

## Protocol 4: RNA interference (RNAi)

RNAi reveals gene function due to the ability of the protocol to destroy the mRNA of the gene of interest resulting in a phenotype. The most challenging aspect of this experiment is the gene cloning and, for the lower level classes, the food preparation.

### 4.1. Gene cloning

As for the WISH protocol, the first steps of the RNAi protocol in planarian are: look for mRNA species-specific sequences that correspond to the genes of interest and design F and R primers (Table 6) as described in Section 3.1, produce the cDNA and clone the gene of interest as described in Section 3.2, transform the bacteria and sequence the insert as described in Section 3.3.

#### REQUIRED MATERIALS 4.1:

Laptop and Internet connection	Phusion DNA polymerase, together with
TRizol <sup>®</sup>	5x High Fidelity buffer, dNTPs mix and
Plastic tubes (15, 1.5 and 0.2 ml) and pestels	MgCl <sub>2</sub>
Chloroform, Isopropanol and Ethanol	Agarose and 1x TAE buffer
Milli-Q water	Electrophoresis chamber
NanoDrop 1000 Spectrophotometer	Loading dye and 1 kb DNA ladder
SuperScript III kit (Oligo(dT) <sub>20</sub> primer, dNTPs Mix, 5x First strand buffer, DTT, RNase Inhibitor and SuperScript III retro transcriptase)	UV transilluminator
Plastic tubes (0.2 ml)	QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (blade and thermoblock)
Thermocycler	Plasmid PR-T4P
Primers F and R (Table 6)	Smal restriction enzyme and 10x NEB buffer #2
	T4 polymerase, its buffer and BSA

GTP and CTP nucleotides	LB-Kan (50 µg/ml) plate and LB-Kan (50 µg/ml) broth
Competent <i>Escherichia coli</i> DH5α and ice	Optional step: Taq DNA polymerase, Taq buffer
Water bath or thermoblock	Primers AA18 and PR244
Luria Broth (LB)	QIAprep® Spin Miniprep Kit
Incubator at 37°C and shaker	

#### 4.2. Food preparation

Fresh calf liver paste is prepared by trimming and homogenizing it in a blender. The liver should be kept on ice during the whole process to preserve it. The steps for the RNAi food preparation are:

1. Remove the capsule (clear outer tissue layer) from the liver (decapsulation) by cutting the surface of the liver with a blade and moving the finger between the capsule and the liver.
2. Cut the liver into big pieces and from these remove all connective tissue and blood vessels by gripping one end and scraping the surface of the liver with a blade.
3. Transfer the liver to a blender or food processor and mix until the liver appears creamy and homogeneous.
4. The blended liver should be filtered as a final step to remove any remaining bits of vasculature. Either a food mill or sieve placed above a container on ice may be used as a filter. The usage of a sieve will require a wooden spoon to force the liver paste through the sieve.
5. Transfer the liver to a Small Petri dish (35 x 10 mm, 5 ml) or make small portion of food wrapped in the plastic transparent wrap.

6. Store at -20°C or -80°C, respectively for 3 or 6 months.

#### **REQUIRED MATERIALS 4.2:**

Calf liver

Ice and containers

Blade and blender

Food mill or sieve

Spoons

Small Petri dish (35 x 10 mm, 5 ml) or plastic wrap

#### **4.3. RNAi food with bacteria**

The first RNAi method involves inducing bacteria transformed with the construct to transcribe the insert into the plasmid to generate double-stranded RNA (dsRNA) and then mixing these bacteria with the liver paste. When the planarians eat the food, planarians cells take up the dsRNA and it activate the pathway that degrades the mRNA with sequence complementary to one of its strands. This method works for *Girardia sp.* and *D. dorocephala*, but *P. morgani* and *P. gracilis* do not eat food mixed with bacteria and the protocol reported in the Section 4.4 is necessary.

Thanks to the strong phenotype showed by the RNAi of the two selected genes (*β-catenin-1* and *ODF2*), 3 RNAi feedings are enough. Following this protocol, 250 ul of RNAi food (3-4 feedings for 30 to 50 worms) can be prepared from 50 ml transformed bacteria culture.

Keeping the construct (3 µl) on ice, 48 µl of competent *Escherichia coli* HT115 strain was added to it. The mix was incubated for 10 min on ice, and then a heat shock was

performed for 35 sec at 42°C after which the tube was moved back into the ice. After 5 min, the transformed *E. coli* were placed at RT and 1 ml of Luria Broth (LB) without antibiotics were added. The cells were placed on a shaker at 250 rpm for 1 h at 37°C to recover and, finally, all the bacteria culture is moved into 4 ml of 2x YT medium-Kan (50 µg/ml)-Tet (12.5 µg/ml) broth culture.

Note: The glycerol stock is a mixture of 50% bacteria in broth culture and 50% glycerol that can be stored at -80°C for long time and used as starter for a new bacteria culture, when required. If a glycerol stock of bacteria HT115 already transformed with the construct of interest is available, few bacteria can be picked up from here and added to a 5 ml of 2x YT medium-Kan (50 µg/ml)-Tet (12.5 µg/ml).

1. Incubate the bacteria culture overnight (ON) at 37°C shaking at 250 rpm.
2. Preheat 45 ml of fresh 2x YT medium for each ON culture at 37 °C.
3. Add 50 µg/ml Kan and 12.5 µg/ml Tet to the warm 2x YT medium and place 45 ml of this in 50 ml centrifuge tube.
4. Transfer 1:50 ON culture into the 45 ml 2x YT medium-Kan (50 µg/ml)-Tet (12.5 µg/ml) broth.
5. Incubate the culture at 37°C shaking at 250 rpm.
6. After 1 h, check the OD600 value by spectrophotometer.
7. When the OD600 measures 0.6-1, add 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce dsRNA synthesis in the bacteria.
8. Incubate the bacteria culture for 2 h at 37 °C shaking at 250 rpm.
9. If one culture finishes with induction before other, place on ice until all are done.
10. Thaw liver paste.

11. Spin the bacteria culture at 3000 xg for 10 min.
  12. Mix together: liver paste, 10 % planarian water and 1:50 red food dye.
  13. Eliminate the bacterial media and place the tubes upside down on paper towel.
  14. Add 50 ml of Milli-Q water and dissolve the pellet, to remove residual salts that can be unappetizing for planarians.
  15. Spin the bacteria culture at 3000 xg for 10 min.
  16. Eliminate the water and place the tubes upside down on paper towel.
  17. Add 150 µl of liver mixture (liver paste/water/red dye) in each 50 ml tube.
- Note: Cut the end of the tip for pipetting the liver.
18. Mix well the liver mixture with the bacteria.
  19. Aliquot the food into 1.5 ml tubes and store it at -20°C or -80°C.

Note: 1 ul of food/feeding/worm is usually required.

### **REQUIRED MATERIALS 4.3:**

Option 1: Construct (Section 4.1), competent *Escherichia coli* HT115, water bath or thermoblock, Luria Broth (LB)

Option 2: Glycerol stock of transformed *Escherichia coli* HT115

2x YT medium-Kan (50 µg/ml)-Tet (12.5 µg/ml) broth

Incubator at 37°C and shaker

Plastic tube (1.5 and 50 ml)

Spectrophotometer

Isopropyl β-D-1-thiogalactopyranoside (IPTG)

Calf liver paste (Section 4.2) and Red food dye

Centrifuge and its adaptor for 50 ml tubes

Instant Ocean<sup>®</sup> Sea Salt or 1x Montjuic water

Paper towel, Milli-Q water and ice

#### 4.4. RNAi food with dsRNA

The second method to induce RNAi is to mix the liver paste with purified dsRNA generated *in vitro* from the construct of interest. When the planarians eat the food, planarians cells take up the dsRNA and it activate the pathway that degrades the mRNA with sequence complementary to one of its strands. This method is best for *P. morgani* and *P. gracilis*, which do not eat food mixed with bacteria.

Once the gene of interest was successfully cloned, the insert was amplified by PCR reaction, using the construct as template, the primer T7 (5'-TAATACGACTCACTATAGGG-3'), the enzyme Phusion DNA polymerase and its GC buffer (New England Biolabs), following the manufacturer's instructions. To obtain the required amount of amplified insert sequence, 240 ul (4 reaction/60 µl total each) of PCR reaction are prepared with 100 ng of template each reaction. The following touchdown PCR program was used:

- 1 cycle: 30 sec at 98°C
- 40 cycles: 10 sec at 98°C, 20 sec at 56°C, 90 sec at 72°C
- 1 cycle: 10 min at 72°C.

A small aliquot of the amplified DNA (1-2 µl) will be run in 1% agarose gel in 1x TAE to check if the reaction worked properly. The DNA contained in the residual aliquot (237-238 µl) is purified with the QIAquick PCR Purification Kit.

The purified DNA was the template for the Transcription Reaction to produce the dsRNA. The reaction was done mixing 2.5-3 µg of the amplified and purified DNA fragment with the transcription buffer, the ribonucleotide mix (ATP, CTP, GTP and

UTP), the T7 RNA polymerase (Invitrogen) and the RNase Inhibitor (Promega) in Milli-Q water to a final volume of 200  $\mu$ l. Incubate the reaction ON at 37°C.

The dsRNA was treated with 5  $\mu$ l RQ1 DNase (Promega). The reaction was incubated for 20 min at RT.

The dsRNA was precipitated following the protocol:

1. Add 1 volume of 5 M Ammonia acetate and 2.5 volumes of 100% Ethanol.
2. Incubate for 30 min -20°C.
3. Spin for 15 min at 4°C at max speed.
4. Remove the supernatant.
5. Wash with 70% Ethanol.
6. Spin for 15 min at 4 °C at max speed.
7. Remove the supernatant and dry the pellet for 5 min at RT.
8. Resuspend the pellet in 75  $\mu$ l Milli-Q water.

A small aliquot of the synthesized dsRNA (2  $\mu$ l) will be run in 0.8% agarose gel in TAE to check if the reaction worked properly. It could happen that the band is not well defined or exactly at the expected size because of the different migration property of the dsRNA compared to the DNA and the RNA. The concentration of the dsRNA should be about 3-4  $\mu$ g/  $\mu$ l following the NanoDrop 1000 Spectrophotometer output. The dsRNA is stored at -80°C.

The day of the feeding, the dsRNA and one aliquot of liver paste are thawed. The dsRNA is mixed with the red food dye and then they are added to the liver paste and mixed together with the proportion liver:red food dye:dsRNA equal to 30:3:5.

#### **REQUIRED MATERIALS 4.4:**

Construct (Section 4.1)	T7 RNA polymerase together with its transcription buffer, NTPs mix (ATP, CTP, GTP and UTP), RNase Inhibitor
Phusion DNA polymerase, together with GC buffer, primer T7, dNTPs mix and MgCl <sub>2</sub>	Milli-Q water
Thermocycler and plastic tube (0.2 and 1.5 ml)	Thermoblock
Agarose and 1x TAE buffer	RQ1 DNase
Electrophoresis chamber	5 M Ammonia acetate
Loading dye and 1 kb DNA ladder	70% and 100% Ethanol
UV transilluminator	NanoDrop 1000 Spectrophotometer
QIAquick PCR Purification Kit	Calf liver paste (Section 4.2) and Red food dye

#### **4.5. RNAi feedings**

The suggested genes for the RNAi protocol have a really strong phenotype that appears after 3 feedings. For each experiment, 3-5 control worms and 3-5 RNAi worms, all of them 4-7 mm long and starved for at least 7-10 days were used.

1. Move the worms in the Petri dishes (1 dish for the control and 1 dish for each treatment) with fresh planarian water.
2. The next day, place the dishes on a surface that is not disturbed.
3. Leave the animals for 30-60 min in the dark.
4. Thaw the food 30 min before the worms are ready for the feeding. The food is liver mixed with the bacteria for *Girardia sp.* and *D. dorotocephala* or liver paste and dsRNA for *P. morgani* and *P. gracilis*.

Note: The food for *Girardia sp.* and *D. dorotocephala* is ready to use; the food for *P. morgani* and *P. gracilis* has to be prepared fresh, mixing the liver paste, the red food dye, and the dsRNA with the proportion 30:3:5.

5. Cut the end of the 200  $\mu$ l pipet tip and use it for adding 1-3  $\mu$ l of food for each worm in the Petri dish.
6. Let the worms eat for 1-2 h.
7. Remove the uneaten food and replace the water with fresh planarian water.
8. Put the worms back where they are usually maintained.
9. The day after the feeding, leave the worms undisturbed.
10. The second day after the feeding, move the worms in a new Petri dish and change the water as described in Section 1.2.
11. The third day after the first feeding, the second feeding will be provided to the worms.
12. Continue with the same schedule until the third feeding.
13. Three days after the last feeding cut the worms in 3 parts (head, trunk and tail fragment) following the protocol described in Section 2.1
14. Check daily the worms to observe the emerging phenotype in the regenerating fragment.

Note: the phenotype for both  *$\beta$ -catenin-1* and *odf-2* gene emerge also in the worms that are not amputated but it takes more time and the efficiency is lower. If the researcher is interested in discovering the function of the gene of interest in the homeostasis of the worms they should not cut the worms and start the observation from the day after the last feeding. If the researcher is interested in discovering the function of the gene of interest in the regeneration of the worms they have to cut them and compare the fragment regeneration process between control and interfered worms.

#### REQUIRED MATERIALS 4.5:

Plastic Pasteur pipettes

Petri dishes

Instant Ocean<sup>®</sup> Sea Salt or 1x Montjuïc water

Option 1: Calf liver paste mixed with bacteria (Section 4.2 and 4.3)

Option 2: Calf liver paste (Section 4.2), Red food dye and dsRNA (Section 4.4)

Squeeze bottle or standard bottle

Waste container

Paper towels or lab wiper KimWipes™

Optional for the amputation: Ethanol, Blade

**Table 6:** Primer for RNAi protocol

Planarian species	Gene Name	Primer Sequence F	Primer Sequence R	Sequence length
<i>Girardia sp.</i>	<i>beta-catenin</i>	TAGTATGTCAAGGCACAACG	GTATGGCCGATGTTAGTCTG	2749
<i>D. dorotocephala</i>	<i>beta-catenin</i>	TTGGAAAGTCACAGTCACAG	GTGTCTAACATGCACACGTC	2669
<i>P. morgani</i>	<i>beta-catenin</i>	GCCTATCGAACATAAGCATC	GTAGTCAATCGAAGGTCGAG	3049
<i>P. morgani</i>	<i>odf2</i>	TCAATTGCTCTAGCTGCTTC	AAAGGATCCAAGTCCACATT	1003
<i>P. gracilis</i>	<i>beta-catenin</i>	TGGATTCAGAAGACCAGAAC	CGATCATGTGTGTTTGCTAC	2613