experimental Outline 9
Protocol 1: Cloning a Target Sequence in LITMUS 9
Protocol 2A: Template Preparation: Linearization by Restriction Digestion . . 10
Protocol 2B: Template Preparation by PCR 12
Protocol 3: *In vitro* Transcription 13

RNA Interference (RNAi) Applications
Introduction 17
Guidelines for RNAi in *C. elegans* 18
Procedures for RNAi in *C. elegans* 18
Insect Cell Culture Information 20

Frequently Asked Questions 21

References 23

Appendix
Supplied Buffer Components 25
LITMUS Polylinker Sequences 26

Kit Components Sold Separately/Companion Products 28

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Kit Components:

All vectors are supplied at a concentration of 500 µg/ml in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). **Store at –20°C except where noted.**

■ LITMUS 28i cloning vector	5 µg
■ LITMUS 38i cloning vector	5 µg
■ LITMUS 28iMal control plasmid	5 µg
■ T7 Minimal Primer (19 MER), 5´-dTAATACGACTCACTATAGG-3´, (10 pmol/µl in water)	250 pmol
■ 10X Transcription Buffer	200 µl
■ 10X NTPs	200 µl
■ 30X High Molecular Weight (HMW) Component Mix	75 µl
■ T7 RNA Polymerase, 150 units/µl	75 µl
■ RNase-Free Water, (can be stored at room temperature)	2 ml
■ 6X SDS-Free Gel Loading Buffer, (can be stored at room temperature)	200 µl
■ 2-Log DNA Ladder (100–10,000 bp), (1 µg/µl in TE)	20 µg

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Introduction:

The HiScribe RNAi Transcription Kit is a complete system for the *in vitro* synthesis of large amounts of double-stranded RNA for RNA Interference (RNAi) experiments. The kit can also be used for traditional synthesis of single-stranded RNA, which can be used in numerous applications including RNA structural studies, ribozyme biochemistry, *in vitro* translation, RNA-protein interactions, antisense technology and aptamer discovery (SELEX). In addition to all reagents for high-yield *in vitro* transcription, the kit includes the LITMUS cloning/bidirectional transcription vectors, which are ideal for all transcription applications, including both *in vitro* (microinjection, soaking and cell culture transfection) and *in vivo* (worm feeding) RNAi experiments.

The kit contains sufficient reagents for synthesis of up to 2 mg RNA in a 2 ml reaction.

RNA Interference (RNAi) Background:

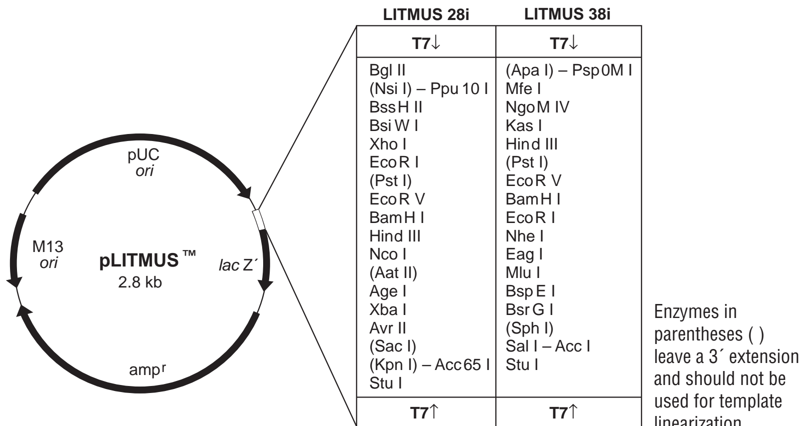
RNA Interference (RNAi) is a recently-discovered mechanism of post-transcriptional gene silencing in which double-stranded RNA corresponding to a gene (or coding region) of interest is introduced into an organism, resulting in degradation of the corresponding mRNA (for recent reviews, see references 1–6). Unlike antisense technology, the RNAi phenomenon persists for multiple cell divisions before gene expression is regained. RNAi is therefore an extremely powerful, simple method for assaying gene function. By making targeted knockouts at the RNA level by RNAi, rather than at the DNA level using conventional gene knockout technology, a vast number of genes can be assayed quickly and efficiently.

Although the exact mechanism of RNAi and the related phenomena of "co-suppression" in plants and "quelling" in fungi is still largely unknown, it is thought to be part of an ancient natural defense response to viral infection and transposon invasion. Related processes are now being described in an increasing number of invertebrate systems from hydra to trypanosomes (7–9). RNA interference has also been demonstrated in insect cell lines and more recently in cultured mammalian cells (10).

In most methods described to date, RNA interference is carried out by introducing double-stranded RNA into cells by microinjection or by simply soaking cultured cells in a solution of double-stranded RNA. *C. elegans* can be fed directly with *E. coli* cells containing the sequence of interest cloned into a vector with opposing T7 promoters. This effectively introduces double-stranded RNA (synthesized *in vivo* in the bacterial cells by a co-expressed T7 RNA polymerase gene) into the gut of the *C. elegans,* where it eventually is incorporated into other cells of the organism (11,12).

Kit/LITMUS Vector Features:

Synthesis of double-stranded RNA using the HiScribe RNAi Transcription Kit, both *in vivo* and *in vitro*, is accomplished by simultaneous transcription of both strands of template DNA with T7 RNA Polymerase. The kit contains sufficient reagents for synthesis of up to 2 mg RNA in a 2 ml reaction. The included LITMUS (Logical *in vitro* Transcription, Multiple Unique Sites) vectors feature extensive polylinkers flanked by opposing T7 promoters. The improved LITMUS 28i and 38i vectors use the consensus wild-type T7 promoter sequence, ensuring maximum yield of transcript. LITMUS 28i and 38i are identical except for the multiple cloning sites (Figure 1). Both feature the pUC origin for high plasmid DNA yields, the M13 origin for production of single-stranded template for sequencing or mutagenesis, and ampicillin resistance. Additionally, the polylinkers are



LITMUS 28i	LITMUS 38i
T7↓	T7↓
Bgl II	(Apa I) – Psp0M I
(Nsi I) – Ppu 10 I	Mfe I
BssH II	NgoM IV
BsiW I	Kas I
Xho I	Hind III
EcoR I	(Pst I)
(Pst I)	EcoR V
EcoR V	BamH I
BamH I	EcoR I
Hind III	Nhe I
Nco I	Eag I
(Aat II)	Mlu I
Age I	BspE I
Xba I	BsrG I
Avr II	(Sph I)
(Sac I)	Sal I – Acc I
(Kpn I) – Acc65 I	Stu I
Stu I	
T7↑	T7↑

Enzymes in parentheses ( ) leave a 3´ extension and should not be used for template linearization.

Figure 1: *LITMUS vectors for dsRNA production.*

inserted in-frame with the *lacZ* α-fragment, allowing screening for cloned inserts by α-complementation.

For *in vitro* transcription, plasmid DNA containing the cloned target of interest is digested (in separate reactions) with enzymes flanking the inserts, generating a template for each RNA strand (Figure 2). Simultaneous transcription of both linearized templates will produce double-stranded RNA of a defined length, with no vector-derived sequence apart from polylinker sequence. RNA is produced in yields up to 500–1000 µg/ml of transcription reaction (Figure 3).

Alternatively, the cloned insert is amplified by PCR using a single T7 promoter-specific primer, generating a double-stranded template whose ends are defined by the T7 promoters themselves (Figure 2). *In vitro* transcription of the PCR product will produce double-stranded RNA directly, without the need for separate restriction digestions. By first cloning the target sequence in LITMUS, one can amplify the sequence using the T7 Minimal Primer, bypassing the need for long insert-specific primers containing T7 promoters which must be designed and synthesized for each target sequence.

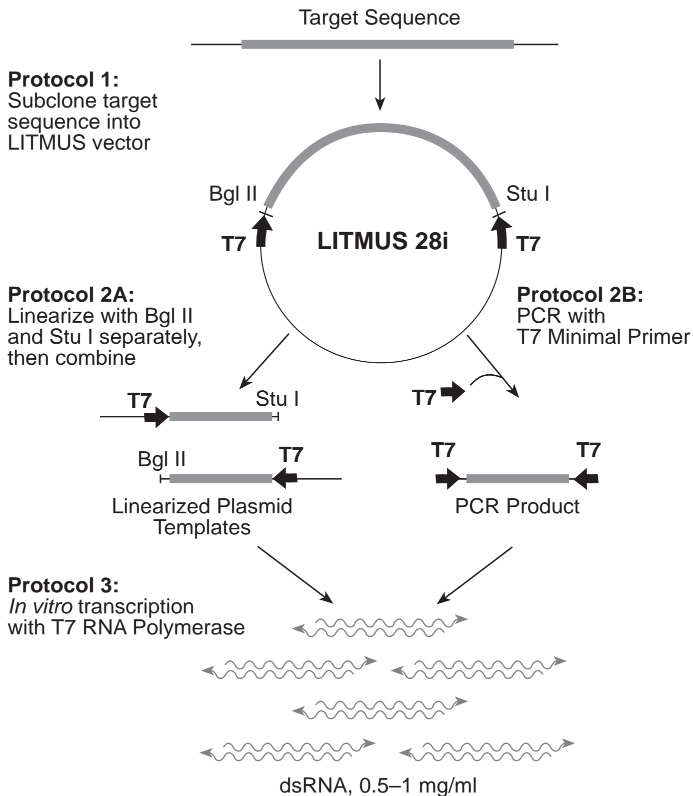


Figure 2: *Production of dsRNA from an insert cloned in LITMUS 28i.*

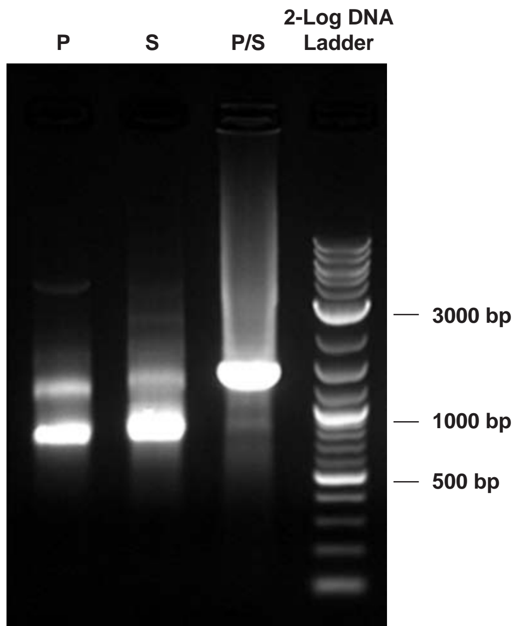


Figure 3: *In vitro production of single and double-stranded RNA using the HiScribe RNAi Transcription Kit. LITMUS 38i containing a 1.3 kb luciferase fragment was digested in separate reactions with PspOM I (P) and Stu I (S), which flank the insert. The resulting linearized templates were then transcribed either separately (lanes P and S, respectively) or together (P/S) to yield the expected single-stranded and double-stranded RNA products. RNA yields were determined to be least 0.5 µg per µl by agarose gel electrophoresis of 1 µl of each reaction.*

HiScribe RNAi Protocols:

Experimental Outline

Production of double-stranded RNA for RNAi involves three sequential protocols (Figure 2):

Protocol 1: The target sequence is cloned directly or subcloned into either of the LITMUS vectors provided.

Protocol 2A: The vector containing the target sequence is linearized in two separate reactions by restriction digestion at sites flanking the insert.

or

Protocol 2B: The insert is amplified by PCR with the supplied T7 Minimal Primer, producing a linear product with the target sequence flanked by T7 promoters.

Protocol 3: Double-stranded RNA is produced in high yield directly from the combined linear templates (from 2A), or PCR product (from 2B). No separate annealing steps are required.

Protocol 1: Cloning a Target Sequence in LITMUS

1. LITMUS 28i and 38i are identical except for their polylinker sequences (see Figure 1 and Appendix). Inserts should be cloned into the LITMUS vector that contains compatible sites. If the restriction site just downstream from either T7 promoter is used for cloning (e.g., Bgl II or Stu I in LITMUS 28i) and the linearization protocol is chosen, it is important that the cloning site is still present and that this site does not occur within the insert.

2. Standard ligation reactions (20 µl) should contain a maximum of 20 ng of vector, 3 molar equivalents of insert and 200 NEB units (= 3 Weiss units) of T4 DNA Ligase. Incubate overnight at 16°C. (Alternatively, the Quick Ligation Kit, NEB #M2200S, can be used.)

3. Transformation of the ligation products into a lacZ α-complementing strain such as ER2267 (NEB #E4103S), ER2738 (NEB #E4104S), XL1-Blue (Stratagene), or DH5α (LTI) will allow blue/white screening for insert-containing clones when plated on media containing the chromogenic β-galactosidase substrate X-Gal and the lac inducer IPTG (13). Cloned inserts will disrupt the lacZα reading frame, resulting in white colonies, while vector without inserts will produce blue colonies. Plates should contain ampicillin at 100 µg/ml.

4. Miniprep DNA should be prepared from individual colonies (1 ml cultures containing 100 µg/ml ampicillin) by standard miniprep procedures and screened for the presence of the insert by appropriate restriction digestion. If the linearization protocol (Protocol 2A) is followed, plasmid DNA from at least one positive clone should be isolated from larger-scale cultures (500–1,000 ml) and purified by chromatographic methods (e.g., Wizard or Qiagen columns) or by ultracentrifugation in a CsCl gradient.

■ Protocol 2A: Template Preparation: Linearization by Restriction Digestion

1. For simultaneous transcription of both strands of the cloned insert for RNAi, LITMUS containing the cloned target DNA (see Note A) should be linearized **in separate reactions** with enzymes flanking the insert, and then pooled prior to transcription (Figure 2). The recommended enzymes for LITMUS 28i are Stu I and Bgl II; and for LITMUS 38i are Mfe I and Stu I (see Note B). The quantity of DNA to be digested depends on the scale of the subsequent transcription reaction (see Protocol 3). As a rough guide, 30 µg of plasmid DNA should be digested per milliliter of transcription reaction. DNA should be digested in a minimum volume of 10 µl per µg of DNA, using the buffer and temperature recommended for each enzyme.

2. As a control for *in vitro* transcription, digest 1–2 µg of the included LITMUS 28iMal control DNA (see Note C) with Bgl II and Stu I (in separate reactions).
3. Purify DNA either by phenol/chloroform extraction and ethanol precipitation, or by an equivalent method (e.g., QiaQuick), and resuspend in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, prepared with Milli-Q water or equivalent) to a final concentration of ~500 µg/ml (see Note D).
4. Confirm cleavage by agarose gel electrophoresis and estimate quantity of DNA by comparison of band intensity to a known quantity of linearized marker DNA. The presence of small amounts (< 5%) of undigested plasmid will have little effect on RNA yield.

Notes:

A. Plasmid DNA containing cloned insert should be purified by ultracentrifugation in a CsCl gradient, or with commercially available chromatographic methods (e.g., Wizard or Qiagen columns). Crude alkaline lysis miniprep DNA contains unacceptable levels of ribonucleases and should ***not*** *be used for in vitro transcription.*

B. Any of the polylinker sites (see Appendix) are acceptable, but the chosen pair of enzymes must not occur within the insert, and ***must*** *leave blunt ends or 5´ overhangs (linearization of template with an enzyme that produces a 3´ overhang will result in aberrant transcripts (14).*

C. The control plasmid LITMUS 28iMal consists of the 808-bp Bgl II-EcoR I fragment of pMal-p2 (containing a nonfunctional portion of the malE gene) cloned in the EcoR I and Bgl II sites of LITMUS 28i. Linearization of LITMUS 28iMal with Bgl II and Stu I (in separate reactions), followed by in vitro transcription of the combined linearized templates, will produce double-stranded RNA 901 bp in length.

D. Linearized DNA can be used straight from the digestion reaction if desired, with only a slight reduction of yield (< 10%). Reactions ***must*** *be heat-killed at 65°C for 20 minutes prior to transcription, however.*

■ Protocol 2B: Template Preparation by PCR

The template for *in vitro* transcription can be prepared by PCR with the included T7 promoter primer, which will hybridize to the T7 promoters on either side of the cloned insert and amplify everything between them. The universal pUC/M13 forward and reverse sequencing primers (e.g., NEB products #S1224S and #S1233S) hybridize outside of the T7 promoters and can also be used for PCR amplification.

1. Set up PCR reactions in 50 µl volumes, containing 25–100 ng of plasmid DNA and 20 pmol (~125 ng, 2 µl) of T7 primer. A typical cycling protocol is as follows:

94°C	3 min	
94°C	30 sec	25 cycles
50°C	30 sec	25 cycles
72°C	30 sec	25 cycles
72°C	5 min	

2. As a control for *in vitro* transcription, set up an amplification reaction using the included LITMUS 28iMal control DNA as a template.
3. Following PCR, analyze 1 µl of the reaction by agarose gel electrophoresis in order to confirm amplification and to provide a rough estimate of quantitation.
4. The PCR reaction can be used directly as a template for transcription, without purification. Alternatively, purify the PCR product by phenol/chloroform extraction and ethanol precipitation, or equivalent chromatographic method (e.g., QiaQuick), and resuspend in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, prepared with Milli-Q water or equivalent) to a final concentration of ~500 µg/ml.

■ Protocol 3: *In vitro* Transcription

This protocol may be used for any large-scale transcription reaction.

Yields of ~500 µg per ml of transcription reaction should be easily obtainable using the HiScribe RNAi Transcription Kit. A 30 µl pilot reaction should be carried out initially to test the quality of DNA template and transcription reagents. Reactions can then be scaled up accordingly as required by the particular application. Pilot reactions should be carried out side-by-side with reactions containing linearized or amplified control template from the LITMUS 28iMal vector (see Protocol 2).

Pilot Reaction

1. Thaw the 10X Buffer and NTPs at room temperature for the minimum amount of time required for complete thawing. Do not thaw at 37°C. If a precipitate is evident following thawing, vortex briefly to resuspend. Keep the 30X High Molecular Weight Component (HMW) Mix and T7 RNA Polymerase at –20°C until needed.

2. Combine the following, in order, taking caution to avoid ribonuclease contamination (see Note A). Set up separate reactions for each plasmid of interest, as well as the control plasmid LITMUS 28iMal.

RNase-Free Water	22 – x µl
10X Transcription Buffer	3 µl
10X NTPs	3 µl
Template(s) (~0.5 µg each)	x µl (see Note B)
30X HMW Mix	1 µl
T7 RNA Polymerase (150 U/µl)	1 µl
	30 µl

If using linearized plasmid template (from Protocol 2A), combine both DNA templates (i.e., linearized on either side of the insert) in the reaction to produce double-stranded RNA. For a PCR template with opposing T7 promoters, only one template is needed.

3. Incubate at 37°–42°C for 90–180 minutes (see Note C).

4. Optional: to improve efficiency of strand annealing, heat reaction to 65°C for 5 minutes and cool to room temperature. It is not necessary to use a separate annealing buffer.

5. For gel analysis, dilute 1 µl of transcription reaction with 9 µl of RNase-Free water (or TE). Add 2 µl of 6X SDS-Free Gel Loading Buffer. Use the included 2-Log DNA Ladder (Figure 4) as a molecular weight standard: the difference in gel migration between DNA and double-stranded RNA is minimal. Electrophorese on 1% agarose gels in TAE buffer (40 mM Tris-acetate, pH 7.5, 1 mM EDTA) containing 0.1 µg/ml ethidium bromide in both the gel and the tank buffer. Both the gel and tank buffer should be prepared with Milli-Q (or equivalent) water to avoid RNase contamination. The expected length of the transcript from the LITMUS 28iMal control template is 901 bp.

Large-scale Transcription Reaction

1. If the yield of RNA from the pilot reaction is sufficient, scale up the reaction appropriately. Use no more than 500 µl per tube; use multiple tubes if necessary.

2. If required for the particular RNAi application, the RNA product can be purified either by phenol/chloroform extraction and ethanol precipitation, or by using a commercial kit for this purpose. It is generally not necessary to remove template DNA (e.g., with DNase I)

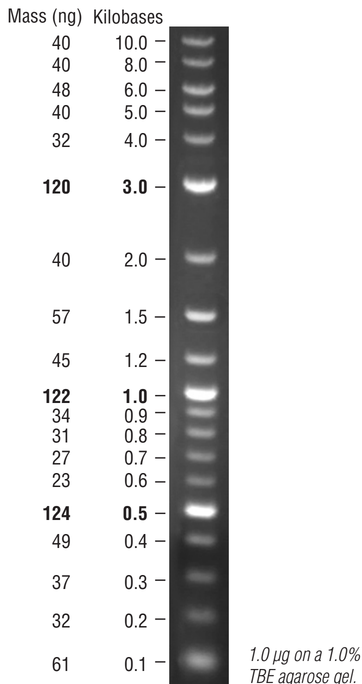


Figure 4: *2-Log DNA Ladder. The 2-Log DNA Ladder is a broad-range molecular weight marker that can be used to estimate both the size and quantity of double-stranded RNA by gel electrophoresis. Bands indicated in* ***bold*** *have increased intensity to serve as reference points. The second column indicates the amounts of DNA in each band when 1 µg (total) of the marker is loaded.*

Notes:

A. While all of the components in the HiScribe RNAi Transcription Kit are RNase free, it is possible to introduce ribonucleases into the transcription reaction from the laboratory environment. This can be avoided by following some very simple precautions: 1) always wear gloves when working with RNA, 2) use either a dedicated set of pipettors for RNA work or aerosol-resistant (barrier) pipette tips, 3) use ultrapure water (Milli-Q or equivalent) and autoclave all solutions if possible, and 4) use disposable plasticware instead of glassware whenever possible. It should NOT be necessary to treat solutions and equipment with diethyl pyrocarbonate (DEPC), and indeed, DEPC can inhibit subsequent reactions if it is not completely inactivated following treatment.

B. The volume of template used in the transcription reaction depends on the method of purification. For unpurified, heat-killed restriction digests, include no more than 5 µl of each template strand (10 µl total) per 30 µl reaction. For unpurified PCR product, include no more than 5 µl per 30 µl reaction. In all cases, the amount of added template DNA should not exceed 1 µg per 30 µl reaction, as RNA yields will not be higher at template concentrations greater than this.

C. Incubation at 42°C may improve yields of RNA transcripts containing substantial secondary structure. As it is difficult to gauge the secondary structure content in a particular transcript, we recommend that all transcription be carried out at 42°C if possible. Transcription yields increase linearly for the first 90 minutes (approximately) and reach maximum after 2–3 hours. Reactions can be carried out overnight if desired, but yields will not be higher. Double-stranded RNA is stable upon prolonged incubation at 37°C.

RNA Interference (RNAi) Applications:

Introduction

RNAi has been employed extensively in *C. elegans* and *Drosophila*, and increasingly in other systems. The method effectively creates transient knockout mutations at times specified by the investigator. It has been used to identify the function of cloned genes by determining the loss-of-function phenotype of genes without known mutations, and to create "double mutants" by injecting dsRNA into mutant worms or by the simultaneous delivery of multiple dsRNA species (reviewed in reference 3). Other applications may include the identification of suppressor genes in a particular mutant host by looking for suppression of the mutant phenotype, or screening for genes that cause resistance or sensitivity to drugs (15).

There can be some distinct advantages to using RNAi over classical knockout methods, because it can be applied selectively at different developmental stages. This would be useful if eliminating the gene of interest causes embryonic lethality, therefore preventing the study of other functions in later stages of development.

RNAi is less time consuming and easier to perform than the isolation of chromosomal mutations. In most cases, the phenotypes resulting from chromosomal mutations or RNAi are the same (15), although neuronal cells appear somewhat resistant to RNAi for unknown reasons (16).

A unique feature of RNAi is its transient nature. In *C. elegans*, transmission of RNAi occurs from the adult hermaphrodite to the first generation (F1) progeny and complete recovery of wild-type gene activity is normally regained in the second (F2) generation. RNA can be absorbed through the gut and distributed to somatic tissues and the germ line. This can occur following injection of adult worms with dsRNA, or after soaking the animals in the nucleic acid, or by feeding the worms an *E. coli* strain engineered to produce the appropriate dsRNA.

Guidelines for RNAi in *C. elegans*

cDNA sequences are the preferred templates since the penetrance of the RNAi mutant phenocopy may be greater when using cDNA templates than when using genomic templates (17,18). However, in their absence, exon-rich genomic sequences can be used. A program is available that provides *C. elegans* gene-specific primer pairs capable of amplifying 1 kb exon-rich regions, which are suitable templates for generating dsRNA (see www.sanger.ac.uk/Projects/C_elegans/oligos.shtml).

The effectiveness of induction of RNAi may be influenced by other factors including the length and homology of the dsRNA used (19). The method used to deliver the dsRNA may also influence the potency of the RNAi response. The most common route for administering dsRNA in *C. elegans* is through microinjection of the germline syncytium (20,21), body cavity or gut (8). Microinjection of adult worms has been considered the most effective means of initiating RNAi in *C. elegans* (3). While other modes of delivering the RNA may be less potent, soaking in dsRNA (22), or feeding worms *E. coli* expressing *C. elegans* dsRNA (23) have been used in whole-genome functional analyses and high-throughput screening formats. Soaking and feeding techniques can easily be applied to immature worms if eliminating the gene of interest causes embryonic lethality, thus preventing the characterization of postembryonic phenotypes.

Procedures for RNAi in *C. elegans*

Note: RNAi protocols are constantly changing. Consult recent literature for the most up-to-date techniques.

Injecting Worms: 0.5–1.0 x 10^6 RNA molecules per gonad arm (8), or 1–5 mg/ml into the intestine (24). The RNA should be purified prior to microinjection by phenol/chloroform extraction followed by ethanol precipitation, or using a commercial RNA purification spin column kit. Protocols for microinjection of *C. elegans* can be found in general methodology books, e.g., ref. 12.

Soaking Worms: Soaking worms in dsRNA was originally described by Tabara et al. (22). Worms were incubated in 0.2 M sucrose, 0.1X phosphate-buffered saline, liposome and 1 mg/ml RNA. The technique was later optimized for high throughput analysis (15): worms were incubated in soaking buffer (10.9 mM Na_2HPO_4, 5.5 mM KH_2PO_4, 2.1 mM NaCl, 4.7 mM NH_4Cl, 3 mM spermidine and 0.05% gelatin) and 1–5 mg/ml RNA. Worms were incubated in RNA solution for 24 hours and transferred periodically to fresh plates.

Feeding Worms: *E.coli* engineered to express double-stranded RNA *in vivo* was originally described by Timmons and Fire (23). A fragment corresponding to the gene of interest was cloned into a feeding vector between two T7 promoters and was transformed into a bacterial strain carrying IPTG-inducible expression of T7 RNA Polymerase. The included vectors LITMUS 28i and 38i are specifically designed for this purpose. It is thought that use of an *E.coli* strain (e.g., HT115[DE3]), which lacks the double-strand-specific RNase III, improves the ability to produce RNAi phenotypes by feeding. The protocol was subsequently adapted for high-throughput screening (25,26): using a panel of various genes, the concentrations of IPTG and bacteria, temperature, length of feeding time and age of worms at time of feeding were optimized. L4 worms placed onto NGM plates containing seeded bacteria expressing dsRNA for each gene were incubated for 36 hours at 22°C or for 72 hours at 15°C. Shorter time intervals were not always sufficient to produce a strong RNAi effect.

■ Insect Cell Culture Information

RNA interference experiments have been carried out with cultured *Drosophila* S2 cells (27). Cells were plated in serum-free medium at a density of 10^6 cells per milliliter of medium, per one 35 mm culture plate. A sufficient quantity of dsRNA to reach 40 nM (typically 15 µg of dsRNA and 10^6 cells per ml) was then added immediately after plating. After 30–60 minutes, 2 ml of complete (serum containing) medium is added to the well. Knock-out effects start after several hours. Complete loss of protein expression was detected after 48 hours (27). An alternative protocol using transfection reagents is described in reference 28. A similar method was used in RNAi experiments performed in mosquito cultured cells (29). A method for RNAi on cultured *Drosophila* organs is described in reference 30.

Frequently Asked Questions:

- *Why does the template have to be linearized prior to transcription? Why can't I simply transcribe the circular plasmid containing my target sequence?*

  T7 RNA Polymerase is an extremely processive enzyme, and will continue to transcribe around a circular template multiple times without disassociating, producing a transcript much longer than the plasmid. Not only will the transcript from each promoter contain mostly vector-derived sequence, but the long length makes coherent annealing into a fully double-stranded product very difficult. Linearization at a point downstream from the cloned target sequence, either by restriction digestion or PCR, will produce an RNA transcript with a length defined by the 3´ end of the template (31), and the resulting RNA duplex will contain only the target sequence flanked by a small amount of vector polylinker sequence (see Figure 2).

- *I already have a "universal" T7 promoter primer. Can I use that for PCR amplification of a target sequence cloned in LITMUS?*

  Many so-called "universal" T7 promoter primers, including NEB product #S1248S, contain 3´ nucleotides which are downstream from the minimized T7 promoters in the LITMUS vectors, and consequently will not anneal to LITMUS. As a result, the supplied T7 Minimal Primer (19 MER), 5´-TAATACGACTCACTATAGG-3´, should be used for PCR amplification of inserts cloned in LITMUS. Alternatively, the universal pUC/M13 Forward and Reverse Sequencing Primers (NEB products #S1224S and #S1233S) hybridize outside of the T7 promoters and can also be used for PCR amplification.

- *What is the shelf-life of the supplied reagents?*

  The T7 RNA Polymerase and High Molecular Weight (HMW) Mix should be stable for at least 1 year when stored at –20°C. The 10X Buffer/NTP Mix should be stable for up to 6 months, but stability will vary greatly depending on how often it is thawed and re-frozen. For maximum stability, aliquot and store at –70°C.

- *I have my own gel loading buffer. Do I have to use the buffer supplied with the kit?*

  The placental RNase inhibitor (RNasin) in the transcription reaction is rapidly inactivated by the SDS present in many gel loading buffers. If your template DNA was contaminated with RNase (as is often the case unless using CsCl-purified DNA), the use of an SDS-containing loading buffer will release the RNase from the inhibitor, resulting in rapid degradation of your transcript, especially if it is single-stranded. The buffer supplied with the kit does not contain SDS.

- *My transcript is only half the expected length.*

  It is possible that transcription is occurring in one direction only, and you are seeing single-stranded RNA, which runs at approximately half the size of the expected double-stranded product. Check to see that you added both template strands, the enzymes used to linearize each template cut on the far side of the insert relative to the promoter and that sites for either enzyme are not present within the cloned insert itself.

- *The yield for my transcription reaction is lower than expected, and/or the bands are smeary.*

  Be sure to carry out a control pilot reaction with the LITMUS 28iMal control plasmid. If the yield from your plasmid is substantially lower than that from the control reaction, it is possible your plasmid has RNase contamination. This problem can be solved either by further purifying your plasmid DNA, carrying out phenol extraction and ethanol precipitation steps following linearization as described in Protocol 2A, step 3, or following Template Precipitation by PCR (Protocol 2B).

  If yields from reactions containing your plasmid and the control plasmid are equally low, double-check the concentration of your template DNA, make sure your gel loading buffer does not contain SDS, and confirm that both the gel and tank buffer were prepared using ultrapure water (Milli-Q or equivalent). It is also possible that the linearization method used (restriction digestion or PCR) introduced RNase into both templates; again, phenol extraction followed by ethanol precipitation prior to transcription should solve this problem.

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Appendix:

Supplied Buffer Components:

10X Transcription Buffer

400 mM Tris-HCl, pH 8.1
190 mM $MgCl_2$
50 mM DTT
10 mM spermidine

10X NTPs

10 mM Tris-HCl, pH 7.8
5 mM DTT
40 mM each NTP

6X SDS-Free Gel Loading Buffer

15% (w/v) Ficoll-400
0.25% bromphenol blue

30X High Molecular Weight (HMW) Mix

20 mM Tris-HCl, pH 8.1
1.5 mg/ml BSA
100 units/ml inorganic pyrophosphatase (yeast)
12,000 units/ml pancreatic ribonuclease inhibitor
50% glycerol

LITMUS 28i Polylinker

```
                    2440          T7 →      2460
                      •                       •
5´...atgaccatgattacgccaAGCTACGTAATACGACTCACTATAGGGCAGATCTTCGA
lacZα                     SnaB I                        Bgl II

2480              2500                2520                 2540
•                   •                   •                    •
ATGCATCGCGCGCACCGTACGTCTCGAGGAATTCCTGCAGGATATCTGGATCCACGAAGCTT
 Nsi I   BssH II  BsiW I  Xho I  EcoR I  Pst I  EcoR V  BamH I  Hind III

                2560                2580
                  •                   •
CCCATGGTGACGTCACCGGTTCTAGATACCTAGGTGAGCTCTGGTACCCTCTAGTCA
  Nco I   Aat II  Age I   Xba I   Avr II  Sac I   Kpn I
                                                  Acc65 I

2600              2620
 •  +1              •
AGGCCTATAGTGAGTCGTATTACGGActg...3´
Stu I                  ← T7
```

LITMUS 38i Polylinker

```
                       2440          T7 →       2460
                         •           ┌───────────•─────────┐
                                                      +1
5´...atgaccatgattacgccaAGCTACGTAATACGACTCACTATAGGGGCCCGTG
lacZα                        SnaB I                    Apa I

2480                  2500                  2520
•                       •                     •
CAATTGAAGCCGGCTGGCGCCAAGCTTCTCTGCAGGATATCTGGATCCAC
Mfe I   NgoM IV  Kas I  Hind III  Pst I  EcoR V  BamH I

            2540                  2560                  2580
              •                     •                     •
GAATTCGCTAGCTTCGGCCGTGACGCGTCTCCGGATGTACAGGCATGCGTCGACCCT
EcoR I  Nhe I   Eag I   Mlu I   BspE I  BsrG I  Sph I  Sal I

                 2600
          +1       •
CTAGTCAAGGCCTATAGTGAGTCGTATTACGGActg...3´
       Stu I └─────────────────┘
                             ← T7
```

Kit Components Sold Separately:

LITMUS 28i Vector
#N3528S 20 µg

LITMUS 38i Vector
#N3538S 20 µg

T7 Minimal Primer (19-mer)
#S1272S 0.5 A_{260} units

Companion Products:

LITMUS U
#N3640S 2 µg

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