Chapter 28

Gene Knockdown Analysis by Double-Stranded RNA Injection

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Abstract

The discovery of RNAi, in which double-stranded RNA (dsRNA) suppresses the translation of homologous mRNA, has had a huge impact on evolutionary genetics by enabling the analysis of loss-of-function phenotypes in organisms in which classical genetic analysis is laborious or impossible.

In this chapter, we discuss an RNAi method via simple dsRNA injection in the red flour beetle, *Tribolium castaneum*. *Tribolium* is gaining popularity in evolutionary genetics due in part to the ease of RNAi application. We describe procedures for dsRNA synthesis and injection and provide a description of the injection apparatus. In addition, we detail two methods to validate the efficacy of RNAi (real-time PCR and western blot analyses). Although this chapter focuses mainly on *Tribolium*, many of the molecular biology and injection procedures described here are applicable to other organisms with some modifications. A few notes regarding dsRNA injection in other species are also included.

Key words: RNA interference, Double-stranded RNA, Injection, Real-time PCR, Western blot

1. Introduction

RNAi is an evolutionarily conserved gene silencing pathway found in diverse eukaryotic species (1–3). This biological process enables the analysis of loss-of-function phenotypes in organisms in which classical genetic analysis is laborious or impossible. The ease of RNAi application in the red flour beetle, *Tribolium castaneum*, has, thus, made this species a popular and powerful model in evolutionary genetics (4, 5).

In brief, RNAi is triggered by dsRNA molecules, which are processed into small interfering RNAs (siRNAs) by a dsRNase, Dicer. siRNA then binds to several proteins including Argonaute, forming a complex called RISC (RNA-Induced Silencing Complex). RISC binds to the target mRNA using complementarity between the

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target sequence and the siRNA sequence and cleaves the mRNA through the action of the catalytic Argonaute protein. This intracellular RNAi machinery is well conserved among eukaryotes; therefore, RNAi can be triggered in many organisms once dsRNA (or siRNA) molecules are delivered inside the cell.

In contrast to the intracellular RNAi response, the mechanism by which the dsRNA enters a cell appears to be less conserved. This cellular dsRNA uptake process, sometimes called "systemic RNAi" (see Note 1), has an important implication for the application of RNAi to an organism of interest, as dsRNA molecules injected at a multicellular stage need to be taken up by cells to trigger an RNAi response. The degree of systemic RNAi effectiveness differs greatly among organisms (6–9), presumably in part because of the less conserved dsRNA cellular uptake machinery. Unfortunately, to date, it has been very difficult to predict whether an organism shows a robust systemic RNAi response without actually testing RNAi in the organism. A multi-transmembrane protein Sid-1 is an essential factor for systemic RNAi found in the nematode *Caenorhabditis elegans* (10, 11) and is suggested to be a determinant for the presence of systemic RNAi in some organisms (11). However, this view is currently under debate because of inconsistencies between the presence of *sid-1* homologs and the efficacy of systemic RNAi in some organisms, as well as the question whether *sid-1* homologs found outside nematodes are true orthologs of the *C. elegans sid-1* (8). Therefore, it is important to assess the efficacy of systemic RNAi carefully in an organism of interest before utilizing RNAi as a genetic tool in the organism.

Tribolium is found to show a robust systemic RNAi response throughout development, which makes it possible to perform RNAi at the postembryonic stage by injecting dsRNA into larval body cavities (larval RNAi) (12), or trigger an RNAi response in offspring embryos by injecting dsRNA into the mother's body cavity (parental RNAi) (13). As such, RNAi phenotypes in *Tribolium* are easy to obtain, highly reproducible, and are able to phenocopy the genetically null phenotypes (for example, *see* (13–15)). In addition, virtually all *Tribolium* tissues can respond to extracellular dsRNA (6). These traits allow researchers to create loss-of-function phenotypes at any desired stage in *Tribolium* by simple larval or pupal injection, making *Tribolium* a good alternative model organism to study gene function outside classic model organisms.

2. Materials

2.1. dsRNA Synthesis

1. A fragment of gene cloned into a plasmid. TOPO TA Cloning Kit for Sequence (Invitrogen) should be used in the case TOPO-RNAi primer is used for dsRNA template synthesis (see Subheading 3.1.1).

2. Go Taq DNA polymerase (Promega).
3. dNTP mixture (Takara Bio).
4. Thermal cycler (e.g., Bio-Rad).
5. DNA loading dye (Promega).
6. 100 bp DNA ladder 6× (Promega).
7. QIAquick PCR purification kit (QIAGEN).
8. DNA/RNA/Genetic analysis-grade agarose.
9. Spectrophotometer (e.g., NanoDrop 2000, Fisher Thermo Science).
10. Heating block (37°C and 70°C).
11. MEGAscript T7 Kit (Ambion). This kit contains TURBO DNase I.
12. MEGAclear (Ambion).
13. 100% ethanol.
14. 70% ethanol (diluted with ddH_2O) (stored at −20°C).

2.2. dsRNA Injection

Items 1–7 describe materials for *Tribolium* culturing (Fig. 1). Injection buffer requires items 8–14. Items 27–32 describe the injection apparatus (two examples of injection settings are shown

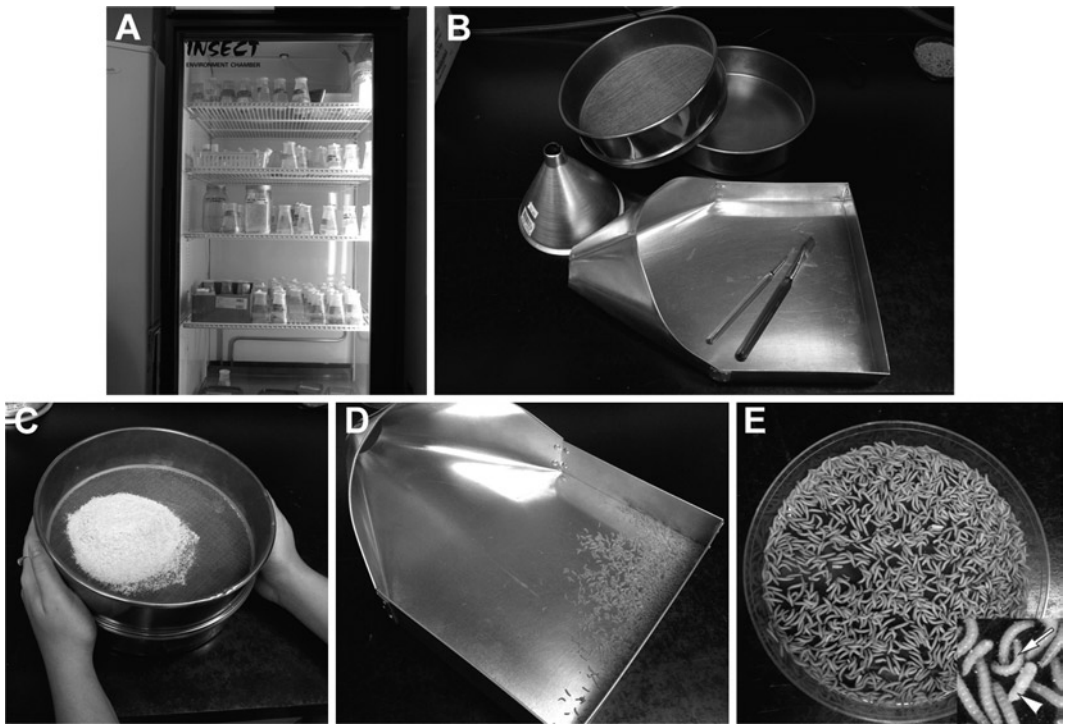


Fig. 1. *Tribolium* culturing and manipulation. (**a**) A beetle incubator. (**b**) Typical beetle equipment. (**c**, **d**) Isolating beetles from culture flour using a sieve (**c**) and a seed pan (**d**). (**e**) Isolated beetles (larvae and pupae) in a plastic Petri dish. A larva and pupa are indicated by *arrow* and *arrowhead* in inset, respectively.

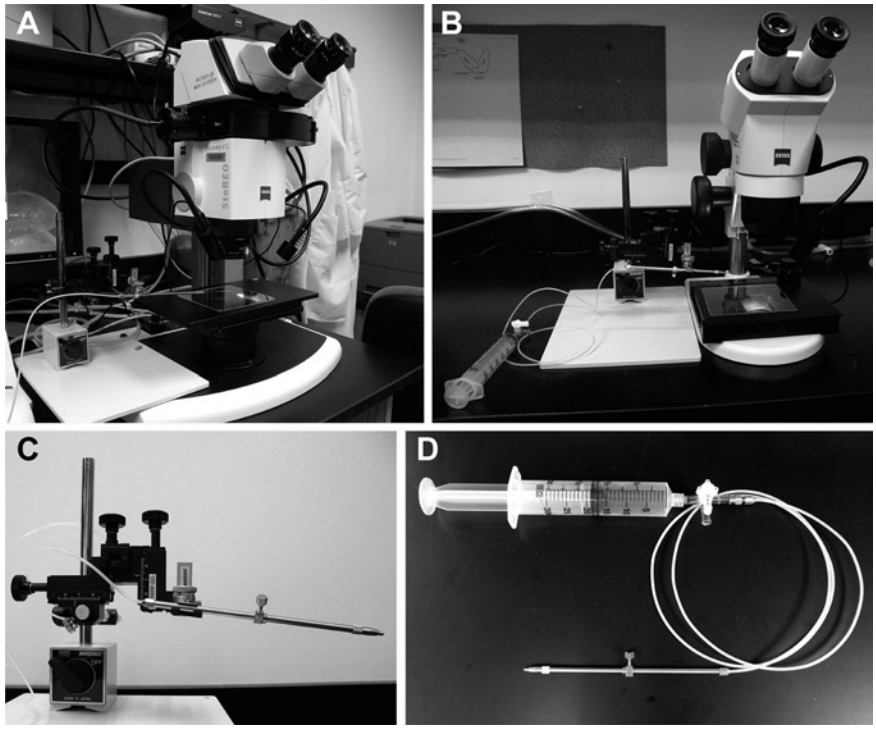


Fig. 2. Injection apparatus. (**a**, **b**) Two injection setups. (**c**) Typical manipulator orientation. (**d**) Injection syringe with a needle holder.

in Fig. 2a, b), and items 33–35 describe the etherization apparatus (Fig. 3c–e).

1. Flour: Organic whole wheat flour (e.g., Golden Buffalo Flour. Heartland Mill Inc. Kansas).
2. Brewer's Yeast (MP Biomedicals).
3. Culture Flour: Add 5% (by weight) of yeast to flour for nutrition supplement. Store at −20°C.
4. Culture bottles (Fig. 1a): 6 oz plastic *Drosophila* stock bottles (Flystuff.com).
5. Sieves: 8 in. diameter sieve (Fig. 1b, c) (#25 for larvae, pupae, and adults, #50 for embryos) (Fisher).
6. Beetle collection pan (Fig. 1b, d): 1.5 quart spouted sample pan (Seedburo Equipment Co.).
7. Incubator (Fig. 1a): A humidity-controlled incubator is preferable, e.g., Insect Chamber (BioCold Environmental Inc). *Tribolium* can be cultured at 30°C with 50% humidity. A lower temperature (20–25°C) can be used for long-term stock maintenance. Detailed *Tribolium* husbandry information can be found at the USDA *Tribolium* home page (16).

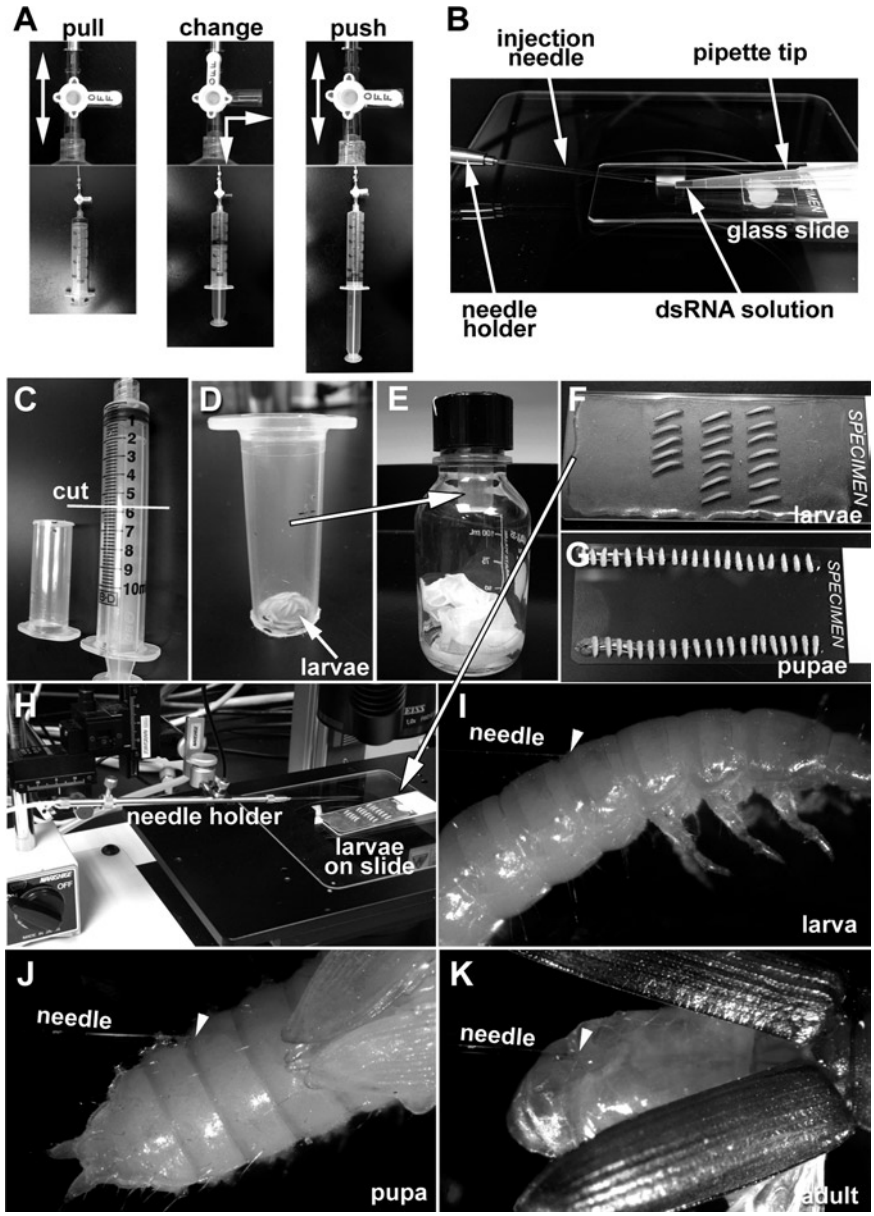


Fig. 3. Injection procedures. (**a**) Injection syringe with different stopcock positions. The air-flow direction is indicated by *arrows*. (**b**) Front loading the injection needle. (**c**–**e**) Etherization apparatus. (**f**, **g**) larvae (**f**) and pupae (**g**) on a sticky glass slide. (**h**) Injection setting. (**i**–**k**) Injection points for a larva (**i**), pupa (**j**), and adult (**k**). *Arrowheads* indicate injection points.

8. 1 M Na_2HPO_4.
9. 1 M NaH_2PO_4.
10. 0.1 M sodium phosphate buffer, pH 7.6 at 25°C: Mix 8.5 ml of 1 M Na_2HPO_4 with 1.5 ml of 1 M NaH_2PO_4 to obtain 10 ml of 0.1 M sodium phosphate buffer. Check the pH with a pH indicator strip and adjust accordingly.

11. 0.5 M KCl.
12. Food dye (green, blue, or red preferable) (Kroger).
13. 10× injection buffer (1 ml): 0.1 M sodium phosphate buffer (10 µl), 0.5 M KCl (100 µl), food dye (100 µl), and double-distilled water (ddH_2O) (790 µl).
14. 2× injection buffer (1 ml): 10× injection buffer (200 µl) and ddH_2O (800 µl). Store at 4°C.
15. Glass slide (Fisher).
16. Repositionable Glue (e.g., Aleen's TACK-IT Over&Over).
17. Plastic CD case (10 mm thick) (Amazon.com).
18. Glass capillary: O.D. 1 mm, I.D. 0.5 mm, without filament (Sutter Instrument).
19. Needle puller: P-87 or P97 Micropipette Puller (Sutter Instrument).
20. Injection needle: Use a needle puller to pull glass capillaries. "Pipette Cookbook" downloadable from the Sutter Instrument Web site (17) is an excellent reference to determine the needle pulling condition. "Adherent Cell, *C. elegans*, *Drosophila*, & Zebrafish – Recommended Programs" described in the cookbook is recommended for *Tribolium* injection. Store pulled needles in a plastic CD case. A strip of removable mounting putty can be used to hold needles in the CD case.
21. Removable mounting putty (e.g., LockTite Fun-Tak, Henkel Consumer Adhesives).
22. Compressed gas duster.
23. Forceps: INOX #1 and #5 (Fine Science Tool or ROBOZ Surgical Instrument).
24. Ethyl ether, anhydrous (Fisher).
25. Nylon mesh: 120-µm pore size/49% open area (Flystuff.com). Nylon mesh available at a fabric store can also be used.
26. Narrow-mouth 250-ml glass bottle (e.g., KIMAX).
27. Stereomicroscope: A stereomicroscope with a magnification range of 10–50× and a working distance of about 5–10 cm will suit the injection procedure (e.g., Stemi2000 or SteREO Discovery V12, Zeiss. Fig. 2a, b).
28. X-Y mechanical stage for stereomicroscopes (see Note 3).
29. Manipulator: M-152 (Fig. 2C) (Narishige).
30. Magnetic stand: GJ-1 (Narishige).
31. Glass capillary holder: IM-H1 Injection Holder Set (Narishige).
32. Injection syringe (Fig. 2d): A 30-ml disposable syringe (e.g., BD syringe) and a four-way stopcock (e.g., Stopcocks with Luer Connections; 4-way; male slip, EW-30600-03,

Cole-Parmer Instrument Co.) connected to a glass capillary holder. The stopcock allows you to change the position of the syringe plunger without applying pressure to the injection needle (see Fig. 3a for stopcock usage).

33. A fume hood for handling ether.
34. Etherization bottle (Fig. 3e): Place two pieces of tissue papers (e.g., Kimberly-Clark Wipes) in a 250-ml narrow-mouth glass bottle and pour about 70 ml of ether onto the paper.
35. Etherization basket (Fig. 3c, d): Remove the syringe plunger from a 10-ml disposable syringe and carefully cut in half along the 6 ml line. Discard the tip. Briefly heat the cut surface with a gas burner until plastic becomes soft and quickly place the heated syringe onto a piece of nylon mesh (the mesh will be glued onto the syringe). Trim off the edge with scissors to make the edge smooth and round. This "basket" will fit on a 250-ml narrow-mouth glass bottle (Fig. 3e).
36. Sticky glass slide: apply repositionable glue on a glass slide. Cover the entire slide for larval injections or make two thin strips along the longer edges for pupal and adult injection (Fig. 3f, g).

2.3. RNA Isolation and Real-Time PCR

1. RNeasy Mini Kit (QIAGEN).
2. RNase Away Spray Bottle (Molecular BioProducts/Thermo Fisher).
3. 20-gauge needle.
4. 1-ml syringe.
5. Disposable pestles for 1.5-ml microcentrifuge tubes (Fisher).
6. Liquid nitrogen.
7. Spectrophotometer, e.g., NanoDrop 2000 (Fisher Thermo Science).
8. 0.5 M EDTA: Add 93.05 g of EDTA to 400 ml of ddH_2O. Mix briefly on a stir plate, then carefully add 8 g of NaOH pellet. Mix until the solution becomes clear. Adjust pH to 8.0 with NaOH if necessary. Adjust the final volume to 500 ml with ddH_2O.
9. RNase-free recombinant DNase I (Roche).
10. Protector RNase Inhibitor (Roche).
11. iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad).
12. Optically clear PCR tubes.
13. Real-time PCR thermal cycler (e.g., iCycler, Bio-Rad).

2.4. Protein Extraction and Western Blot

2.4.1. Total Protein Extraction

1. Protein extraction buffer: 150 mM NaCl, 10 mM Tris–HCl, 0.1% (w/v) sodium deoxycholate, pH 7.2. To make 100 ml of this buffer, add 0.876 g of NaCl and 0.156 g of Tris–HCl to 100 ml of ddH_2O. Bring the solution to pH 7.2 with NaOH and add 0.1 g of sodium deoxycholate.
2. Protease Inhibitor Cocktail (e.g., Sigma # P8340).
3. Disposable pestles for 1.5-ml microcentrifuge tubes (Fisher).

2.4.2. Electrophoresis and Transfer

1. 10× TGS buffer: 10× Tris/Glycine/SDS stock solution (Bio-Rad).
2. Transfer buffer: mix 100 ml of 10× TGS, 700 ml of ddH_2O and 200 ml of methanol (see Note 5).
3. Methanol.
4. β-Mercaptoethanol, 14.2 M stock solution (Bio-Rad).
5. Laemmli Sample Buffer (Bio-Rad).
6. Protein loading buffer: Laemmli + 5% β-mercaptoethanol (95 µl Laemmli + 5 µl β-mercaptoethanol).
7. Protein standard: MagicMark XP (Invitrogen).
8. Ready Gel Tris–HCl Gel: precast 4–15% linear gradient polyacrylamide gel (Bio-Rad).
9. Hybond ECL Nitrocellulose Membrane (GE Healthcare).
10. Power supply: PowerPac 1000 (Fig. 4c) (Bio-Rad).

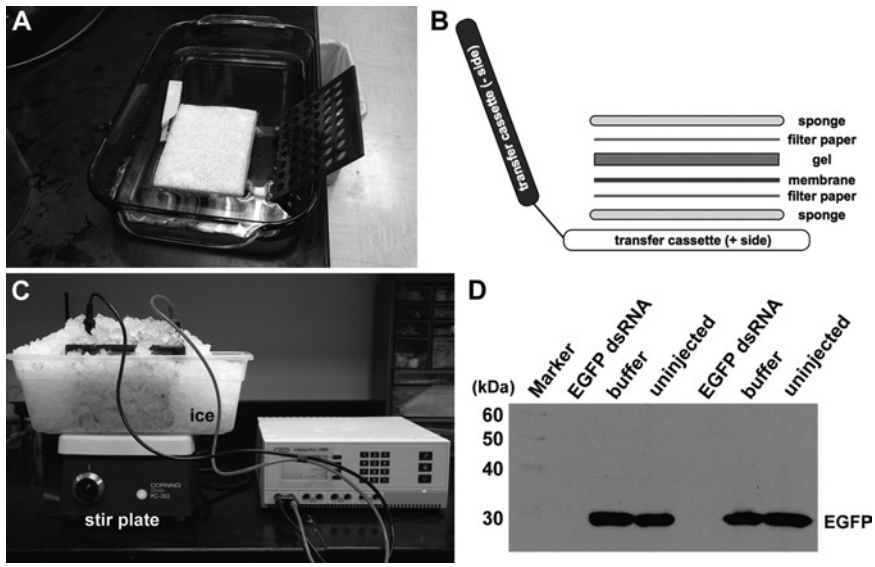


Fig. 4. Monitoring EGFP RNAi efficacy by Western blot. (**a**) A transfer cassette in a large shallow dish filled with transfer buffer. (**b**) A scheme of transfer cassette assembly. (**c**) A typical western blot setup. (**d**) Reduction of EGFP protein by EGFP RNAi shown by western blot. dsRNA for EGFP (500 ng/µl) was injected into last-instar *Pu11* larvae. Injection buffer was used for a control group. Another set of larvae was isolated for an uninjected control. Total proteins were isolated 7 days after injection from the three experimental groups. One pupa was used from each experimental group (experiments were triplicated). GFP antibody (1:500, ab6673, abcam) and HRP-conjugated Donkey anti-Goat antibody (1:50,000) were used for immunoblotting.

11. Bio-Rad Mini-PROTEAN vertical gel electrophoresis and blotting system. Other suitable systems can be substituted.
12. Gel loading tips.
13. Blot absorbent filter paper (Bio-Rad).
14. Stir plate.
15. Rocking platform.

2.4.3. Immunoblotting

1. 10× Tris-buffered saline (10× TBS): 100 mM Tris, 1 M NaCl, pH 7.5. To make 2 L of 10× TBS, add 24 g of Tris and 116 g of NaCl to 2 L of ddH_2O. Adjust the pH to 7.5 with HCl.
2. Tris-buffered saline with Tween (TBS-T): 10 mM Tris, 100 mM NaCl, pH 7.5, with 0.1% Tween20. To make 1 l of TBS-T, mix 100 ml of 10× TBS, 899 ml of ddH_2O, and 1 ml of Tween-20.
3. Nonfat dry milk (available in a grocery store).
4. Blocking buffer: 10% (w/v) nonfat dry milk in TBS-T (10 g of nonfat dry milk in 100 ml of TBS-T).
5. Antibodies buffer: 5% (w/v) nonfat dry milk in TBS-T (5 g of nonfat dry milk in 100 ml of TBS-T).
6. Primary antibodies.
7. Horseradish peroxidase (HRP)-labeled secondary antibodies.
8. Enhanced Chemiluminescence (ECL) Detection kit (GE Healthcare).
9. Blue Basic Autoradiography Film (ISC BioExpress).
10. Autoradiography film processing equipment.

3. Methods

3.1. dsRNA Synthesis

A summary of the dsRNA synthesis process is shown in Fig. 5. Initially, a gene fragment with the T7 polymerase promoter site at both ends is amplified by PCR with primers that have T7 sequence at their 5′ ends (T7 tail) (see Fig. 5a and the next section). This PCR product will be used as a template in an in vitro transcription (Fig. 5b). Sense and antisense strands of RNA are synthesized and annealed together in an in vitro transcription process. dsRNA is then purified using an RNA purification kit for subsequent injection procedures.

3.1.1. Primer Design for dsRNA Synthesis

1. Prepare gene-specific primers with the T7 sequence (taatac gactcactataggg) at their 5′ ends. About 15 mer sequence should be sufficient for the gene specific portion of the primers. These primers amplify a gene fragment with the T7 polymerase

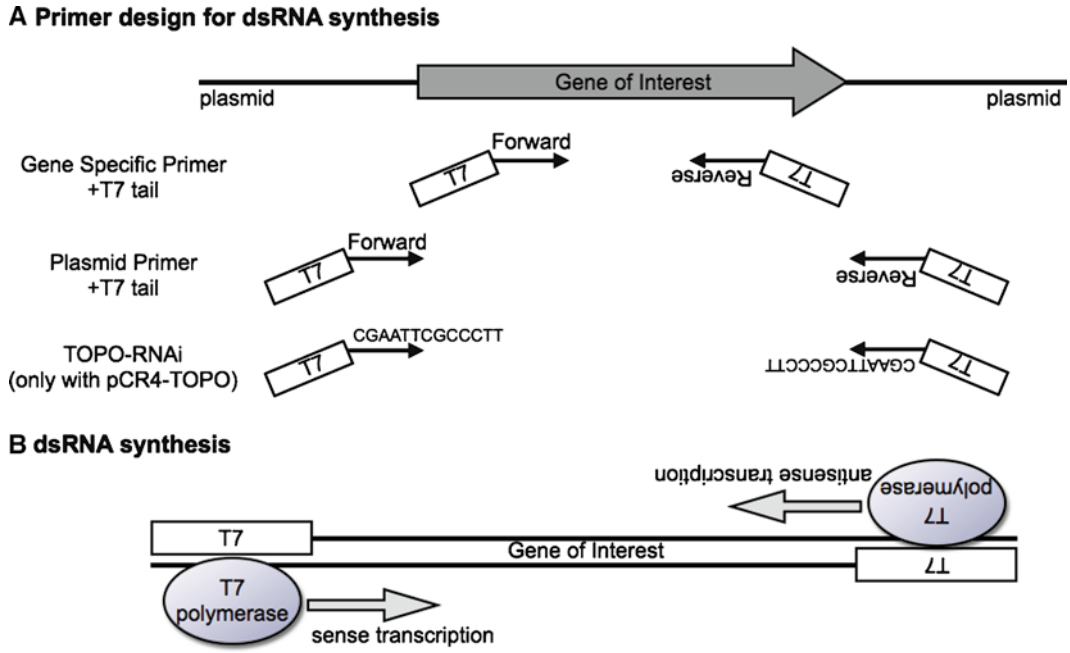


Fig. 5. An outline of dsRNA synthesis (**a**) Primer design for dsRNA synthesis, and (**b**) dsRNA synthesis scheme.

promoter site at both ends (see Note 2 for the primers used here to knock down EGFP).

5′-taatacgactcactataggg---------- -3′

T7 ~15 mer gene-specific sequence

2. Alternatively, primers designed to prime on two plasmid regions that flank the inserted gene fragment can also be used, which eliminates the process of ordering gene-specific primers with T7 tail for each gene.
3. Only one primer is necessary if TOPO TA Cloning Kit for Sequence (pCR4-TOPO vector, Invitrogen) is used. This primer is designed to prime on two pCR4-TOPO vector regions that flank the inserted gene fragment (8) (Fig. 5a).

   TOPO_RNAi_T7 primer: 5′-taatacgactcactatagggcgaattcgccctt-3′

   T7
4. The dsRNA length coincides with the gene fragment amplified with the primers described above. dsRNA shorter than 100 bp appears to have weaker effect (Tomoyasu et al., manuscript in preparation), so dsRNA longer than 100 bp is recommended. A dsRNA length of 400–1,000 bp is routinely reported in the *Tribolium* literature.

5. Off-target effect (OTE) is a major concern in RNAi experiments (18). Targeting several nonoverlapping portions of a gene in separate experiments helps evaluate whether an observed phenotype is caused by depletion of the targeted gene or by an OTE. Phenotypes caused by depleting the target should be observed regardless of the region of the mRNA targeted, while phenotypes caused by an OTE are likely to be seen only when a certain sequence within the target mRNA is targeted.

3.1.2. Template Preparation for dsRNA Synthesis

In the following procedures, the DNA template for dsRNA synthesis is synthesized by PCR. After specificity of the PCR is confirmed on an agarose gel, the DNA template is then purified using the QIAGEN QIAquick PCR purification kit.

1. Prepare a gene of interest (either a full-length or a partial fragment) cloned into a plasmid (see Note 6).
2. Adjust the plasmid concentration to 10 ng/μl using ddH_2O.
3. Set up the PCR reaction as follows:

ddH_2O	102 μl
5× GoTaq buffer with Mg^{2+}	40 μl
dNTP mix (2.5 mM stock)	16 μl (0.2 M)
primer 1 (10 M stock)*	15 μl (0.75 M)
primer 2 (10 M stock)*	15 μl (0.75 M)
plasmid DNA (10 ng/μl)	10 μl
Go Taq polymerase (5 U/μl)	2.5 μl
Total	200 μl

*Both primers can be replaced with 30 μl of TOPO-RNAi primer (10 M stock) when the template fragment is cloned into pCR4-TOPO plasmid.

4. Split the PCR reaction mix into eight tubes of 25 μl for heat transfer efficiency.
5. Run the following program.

	Denature:	94°C × 2 min
	Denature:	94°C × 30 s
35 cycles	Annealing:	57°C × 30 s
	Extension:	72°C × ___min/s*
	Extension:	72°C × 5 min
	Hold:	4°C

*depends on the length of the PCR product (60 s/1,000 bp).

6. Take 1 μl from one of the tubes and mix with 4 μl ddH_2O and 1 μl 6× DNA loading dye.

7. Run a gel to confirm the specificity of the PCR reaction (see Note 7).
8. Combine the PCR reactions into a 1.5-ml microcentrifuge tube.
9. Add 1 ml of buffer PBI (5 volumes of the sample) and mix well by vortexing.
10. Place a QIAquick spin column in a provided 2-ml collection tube.
11. Apply half of the sample (600 µl) to the QIAquick column and centrifuge for 1 min at 8,000 rpm. All the centrifugation steps are done in a microcentrifuge (the *g*-force value for 8,000 rpm and 12,000 rpm is 5,000 × *g* and 11,000 × *g*, respectively).
12. Discard the flow-through and place the QIAquick column back into the same tube.
13. Apply the other half of the sample to the QIAquick column and repeat steps 11 and 12.
14. Add 750 µl of buffer PE to the column and centrifuge for 1 min at 8,000 rpm.
15. Discard flow-through and place the QIAquick column back in the same tube.
16. Centrifuge the column for 1 min at 12,000 rpm to completely remove the residual buffer PE.
17. Place QIAquick column in a clean 1.5-ml microcentrifuge tube.
18. Add 30 µl of buffer EB to the center of the QIAquick membrane and allow it to stand for 1 min.
19. Centrifuge the column for 1 min at 12,000 rpm to collect the template DNA sample.
20. Quantitate the amount of DNA with a Spectrophotometer . A minimum of 125 ng/µl is required for the next step (see Note 8). Usually, a concentration of 300–500 ng/µl is obtained.
21. The sample can be stored at –20°C for at least several months.

3.1.3. In Vitro Transcription

The following protocol uses Ambion MEGAscript T7.

1. Prepare the reaction mix as follows:

NTP mix solution	8 µl
10× Reaction buffer	2 µl
Enzyme mix	2 µl
Template	µl (1.5 µg)
Add nuclease-free water to	20 µl

The nucleoside triphosphate solutions (ATP, CTP, GTP, and UTP) come separately in the MEGAscript T7 kit. They can be premixed (1:1:1:1) and stored at –20°C (NTP mix solution). Vortex the 10× Reaction Buffer well, as it is very viscous, and keep it at room temperature while assembling the reaction.

2. Incubate the reaction mix at 37°C for 5–6 h. Although a shorter incubation time is recommended in the MEGAscript manual, a longer incubation appears to give a higher dsRNA yield.
3. Add 1 µl of TURBO DNase I. Mix well by pipetting.
4. Incubate at 37°C for 30 min.
5. Turn on a heat block and set the temperature at 70°C for subsequent procedures.

3.1.4. dsRNA Purification

1. Bring the dsRNA sample to 100 µl with 79 µl of elution solution. Mix gently but thoroughly.
2. Add 350 µl of Binding Solution Concentrate to the sample and mix gently by pipetting.
3. Add 250 µl of 100% ethanol to the sample and mix gently by pipetting.
4. Insert the Filter Cartridge into the Collection Tube provided.
5. Pipette the sample mixture (700 µl) onto the Filter Cartridge.
6. Centrifuge for 1 min at 12,000 rpm.
7. Discard the flow-through and place the Filter Cartridge back in the same tube.
8. Apply 500 µl of Wash Solution Concentrate (make sure ethanol has been added).
9. Centrifuge for 1 min at 12,000 rpm.
10. Discard the flow-through and place the Filter Cartridge back in the same tube.
11. Repeat steps 8–10 with a second 500-µl aliquot of Wash Solution Concentrate.
12. Centrifuge again for 1 min at 12,000 rpm to make sure that the Filter Cartridge is dry.
13. dsRNA elution:
    (a) Place the Filter Cartridge in a new Collection Tube.
    (b) Apply 50 µl of Elution Solution to the center of the Filter Cartridge.
    (c) Close the cap of the Collection Tube and incubate in a heat block at 70°C for 10 min.
    (d) Recover eluted dsRNA by centrifuging for 1 min at room temperature at 12,000 rpm. Keep the collected dsRNA solution in the Collection Tube.

(e) Repeat steps b–d with another 50-µl aliquot of Elution Solution.

(f) Discard the Filter Cartridge.

14. Ethanol precipitation:

(a) Add 10 µl of 5 M Ammonium Acetate (provided) and 275 µl of 100% ethanol to the sample.

(b) Mix well by vortexing and incubate at –80°C overnight.

(c) After overnight incubation, centrifuge the sample at 12,000 rpm for 15 min at 4°C.

(d) Carefully remove the supernatant and discard it (do not disturb the dsRNA pellet).

(e) Wash the pellet by adding 500 µl of 70% cold ethanol (stored at –20°C) and gently inverting the sample tube several times (but it is not necessary to disturb the dsRNA pellet).

(f) Centrifuge at 12,000 rpm for 10 min at 4°C.

(g) Carefully remove the 70% ethanol and discard it (do not disturb the dsRNA pellet).

(h) Air-dry the pellet briefly by leaving the tube lid open for 15 min at room temperature (do not overdry the pellet, as it may be difficult to get dissolved).

15. Resuspend the pellet in 16 µl of nuclease-free water (provided in the MEGAscript T7 kit).

16. Take 0.5 µl from the purified dsRNA sample and mix with 9.5 µl of ddH_2O in a new 1.5-µl microcentrifuge tube (20× diluted sample).

17. Run 5 µl of the 20× diluted sample mixed with 1 µl of 6× DNA loading dye on gel electrophoresis. A regular agarose gel for DNA is sufficient for this step.

18. Use the rest of the 20× diluted sample for sample quantification by a Spectrophotometer. Usually, a concentration of 150–300 ng/µl (therefore, the sample concentration is 3,000–6,000 ng/µl) is obtained.

3.2. dsRNA Injection

3.2.1. dsRNA Preparation for Injection

1. Various concentrations of dsRNA can be used to achieve different degrees of gene knockdown (from close to null to very week hypomorphic) (see Supplemental Data of ref. 19 for example). 1 g/µl (final concentration after mixing with injection buffer) is a reasonable starting concentration. RNAi with dsRNA concentration of as high as 7–8 g/l, and as low as 5 pg/l have been tested, producing various degrees of phenotypes in *Tribolium* ((12, 19), and Tomoyasu Y., unpublished data).

2. Adjust the concentration of the dsRNA solution to the desired concentration (e.g., 2 g/l to obtain 1 g/l final concentration) and mix the dsRNA solution 1:1 with 2× injection buffer. Keep the sample on ice during the injection procedures.

3.2.2. Needle Preparation

1. Break the tip of an injection needle with sharp forceps #5 under a stereomicroscope to create a sharp and stiff tip.
2. Place the needle in the needle holder and place the needle holder onto the manipulator (Fig. 2c). Adjust the angle of the needle close to horizontal (Fig. 2c).
3. Adjust the position of the injection needle by changing the position of the manipulator to place the tip of the injection needle at the center of the stereomicroscope field.
4. Cut the tip of a 20-µl disposable micropipette tip using scissors.
5. Pipette 10 µl of dsRNA solution into the micropipette tip prepared above using a micropipettor. Carefully remove the tip from the micropipettor so that the dsRNA solution remains.
6. Place the micropipette tip horizontally on a glass slide using a piece of removable mounting putty (Fig. 3b).
7. Set the injection syringe to the pulling position (Fig. 3a).
8. Under a stereomicroscope, carefully insert the injection needle into the dsRNA solution in the micropipette tip (Fig. 3b).
9. Slowly pull the injection syringe plunger to load the dsRNA solution into the injection needle. Do not overload the injection needle to avoid possible contamination of the needle holder (see Note 9).
10. Control the filling by gently pushing the injection syringe plunger to neutralize the pulling pressure.
11. Carefully remove the injection needle from the micropipette tip. Raise the position of the injection needle using the *z*-axis of the manipulator to avoid any accidental contact to the injection needle.
12. Remove the glass slide with the micropipette tip from the microscope stage (see Note 10 for alternative injection settings for other organisms).

3.2.3. Beetle Preparation

1. Isolate beetles of the desired stage (such as larvae, pupae, or adults) using a #25 sieve and a collecting pan (Fig. 1c–e).
2. Place beetles in the etherization basket and then place the basket in the etherization bottle (Fig. 3d, e). Close the bottle lid and etherize beetles for about 4 min (see Note 11). Do not let beetles touch ether, as it will kill beetles. A fume hood must be used when handling ether.

3. There is no need to etherize pupae. Pupae can be laid on a sticky slide (Fig. 3g) prior to all of the above injection procedures (or even a day before).
4. Lay the etherized beetles on a sticky slide under a separate stereomicroscope using forceps #1 (Fig. 3f). This procedure should be performed as quickly as possible (preferably less than 5 min) to avoid beetles awakening during the following injection process. You may have to reduce the stickiness of the glue by briefly touching the glue several times with your finger (see Note 12 for dipteran larvae).

3.2.4. Injection

1. Place the sticky glass slide with beetles under the injection microscope (Fig. 3h).
2. Switch the position of the injection syringe to the pushing position (Fig. 3a).
3. Carefully insert the injection needle into a beetle. Injection points for a larva, pupa, and adult are shown in Fig. 3i–k; however, the injection point does not appear to significantly affect RNAi efficiency; the circulatory system in the beetle allows the dsRNA solution to quickly spread out within the entire beetle body, causing a systemic effect.
4. Gently apply pressure onto the injection syringe plunger to inject dsRNA solution into the beetle. Approximately 0.5–0.8 μl of the dsRNA solution can be injected into one last-instar larva, pupa, or adult (see Note 13).
5. Slowly remove the injection needle from the beetle. Briefly pull the plunger to counter the pushing pressure as you pull out the needle from the beetle (see Note 9).
6. Repeat steps 3–5 until all the beetles on the sticky slide are injected. Make sure to remove beetles that were unable to be injected.
7. Leave the beetles on the sticky slide until the beetles wake up.
8. For larvae and adults, carefully remove the injected beetles from the slide using forceps #1 under a stereomicroscope. Leave the beetles in a plastic Petri dish without culture flour for 15 min to let the injection wound dry and then place the beetles in a culture bottle.
9. For pupae, leave the injected pupae on the sticky slide. Flip the slide upside down and place it on culture flour in a large plastic Petri dish (100 mm diameter).
10. Culture the beetles in an incubator at 30°C with 50% humidity until the desired stage for phenotype analysis (see Note 14 regarding the survival rate).

11. For parental RNAi, let the injected pupae close to adults. Culture adults for several days for sexual maturation. Analyze phenotypes in offspring embryos.
12. Alternatively, adults can also be used for parental RNAi. Injection at the adult stage allows to bypass the RNAi effect on oogenesis, as some oocytes have already matured at the time of injection. Adult injection is ideal when knocking down the gene of interest at the pupal stage has a drastic effect on oogenesis and prevents the production of embryos.

3.3. RNA Isolation and Real-Time RT-PCR

There are several methods available to monitor RNAi efficacy. Real-time PCR is one way to validate RNAi effect by directly measuring the amount of targeted mRNA molecules. First, total RNA needs to be isolated from beetles. Then, the total RNA is used as a template for one-step reverse-transcription (RT) real-time PCR to monitor the amount of target mRNAs. The following protocol describes a total RNA isolation method as well as one of the quantification methods using real-time PCR, comparative Ct method. See Chapter 17 for additional information.

3.3.1. Primer Design for Real-Time RT-PCR

1. Two sets of primers are required for quantitative real-time PCR. The first set is designed against the gene of interest. The second set should be designed against an internal control gene (reference) that maintains a relatively uniform expression level throughout development (such as housekeeping genes). *Polyubiquitin* and ribosomal protein genes have been used as internal controls in *Tribolium* (20, 21) (see Note 4 for example primers).
2. Assuming that the injected dsRNA corresponds to a portion of the gene of interest, at least one of the primers used for real-time PCR should complement an area of the gene that is outside the dsRNA sequence. This prevents real-time PCR from inadvertently measuring the dsRNA as endogenous gene expression.
3. PCR amplicons shorter than 100 bp are preferable to minimize the effect on amplification efficiency.
4. Primer design programs exist that assist users in developing primers of high quality (e.g., Integrated DNA Technologies Web site (22)).

3.3.2. Total RNA Isolation

The following protocol uses QIAGEN RNeasy Mini Kit (see Note 15). Also, see Note 16 about RNA work in general.

1. Place individual or groups of larvae, pupae, or adults (see Note 17) in a 1.5-ml microcentrifuge tube.
2. Freeze the sample in liquid nitrogen and grind the sample thoroughly using a disposable pestle. Freeze the pestle in liquid nitrogen before use.

3. Add 350 µl of buffer RLT.
4. Homogenize the sample by passing lysate 20 times through a 20-gauge needle with a 1-ml disposable syringe. This step is essential to avoid genomic DNA contamination.
5. Centrifuge for 3 min at 12,000 rpm at room temperature to pellet debris.
6. Transfer the supernatant to a new 1.5-ml microcentrifuge tube.
7. Add 350 µl of 70% EtOH and mix well by pipetting.
8. Transfer the whole solution (~700 µl) to an RNeasy Mini Column.
9. Centrifuge for 30 s at 12,000 rpm at room temperature. Discard flow-through.
10. Add 700 µl of buffer RW1 to the RNeasy Mini Column.
11. Centrifuge for 30 s at 12,000 rpm at room temperature.
12. Transfer the RNeasy Mini Column to a new 2-ml Collection Tube (supplied). Do not allow the RNeasy Mini Column to touch the flow-through.
13. Add 500 µl of RPE to the RNeasy Mini Column.
14. Centrifuge for 30 s at 12,000 rpm at room temperature. Discard flow-through.
15. Add 500 µl of RPE to the RNeasy Mini Column.
16. Centrifuge for 2 min at 12,000 rpm at room temperature.
17. Transfer the RNeasy Mini Column to a new 1.5-ml tube (supplied).
18. Apply 30 µl of RNase-free water (supplied) directly to the column membrane.
19. Centrifuge for 1 min at 12,000 rpm at room temperature to collect the total RNA solution.
20. Measure RNA concentration in the sample with a spectrophotometer. Usually, a concentration of 200–500 ng/µl is obtained.
21. DNase treatment (optional. See Notes 15 and 18). Prepare the reaction mix as follows:

RNA solution	µl (corresponding to 3 g)
10× DNase buffer	5 µl
DNase	1 µl
RNase inhibitor	1 µl
Add Nuclease-free water to	50 µl

Incubate the reaction mix at 37°C for 20 min. Then, add 1 µl of 0.5 M EDTA and inactivate DNase by incubating the reaction mix at 75°C for 10 min.

22. Proceed to the next step or store the sample at –80°C.

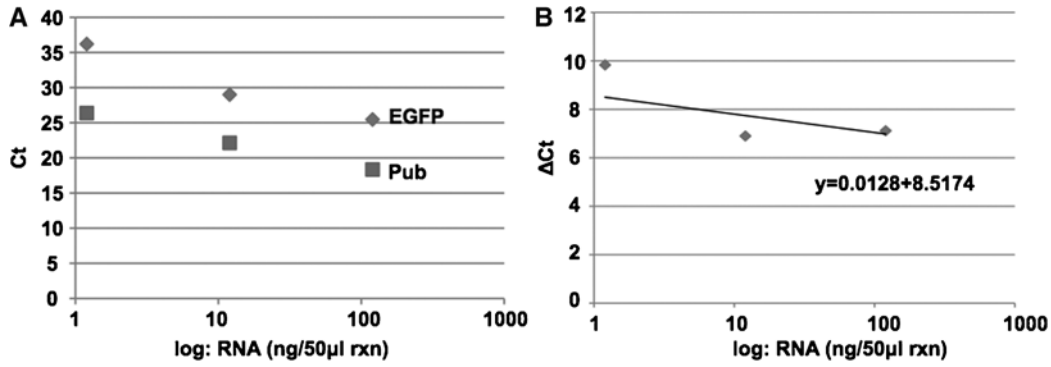
3.3.3. Real-Time RT-PCR

Real-time PCR uses a fluorescence molecule to monitor the amplification of PCR products in real time. One way to monitor the amount of PCR products is to use a DNA binding dye (DNA intercalator such as SYBR Green), which emits fluorescence upon excitation only when it binds to DNA. The intensity of the fluorescence corresponds to the amount of DNA molecules, allowing quantification of PCR products during PCR. Regular PCR primers can be used in this Intercalation method, making this method relatively easy and inexpensive. However, caution must be taken, as a DNA intercalator binds to any DNA molecules in the reaction, emitting fluorescence even when it binds to DNA amplified nonspecifically. A melt curve analysis allows one to assess the specificity of the PCR (multiple T_m may indicate the amplification of more than one kind of PCR products). An alternative to the Intercalation method is the Fluorescence probe method, in which a fluorescence probe is used to detect PCR products in a sequence specific manner. This method has an advantage over the intercalation method, as it monitors the amount of target PCR products specifically, excluding other DNA molecules amplified nonspecifically. However, it requires a specific fluorescence probe, making this method more expensive to perform.

The following protocol details the Intercalation method using SYBR Green as a reporter dye. There are several different methods to quantitate DNA or RNA using Real-time PCR. The following protocol uses the comparative Ct (Threshold Cycle) method, which is able to determine the relative amount of target RNA molecules in samples. A guide for the comparative Ct and other methods is detailed in the *User Bulletin #2: ABI PRISM 7700 Sequence Detection System* manual (23).

The comparative Ct method requires that the amplification efficiencies of the two sets of primers (for the target and reference) are relatively equal; otherwise, the results are uninterpretable. Therefore, a standard curve analysis should first be performed to validate that the two sets of primers show similar amplification efficiency.

1. Assay validation. Primer concentrations should be empirically tested to determine which concentration is used for the standard curve measurements (between 50 and 300 nM concentration is recommended).
2. The standard curves are made for both the target and reference by diluting total RNA over a range of concentrations (at minimum 100-fold) with the primer concentration determined above.



C

	average Ct for EGFP (target)	average Ct for Pub (reference)	ΔCt $Ct_{(target)}$-$Ct_{(ref)}$	ΔΔCt ΔCt-$\Delta Ct_{(ref)}$	$2^{-\Delta\Delta Ct}$	relative expression %
uninjected	31.30	18.60	31.3-18.6=12.7	12.7-12.7=0	1	100
EGFP dsRNA	34.00	17.95	34-17.95=16.05	16.05-12.7=3.35	0.098	9.8

Fig. 6. Monitoring EGFP RNAi efficacy by real-time PCR in a *Tribolium* strain that expresses EGFP (Enhanced Green Fluorescence Protein) in the eyes and the future wing-related tissues (*Pu11*) (12). dsRNA for EGFP (500 ng/µl) was injected into last-instar *Pu11* larvae. Total RNA was isolated 7 days after the injection from both injected and uninjected groups of three pupae. (**a**) Ct values for *EGFP* and *Tc-polyubiquitin* (*pub*, internal control) with three different RNA concentrations (1.2 ng, 12 ng, and 120 ng/50 µl reaction mix: rxn) plotted against the log input RNA. (**b**) The difference between Ct values of *EGFP* and *pub* PCR (ΔCt) plotted against the log input RNA. The slope of the linear regression line is 0.013, indicating that the amplification of both products is approximately equal. (**c**) ΔCt and ΔΔCt calculated from the Ct values obtained from *EGFP* and *pub* PCR using RNA isolated from uninjected or dsRNA injected beetles. The EGFP dsRNA injected group shows a 90.2% reduction in EGFP mRNA expression compared to the uninjected group.

3. The difference between the Ct of both target and reference (ΔCt) within each input amount of total RNA is plotted against the log input RNA (see Fig. 6a as an example). The slope of the line should be inferior to 0.1, which ensures the amplification of the products is approximately equal (Fig. 6b).
4. Once this is verified, the experimental samples can be tested with these primer sets.
5. *Quantification*. Run one-step real-time PCR with the total RNA isolated from the experimental samples, using the primer sets validated above. The concentration of total RNA can be determined based on the standard curve analysis above (e.g., the RNA concentration that gives a Ct value between 15 and 30). The reactions should be run in triplicate.
6. A master mix of all the components, except the input RNA, should be assembled to minimize pipetting errors.
7. Additional samples should be run with no reverse transcriptase to verify if the RNA is free of genomic DNA contamination (see Note 18).

8. An example reaction protocol for one-step real-time PCR is as follows:

cDNA synthesis	10 min at 50°C
RT inactivation	5 min at 95°C
PCR (45 cycles)	10 s at 95°C 30 s at 60°C (data collection)
Melt curve analysis	1 min at 95°C 1 min at 55°C 10 s at 55°C (80 cycles, increasing by 0.5°C each cycle)

9. The relative quantity of target, normalized to a reference, is calculated by $2^{-\Delta\Delta Ct}$ (see Note 19). The ΔCt is the difference between the Ct of the target and reference for each sample. Once this is determined, a "calibrator" group is identified (such as uninjected control), which will serve as the group to which all other measurements are compared (see Fig. 5c as an example).

3.4. Protein Extraction and Western Blot

Another way to validate RNAi efficacy is to monitor the amount of the protein products of the targeted gene by Western blot. The following protocol details a protein isolation method and western blot using *Tribolium*.

3.4.1. Total Protein Isolation

1. Place individual or groups of larvae, pupae, or adults on ice in a 1.5-ml microcentrifuge tube.
2. Homogenize samples in the protein extraction buffer with Protease Inhibitor Cocktail (1:100 dilution) with a disposable pestle. Use approximately 8–10 µl of protein extraction buffer per 1 mg of tissue. For one *Tribolium* pupa, we typically use 100 µl of protein extraction buffer.
3. Centrifuge the protein extract in a microcentrifuge for 10 min at 1,000 × *g* at 4°C.
4. Take out the supernatant, containing the protein fraction, and make aliquots of approximately 90 µl. The aliquots can be used immediately in downstream applications, including gel electrophoresis, or should be frozen at −80°C for future use.

3.4.2. Electrophoresis and Transfer

1. Prepare the 1× Electrophoresis running buffer and the transfer buffer. Store at 4°C.
2. Assemble the Bio-Rad Mini-PROTEAN gel apparatus with the lanes of the gel facing inward and lock into place. Verify that there are no leaks in the electrode assembly by placing running buffer between the gel and buffer dam (or two gels). Once

confirmed, place the electrode assembly into the buffer tank and fill with the remainder of electrophoresis running buffer.

3. Determine the total amount of protein to be added to the lane and mix 1:1 with protein loading buffer. It is important to verify that the volume of the sample and loading buffer does not exceed the capacity of the well.
4. Denature proteins by incubating the samples at 95°C for 3 min and briefly centrifuge tubes to recover sample.
5. Load samples and standards (see Note 20) into wells using gel loading tips and place the lid onto the buffer reservoir, ensuring that the correct terminals are connected.
6. Run the gel at 120 V for 5 min and then at 180 V for 45 min.
7. Equilibrate the sponges, filter paper, and nitrocellulose membrane for transfer in a large shallow dish filled with transfer buffer.
8. Once the electrophoresis is completed, remove the electrode assembly from the buffer reservoir and carefully place the gel into the transfer cassette, which should contain, in the following order, sponge, filter paper, nitrocellulose membrane, gel, filter paper, and sponge (Fig. 4a, b). It is important to remove bubbles from spaces between the different layers, as they prevent proper transfer of proteins to the nitrocellulose membrane. Place the membrane between the gel and the anode to ensure proper transfer.
9. Place the transfer cell into the buffer reservoir, which contains the remaining transfer buffer. Add a magnetic stir bar into the reservoir and place the reservoir on a stir plate, surrounded with ice (Fig. 4c). Alternatively, perform the transfer at 4°C.
10. Let the proteins transfer to the nitrocellulose membrane over 1.5–2 h at 80 V.

3.4.3. Immunoblotting

1. Remove the membrane from the transfer cassette and place it into blocking buffer overnight at 4°C, with gentle agitation on a rocking platform.
2. Dilute primary antibodies in the antibody buffer and add the antibodies solution onto the membrane in a shallow dish (see Notes 21 and 22). The dish can be placed into a larger container with a lid to prevent evaporation. Incubate for 1.5–2 h at 21°C with gentle agitation.
3. Remove the antibodies solution and wash the membrane in copious amounts of TBS-T four times for over 45 min.
4. Repeat step 2 with HRP-conjugated secondary antibodies.
5. Remove the secondary antibodies solution from the membrane and wash the membrane in TBS-T four times for over 45 min.

6. Mix the two components of the ECL kit (1:1). Place the membrane into a shallow dish and cover with the ECL reagent for 2 min.
7. Remove excess reagent and wrap the membrane with plastic wrap. Place the membrane inside a film cassette, expose to autoradiography film in a dark room, and develop using the autoradiography film processing equipment of choice (see Fig. 4d as an example).

4. Notes

1. Systemic RNAi was initially described in plants as spread of posttranscriptional gene silencing (24–26). *Caenorhabditis elegans* was the first animal in which RNAi was shown to work systemically (1, 11). The phenomenon can be subdivided into at least three distinct (but overlapping) processes: uptake of dsRNA by cells (cellular dsRNA uptake), systemic spreading of the RNAi effect (RNAi spreading), and uptake of dsRNA from the outside environment (feeding RNAi or environmental RNAi). For reviews of systemic RNAi, see (8, 9, 27–31).
2. The following primers were used to create an EGFP dsRNA template (520 bp). GFPiF2: taatacgactcactatagggcgatgccacct, GFPiR5: taatacgactcactatagggcggactgggtg (the T7 site is underlined) (8, 12).
3. An X-Y Mechanical stage for stereomicroscopes can be purchased from major microscope companies. However, an inexpensive mechanical stage (e.g., A512, MicroscopeNet.com), which is compatible with most stereomicroscopes (some modifications might be necessary), can also be used for injection (Fig. 2b).
4. The following primers were used for real-time PCR (EGFP and *Tc-polyubiquitin*). EGFPqF2: gacaaccactacctgagcac, EGFPqR2: caggaccatgtgatcgcg, PUBqF1: ggccgtactctttccgatta, PUBqR1: tgtctgagggttctacttcc.
5. 10× Tris/Glycine/SDS stock solution should be diluted down to 1× before adding methanol.
6. Uncloned PCR products can be used as an alternative source to make templates for the subsequent in vitro transcription, but this method is not recommended. A possible contamination of undesired PCR products in the PCR sample could produce dsRNA molecules that are unrelated to the targeted gene, causing unintended genes to be knocked down. However, uncloned PCR products may be ideal when a significant number of genes are required to be assayed (such as in a high-throughput screening), as it can bypass the cloning step.

7. It is critical to observe a single specific PCR band, as any nonspecific PCR products in this step will lead to the contamination of nonspecific dsRNA in the final product.
8. If the concentration is lower than 125 ng/μl, an additional PCR can be run with a regular T7 primer using the dsRNA produced as a template.
9. The needle holder should be thoroughly cleaned out if the RNA solution is sucked into the holder. Remove the needle holder from the teflon tube by carefully unscrewing the back screw. Prepare two wash bottles, one with water and the other with 100% ethanol. Place the nozzle of the water wash bottle to the backside of the needle holder, and squeeze the wash bottle to let the water run through the holder. Be careful not to suck in the liquid in the holder into the wash bottle. Repeat the same procedure with ethanol. After the needle holder is thoroughly washed, a compressed gas duster can be used to remove any residual ethanol in the holder.
10. Instead of using the injection needle (and the needle holder/injection apparatus) described in this protocol, a Hamilton syringe (e.g., Hamilton Syringe 1800 Series Gastight) or auto-nanoliter injector (e.g., Nanoject, Drummond) can be used for a bigger organism (such as bigger coleopteran larvae or cricket nymphs) (32, 33).
11. A time ranging between 3.5 and 4.5 min is recommended at a regular room temperature (~23°C). However, the optimal duration for etherization varies depending on temperature and humidity.
12. For dipteran larvae, a slide with a piece of double-sided sticky tape works better than the sticky glass slide. Larvae without etherization can be placed onto a piece of double-stick tape on a glass slide. Let the larva crawl on the tape until they stick themselves on the tape. Adding a drop of water will easily detach them from the tape after injection.
13. An approximate amount of RNA solution injected in each beetle can be estimated by dividing the total amount of the solution loaded in the injection needle by the number of beetles injected.
14. Injection procedures do not significantly affect the survival rate of *Tribolium* (survival rate with injection buffer is usually above 90%). The following possibilities should be considered if a lower survival rate is observed.
    (a) Inappropriate culture condition (such as overcrowded condition or lower humidity) might be causing lethality.
    (b) Injection buffer might be too old. Injection buffer older than 6 months should not be used.

(c) The needle tip might be too blunt or too big, making a big wound. Make sure to use a needle with a sharp tip.

(d) The sticky glass slide might be too sticky, causing damage upon removing larvae from the slide. You may have to reduce the stickiness of the glue by briefly touching the glue several times with your finger.

15. Different techniques can be used to isolate RNA from samples; however, the yield should be free of genomic DNA. Therefore, it is often advantageous to treat RNA extracts with Dnase.

16. For RNA work, always wear disposable gloves while handling samples and reagents. Avoid touching your skin or hair during the procedures. Decontaminate the bench area, pipette and tube stands with RNase Away Spray Bottle (Molecular BioProducts/Thermo Fisher). Use RNase-free, filtered pipette tips. Maintaining a separate area for RNA work with its own set of pipettes and tube stands is recommended.

17. As few as two beetles (last-instar larvae, pupae, or adults) are sufficient for total RNA isolation using QIAGEN RNeasy Mini kit.

18. The reactions without reverse transcriptase should not result in a measurable amount of product. If there is a measurable Ct, the sample is probably contaminated with genomic DNA, which is being amplified in the reaction. Therefore, all of the experimental reactions for that sample are contaminated, and the Ct values are influenced by the genomic DNA. In this case, it is advisable to treat the RNA samples with DNase.

19. For a detailed description of the theory behind $2^{-\Delta\Delta^{CT}}$, or the alternative standard curve method, see Chapter 17 or consult the Applied Biosystems User Bulletin #2 (23).

20. The MagicMark XP standard, which reacts to the secondary antibody, is only visible on the developed film. Therefore, other prestained standards can be used if visible bands are desired on the membrane.

21. Shallow dishes are required to incubate the antibody and membrane. It is desirable for the dish to be flat-bottomed and slightly larger than the membrane, to reduce the amount of antibodies needed for incubation. Depending on the size of the blot, slide or coverslip box lids can be used.

22. Primary antibodies require different dilutions to be effective in immunoblots. Therefore, one can run multiple lanes of the same protein, cut the membrane into strips and empirically determine which concentration of antibody is most effective. It is easiest to cut the membrane when the transfer is finished and the gel is still on the membrane. Small notches in the corners can be used to track gels and their proper orientation.

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