# Chapter 17

# Systemic RNA Interference in Planarians by Feeding of dsRNA Containing Bacteria

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## Abstract

RNA interference (RNAi) is currently the only method available in planaria for assessing the function of particular genes. We describe here a method for performing body-wide gene knockdown, relying on dsRNA production in bacteria and subsequent delivery to planaria by feeding a liver–bacteria mixture. This method is ideal for screening many genes in parallel, in a cost-effective and reliable manner. We also describe a ligation-independent cloning strategy, which is used to rapidly transfer single genes into an RNAi vector that is also appropriate for downstream applications such as in situ hybridizations. Together, these protocols represent useful components of the current planarian molecular tool kit.

**Key words** RNA interference (RNAi), Ligation-independent cloning, Planaria, Systemic RNAi

## 1 Introduction

The ability to inhibit expression of specific genes has facilitated functional genetic studies in animals that were previously inaccessible to this kind of analysis. Interfering with the expression of single genes has also revolutionized investigations into the molecular mechanisms responsible for planarian regeneration, allowing identification of genes involved in stem cell function [1, 2] and regulation of regeneration polarity [3–6], as well as many other processes. Combined with the ease and ever-decreasing costs of generating transcriptome data, determining the function of any gene in planaria is now within reach. Early versions of RNAi administration in planaria involved injection of dsRNA [7, 8], but to reduce the cost and throughput for screening large libraries of genes, bacterial feeding protocols were developed [9, 10]. Here, we discuss the RNAi food strategy informally referred to as "**soft-serve**" in the field, which is simply a mixture of dsRNA-containing bacteria, liver, and food coloring.

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To perform RNAi, the first step is to clone a gene fragment of interest between two T7 promoters in an appropriate vector. This plasmid is then transformed into the HT115(DE3) *E. coli* strain, which expresses T7 polymerase under the control of an IPTG-inducible promoter. Addition of IPTG to the bacterial culture stimulates production of dsRNA from the two T7 promoters. Although planarians do not find bacteria palatable, they readily eat mixtures of dsRNA containing bacteria with their preferred food (calf liver), which initiates gene-specific, body-wide knockdown. As compared to in vitro-synthesized dsRNAS, bacterially supplied RNAi produces equivalently penetrant phenotypes and efficient knockdown even though the concentration of dsRNA is unknown [11, 12]. The off-target effects of using large regions of genes have not been assessed, and the minimum sequence requirements for knockdown have not been thoroughly tested. Not every gene will produce detectable phenotypes after RNAi, and in fact the number of feedings required can vary dramatically [13]. Nonetheless, this strategy works well for large-scale screens [14, 15] and is an essential component of the current planarian tool kit.

The initial planarian RNAi feeding strategies utilized the Gateway-based L4440 vector adapted from *C. elegans* [9], which allowed mass cloning of inserts into a vector backbone (pPR-244). We have recently adopted a ligation-independent cloning protocol [16], which allows cheap and directional cloning of PCR-amplified

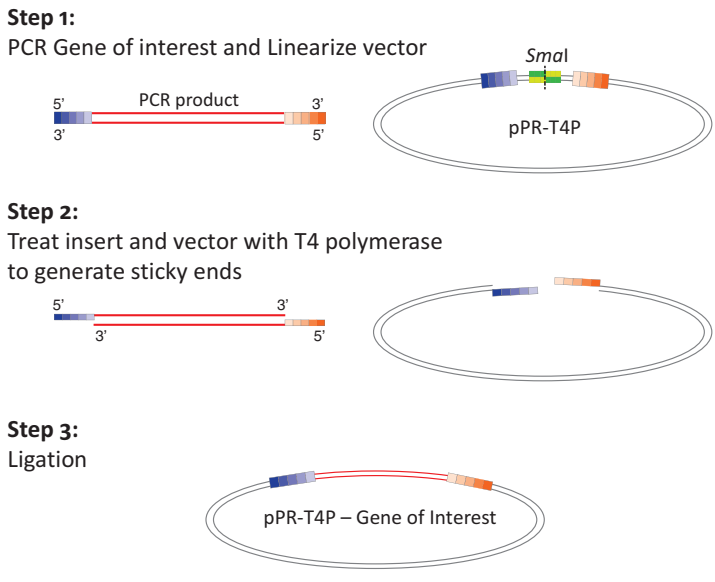


**Fig. 1** Schematic of ligation-independent cloning [1]. Gene of interest (red) is PCR-amplified with oligos containing homology to the vector. pPR-T4P vector is linearized by digestion with *Sma*I [2]. Separate treatment of vector and insert produces sticky ends after exposure to T4 polymerase 3′ ➔ 5′ exonuclease activity [3]. Upon mixing, insert and vector rapidly ligate to produce a plasmid containing the gene of interest

inserts into a slightly modified version of the original vector (now called pPR-T4P). This cloning strategy relies on the 3′ ➔ 5′ exonuclease activity of T4 polymerase, which chews back one strand of DNA, revealing single-stranded DNA. Incubation with an excess of a single nucleotide halts the chewback at the first encounter of this nucleotide, limiting the length of the sticky end. PCR products and linearized vector with complementary sticky ends can then be rapidly and efficiently annealed and transformed without the need for ligation (Fig. 1) (*insert* Fig. 1 *somewhere around here*). This cloning strategy allows systematic and straightforward cloning of genes into the RNAi vector, facilitating large RNAi screens.

## 2 Materials

### 2.1 Molecular Biology

1. Gene-specific oligonucleotides with additional 12-nucleotide sequence complementary to pPR-T4P vector. Forward primer, append 5′—CATTACCATCCCG-NNN…—3′; Reverse primer, append 5′—CCAATTCTACCCG-NNN…—3′. (-NNN = gene-specific primer sequence).
2. cDNA generated from total RNA and produced using a reverse transcriptase (e.g., SuperScript III).
3. PCR polymerase of choice (e.g., Phusion High-Fidelity DNA polymerase).
4. PCR machine.
5. DNA electrophoresis equipment.
6. PCR purification or gel-purification columns.
7. pPR-T4P vector (kanamycin-resistant).
8. *Sma*I restriction enzyme and suitable digestion buffer.
9. 50 mM dCTP and dGTP solutions.
10. NEB 10× Buffer 2 (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$,1 mM DTT, pH 7.9).
11. 10× BSA (2 mg/mL in $ddH_2O$).
12. T4 DNA polymerase.
13. Molecular-biology grade water.
14. Chemically competent *E. coli* (XL1-Blue, DH5α, etc.).
15. LB broth: Dissolve 5 g Bacto yeast extract, 10 g Bacto tryptone, 10 g NaCl in 950 mL $ddH_2O$. Adjust pH to 7.0 with 10 N NaOH, then bring volume up to 1 L with $ddH_2O$. Autoclave for 20 min.
16. LB-agar plates: 5 g Bacto yeast extract, 10 g Bacto tryptone, 10 g NaCl, and 15 g Bacto agar in 950 mL water in a 2 L flask. Adjust pH to 7.0 with 10 N NaOH, then bring volume up to 1 L with $ddH_2O$. Autoclave for 20 min. Put flask onto stir

plate and stir until temperature is reduced to ~50 °C. Add 1 mL of 50 mg/mL kanamycin (or kanamycin plus tetracycline to 12.5 μg/mL final concentration), and mix. Pour into petri dishes.

17. HT115(DE3) bacterial strain (tetracycline-resistant, lacking RNAseIII), made competent.

### 2.2 RNAi Food Production

1. Gene of interest cloned into an appropriate bacterial vector with flanking T7 promoter sites (e.g., pPR-T4P or pPR-244) and transformed into HT115(DE3) *E. coli*
2. 2XYT broth: To make 2XYT, dissolve 16 g Bacto tryptone, 10 g Bacto yeast extract, and 5 g NaCl into 1 L $dH_2O$. Transfer to bottle and autoclave for 20 min.
3. Flasks or aerated culture tubes.
4. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution (1 M): Dissolve 2.38 g IPTG in 9 mL $ddH_2O$. Adjust volume to 10 mL and filter-sterilize with 0.22 μm filter. Aliquot (e.g., 1 mL each) and store at −20 °C.
5. Bacterial shaker, 37 °C.
6. Spectrophotometer for measuring $OD_{600}$.
7. Centrifuge and centrifuge bottles.
8. Liver paste, passed through fine filters and stored at −80 °C [*see* Chapter 5].
9. Red food coloring.
10. Tubes for storage of RNAi food aliquots: Eppendorf tubes, 8-strip PCR tubes, or 96-well PCR plates (for screening).

### 2.3 RNAi Feeding

1. Petri dishes (35 mm, 60 mm, 10-cm) or small Ziploc containers.
2. RNAi food, thawed.
3. Planaria (5–7 days starved).

## 3 Methods

### 3.1 Cloning Genes of Interest

1. PCR amplify genes of interest from planarian cDNA, using primer pairs with T4P-overhangs (*see* **Note 1**).
2. Column-purify or gel-purify PCR product (*see* **Note 2**).
3. To linearize the pPR-T4P vector, digest plasmid with *Sma*I overnight at room temperature. Gel-purify and store at −20 °C.
4. Treat vector and insert with T4 polymerase.
   - 1 μL 10× buffer (e.g., NEB 2).
   - 1 μL 20× BSA.

- 0.5 μL 50 mM dCTP (for inserts) or dGTP (for vector) (*see* **Note 3**).
- 0.5 μL T4 DNA polymerase.
- 5 μL purified PCR-product (or linearized vector).
- 2 μL water.

5. Always perform a control ligation in parallel, substituting water for DNA.
6. Incubate in PCR machine, 30 min at 22 °C for digestion, and 20 min at 75 °C to inactivate T4 polymerase.
7. Mix 2 μL of treated insert with 1 μL of treated vector, mix and spin quickly.
8. Incubate for 10–60 min at room temperature to allow annealing.
9. Mix entire reaction with 30 μL competent cells, incubate on ice for 25 min, then heat shock at 42 °C for 40 s. Add 250 μL LB broth and incubate at 37 °C for 45–60 min in shaking incubator. Plate onto LB/kanamycin plates and incubate overnight at 37 °C.
10. Transformations with inserts should cause significantly more colony formation than controls. Screen for positive colonies by PCR with flanking primers, or by direct sequencing of purified plasmids (*see* **Note 4**).
11. Retransform purified plasmids containing correct clones into HT115(DE3) competent cells, plate onto LB plates containing kanamycin (50 μg/mL) and tetracycline (12.5 μg/mL) (*see* **Note 5**) and make glycerol stocks for permanent storage (*see* **Note 6**).

### *3.2 Preparation of RNAi Food*

1. Estimate the volume of RNAi food and thus bacterial culture volume needed for the specific purposes of the experiment (*see* **Note 7**).
2. The night before the food prep, inoculate HT115 bacteria containing the gene of interest into an appropriately sized culture tube with 2XYT broth. Start from a single colony on a bacterial plate or inoculate directly from glycerol stock.
3. Grow overnight in bacterial shaker at 37 °C, shaking at 200–250 rpm.
4. The next morning, dilute bacterial culture 1:10 into larger volume of fresh 2XYT.
5. Grow approximately 1 h with shaking.
6. Check $OD_{600}$ in spectrophotometer after blanking with autoclaved 2XYT.

7. When culture has reached OD ~0.6–0.8 (*see* **Note 8**), add IPTG to 1 mM final concentration, and return to bacterial shaker for 2 h (*see* **Note 9**).
8. During induction, thaw an aliquot of liver (*see* **Note 10**). Once it is thawed, transfer with a spatula to a 15 mL conical tube. Spin briefly to collect at the bottom of the tube and estimate volume of liver based on gradations on the tube. Add ~10% volume $ddH_2O$, and roughly 1:30 dilution of red food coloring (*see* **Note 11**). Mix well with a spatula until the paste is homogeneous, spin briefly and store on ice until induction is complete.
9. Spin cultures at 5000 × *g* for 15 min. If cultures were spun in large bottles, resuspend in $ddH_2O$ and transfer to 50 mL conical tube for another spin at 5000 × *g*, 15 min.
10. Pour off all media without disturbing the pellet, and then invert 50 mL conical tubes on paper towels for a few minutes. Extra drops of media can be removed with a vacuum aspirator (*see* **Note 12**).
11. Trim narrow ends off of pipet tips to widen the bore for pipetting viscous liver paste. Use these tips to add liver paste to each aliquot. Add 1/400th of the initial culture volume in liver paste. In other words, if the initial volume is 50 mL, add 125 μL of the red liver paste (*see* **Note 13**). To dispense the appropriate volume of liver paste, coat the inside of the tip with liver by slowly drawing up the plunger button, then dispensing once, and drawing up the plunger again (*see* **Note 14**).
12. Mix until homogeneous with a pipet tip, either by stirring or pipetting up and down. Avoid introducing bubbles.
13. Aliquot into appropriately sized aliquots (estimate 1 μL of RNAi food per worm) and create sufficient aliquots for the duration of the experiment.
14. Store at −80 °C for up to 6 months.

### 3.3 RNAi Feedings

1. Select 7-day starved, size-matched worms (*see* **Note 15**) and transfer into an appropriately sized dish (e.g., 10–12 animals/35 mm dish, 30 animals/60 mm dish, etc.) with 4–5 mm of planarian water.
2. Place worms on a solid surface in a quiet corner of the lab (planarians respond sensitively to vibrations) and cover worms with an opaque box for 2 h. Removal of the box and the ensuing switch to daylight just before food addition increases the reliability of feeding throughout the cohort of animals (*see* **Note 16**).
3. Thaw aliquots of RNAi food and spin to remove air bubbles.

4. Using a pipet tip with the narrow end trimmed off, transfer RNAi food into the dish. Make sure that the pipet tip is submerged in the water so that when the RNAi food exits the tip it remains cohesive and does not disperse on the surface of the water.
5. Allow worms to eat for at least 1 h or until all animals have moved off the food droplet again. Worms that have eaten will be visibly red from the ingested food coloring (often 100%). Some experiments may require the removal of worms that have not eaten to ensure uniform RNAi delivery within the experimental cohort.
6. Remove excess food and rinse dish 1–2 times until no more liver pieces are visible. The day after, exchange water.
7. Repeat every 3 days for 3–4 feedings total (*see* **Note 17**). Prior to each feeding, transfer worms to a new dish.
8. Score for visible phenotype, or test knockdown efficiency by in situ hybridization or qPCR.

## 4 Notes

1. For RNAi experiments, we typically clone fragments of the coding sequence of unique genes and have obtained strong knockdown phenotypes with insert sizes between 300 bp and 3 kb. We generally recommend cloning the longest possible gene fragment up to an upper limit of 3 kb because larger insert sizes may be inhibitory to bacterial growth.
2. Insert concentration is not critical. We have successfully cloned bands that were barely visible on agarose gels.
3. Large batches of T4P-digested vector can be prepared in advance and stored at −20 °C indefinitely, preferably in aliquots to avoid excessive freeze/thaw cycles. Simply scale up the vector digestion reaction accordingly and confirm that the digest batch produces no background. The final concentration of the vector should be ~15–20 ng/μL.
4. Colony screening by PCR is a rapid way to screen for the presence of inserts but we always verify the insert by direct sequencing as well. We typically expect 80–90% of the colonies to contain inserts. For colony PCR we use the following primers:

   M13-Forward ACGTTGTAAAACGACGGCCAGT

   M13-Reverse CAGGAAACAGCTATGACCATG

   For sequencing, we use the following primers:

   CA100 TCGATGAATTCGAGCTCCACC

   PR244F GGCCCCAAGGGGTTATGTGG

5. The HT115(DE3) bacterial strain contains a transposon insertion that disrupts the double-strand-specific RNaseIII of *E. coli*, therefore increasing stability of dsRNA. Because this transposon also has a tetracycline resistance gene, selection with tetracycline is required to maintain RNaseIII deficiency.
6. These plasmids can also be used for riboprobe synthesis. Because of the directional insertion, antisense riboprobes are always synthesized with T7 polymerase after PCR with AA18 and PR244F primers.

   AA18 CCACCGGTTCCATGGCTAGC
7. Estimate the number of worms and feedings that will be required for your experiment: Budget roughly 1 μL of RNAi food per worm per feeding. For example, a good initial experiment to test whether a given gene causes a phenotype would be four RNAi feedings of 30 worms, thus requiring a total RNAi food volume of 120 μL in four 30 μL aliquots. To calculate the required bacterial culture volume, use the following formula:

   Desired RNAi food volume (mL) × 400 ~ required bacterial culture volume (mL). In case of our example, this would amount to 0.12 mL × 400 ~ 48 mL. Hence, you might want to budget 50 mL initial bacterial culture, resuspension of the bacterial pellet in 125 μL of liver paste (50 mL/400) and subsequent aliquoting into four aliquots of ~30 μL each.

   Generating a 50 mL bacterial culture by 1:10 dilution of a starting culture will require inoculation of a 5 mL overnight starter culture the evening before the food preparation. For screening large numbers of genes we typically grow 1.8 mL overnight cultures in 96-well square-well plates and grow 48 cultures/day in 30 mL volume (1:20 dilution) in 50 mL bioreactor tubes.
8. Every bacterial strain grows at a distinct rate, which can complicate matters when growing multiple cultures in parallel. Induction density and the following 2-h expression time interval should not be varied, but thereafter, you can store "fast" cultures on ice while waiting for slower ones to catch up.
9. Induction timing has been optimized based on phenotypic outcome rather than absolute dsRNA levels.
10. Thaw sufficient volume for the batch of RNAi food to be made. For example, a 50 mL culture will require 125 μL of liver; multiply this by the number of cultures that are growing. Overestimate the volume necessary to avoid running out—liver paste is viscous, and it is difficult to pipet exact volumes.
11. Food coloring is a helpful addition and allows the easy identification of animals that have indeed ingested the RNAi food.

12. This is a potential stopping point. Pellets can be stored at −80 °C in air-tight containers, and liver paste can be added on another day.
13. Increasing the ratio of bacteria to liver *decreases* palatability of RNAi food to planarians, reducing the amount of food ingested. The concentrated food as described in this protocol is difficult to make due to viscosity, but produces robust and reliable phenotypes.
14. Coating the inside of the tip improves accuracy in liver paste volume. Without it, first-stroke dispensing results in too little liver transfer, making the RNAi food nonpalatable.
15. With each successive feeding, animals increase in size. Therefore, we typically start with smaller animals (e.g., 3 mm). Size can be optimized according to the final experiments to be performed.
16. For the first feeding, this "sleeping" period is typically unnecessary, as the worms are sufficiently hungry after the 7-day starvation period. Feedings are best performed on an isolated bench that is free of vibrations or disturbances.
17. Every gene requires a different number of feedings to produce a phenotype, so this must be determined empirically. As extreme examples, *Smedwi-2* knockdown effects are obvious after two feedings; *Smed-ptc* requires 12 feedings. Sexual animals often require additional feedings to reveal phenotypes. Feedings are typically performed 2× per week, with 2–3 days in between feedings. Feeding every second day is the maximal rate of RNAi delivery, since the animals simply stop eating when fed daily. Moving the animals to clean dishes the evening before an RNAi feed is critical for efficient feeding, since the animals will eat poorly or not at all when situated in dirty dishes.

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