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Gene Knockdown in Planarians Using RNA Interference

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INTRODUCTION

This protocol describes how to produce gene knockdown in planarians using RNA interference (RNAi). It is a standard technique to evaluate gene function during regeneration and tissue maintenance in planarians. The procedure involves microinjecting double-stranded RNA (dsRNA) synthesized in vitro. Depending on the gene target, this technique can produce robust phenotypes that can be further evaluated by diverse macroscopic or microscopic procedures.

RELATED INFORMATION

This procedure was described by Sánchez Alvarado and Newmark (1999). For an introduction to planarians as a model system, see **Planarians: A Versatile and Powerful Model System for Molecular Studies of Regeneration, Adult Stem Cell Regulation, Aging, and Behavior** (Oviedo et al. 2008a). Protocols for **Establishing and Maintaining a Colony of Planarians** (Oviedo et al. 2008b) and **Live Imaging of Planarian Membrane Potential Using DiBAC$_4$(3)** (Oviedo et al. 2008c) are also available.

METHOD

RNA Synthesis

1. Assemble two transcription reactions, one with T3 polymerase and another with T7 polymerase, in separate microcentrifuge tubes. To each tube, add the following:

DNA	1 µg
DTT	10 mM
Ribonucleotides	1%

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RNA transcription buffer	20%
RNasin	60 units
T3 or T7 polymerase	17 units
H_2O, nuclease-free	to 20 µL

Alternatively, if the multiple cloning site of the vector that has the clone inserted is flanked with the same promoter (e.g., double T7 vector), reactions can be performed with T7 RNA polymerase only, but in separate tubes after the clone is linearized by digestion with appropriate DNA restriction enzymes.

2. Incubate the reactions in a water bath for 2 h at 37°C.
3. Treat each reaction with DNase I by adding 1 unit to each tube.
4. Incubate the reactions in a water bath for 15 min at 37°C.
5. Transfer 1 µL from each reaction to fresh microcentrifuge tubes. Keep the tubes at −20°C.
6. Combine the remaining 19 µL of each reaction into one tube.
7. Add 360 µL of solution A for RNAi to the tube from Step 6. Leave it for 10 min at room temperature.
8. Add 200 µL of phenol:chloroform mix, and vortex vigorously.
9. Microcentrifuge the tube at room temperature at 14,000 rpm for 2 min.
10. Transfer the aqueous phase to a fresh microcentrifuge tube.
11. Add 200 µL of chloroform, and vortex vigorously.
12. Microcentrifuge at 14,000 rpm for 2 min at room temperature.
13. Transfer the aqueous phase to a fresh microcentrifuge tube.
14. Incubate in a water bath for 10 min at 68°C to denature the RNA.
15. Incubate in a water bath for 30 min at 37°C to reanneal the RNA.
16. Add 1 mL of cold 100% ethanol.
17. Microcentrifuge at 14,000 rpm for 15 min at 4°C.
18. Discard the supernatant.
19. Add 1 mL of cold 80% ethanol.
20. Microcentrifuge at 14,000 rpm for 10 min at 4°C.
21. Discard the supernatant.
22. Resuspend the pellet in 10 µL of nuclease-free H_2O. Keep the sample on ice.
23. Confirm single-stranded RNA (ssRNA) transcription and dsRNA formation by separating 1 µL of each ssRNA sample from Step 5 and 0.5 µL of dsRNA from Step 22 on a 1% agarose gel under nondenaturing conditions (see Fig. 1 for an example).

See Troubleshooting.

Microinjection of dsRNA

24. Prepare microinjection needles. Use a micropipette puller to form an elongate end. Then, while watching through a dissecting microscope, break the tip of the needle to a diameter that will allow exit of liquid.

Usually, eliminating 10%−25% of the tip of the needle is enough.

Important: Do not make the opening of the tip too wide because it will be difficult to prevent the liquid from coming out of the animal during the injection.

25. Fill the needle with mineral oil.

Important: Do not allow air bubbles inside.

26. Attach the microinjector to the base of the dissecting microscope, and adjust it to a position that allows you to visualize the tip of the needle during the microinjection procedure. In addition, at this point, it is convenient to adjust the controlling box of the microinjector to dispense 32 nL per pulse.

27. Load the needle onto the microinjector, and proceed to aspirate 1–2 μL of dsRNA from Step 22.

28. Watching through the microscope, place the worm on top of cold wet tissues.

You may place ice underneath the tissues to keep the worm moist and cold.

29. Carefully introduce the needle into the worm. Use enough pressure to make sure the tip of the needle will be inside the worm.

Different anatomical areas can be targeted, but usually, microinjections around the prepharyngeal area produce better results. Remember, planarians are flat, and if the animal moves, it is easy to stick the needle out the other end.

30. Press the injection key, which will automatically dispense 32 nL. This should be repeated several times (three to five).

Usually this amount (i.e., 32 × 3 nL) of liquid fills up the gastrovascular system of the worm, which confirms that dsRNA is getting into the worm.

See Troubleshooting.

31. Transfer the injected worm to a Petri dish with fresh planarian water at room temperature.

32. To increase the strength of the phenotype, repeat injection procedures (Steps 24–31) several times (e.g., consecutive days or weeks).

See Troubleshooting.

TROUBLESHOOTING

Problem: ssRNA and/or dsRNA are not visualized on the gel.

[Step 23]

Solution: Consider the following:

1. Confirm that all reagents are good quality (i.e., not expired and have undergone few freeze-thaw cycles).
2. Check that the DNA template included the T3 and T7 polymerase promoters.
3. Make sure to work with clean equipment and surfaces (e.g., previously wiped with RNase and DNase decontaminants).

Problem: No liquid comes out of the needle during injection.

[Step 30]

Solution: Consider the following:

1. Make sure that no air bubbles are in the needle.
2. Confirm that the needle is properly attached to the microinjector.

Problem: You are not sure if the injected liquid is getting inside the animal.

[Step 30]

Solution: This requires practice. Sometimes, adding a few microliters of food coloring to the injection solution helps to ascertain the effectiveness of the microinjection.

Problem: Animals fail to display any phenotype.

[Step 32]

Solution: Keep in mind that not all genes produce observable phenotypes when tested by RNAi. Consider the following:

1. Check whether the procedure is effectively reducing the expression of the target gene by performing in situ hybridization using probes to the target gene.
2. For more sensitive evaluations, perform RT-PCR or quantitative RT-PCR (qRT-PCR).
3. Sometimes it is possible to elicit a phenotype by adjusting the injection schedule (shorter or longer periods of time) or by simultaneously targeting two genes that may compensate for each other’s activity.

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MATERIALS

CAUTIONS AND RECIPES:

Please see Appendices for appropriate handling of materials marked with **<!>**, and recipes for reagents marked with **<R>**.

Reagents

Agarose gel (1%)

<!>Chloroform

DNA (linearized or PCR-amplified and flanked with T3 and T7 promoters)

DNase I

<!>DTT (dithiothreitol)

Ethanol (100% and 80% in H_2O, cold)

H_2O (nuclease-free)

<!>Phenol:chloroform (50:50)

Planarian water (see **Establishing and Maintaining a Colony of Planarians** [Oviedo et al. 2008b])

Planarians of any species

Polymerases T3 and T7

Reagents for agarose gel electrophoresis

Ribonucleotides

<R>RNA transcription buffer

RNasin

<R>Solution A for RNAi

Equipment

Capillaries (glass, 3.5-in. micropipettes) (Drummond Scientific)

Equipment for agarose gel electrophoresis

Gel unit (horizontal)

Ice

Microcentrifuges (at room temperature and 4°C)

Microcentrifuge tubes

Microinjector (Nanoject; Drummond Scientific)

Micropipette puller

Microscope (dissecting)

Mineral oil

Petri dishes

Tissue paper (cold and wet)

Tubes (microcentrifuge, nuclease-free)

Tweezers

Vortex

Water baths (at 37°C and 68°C)

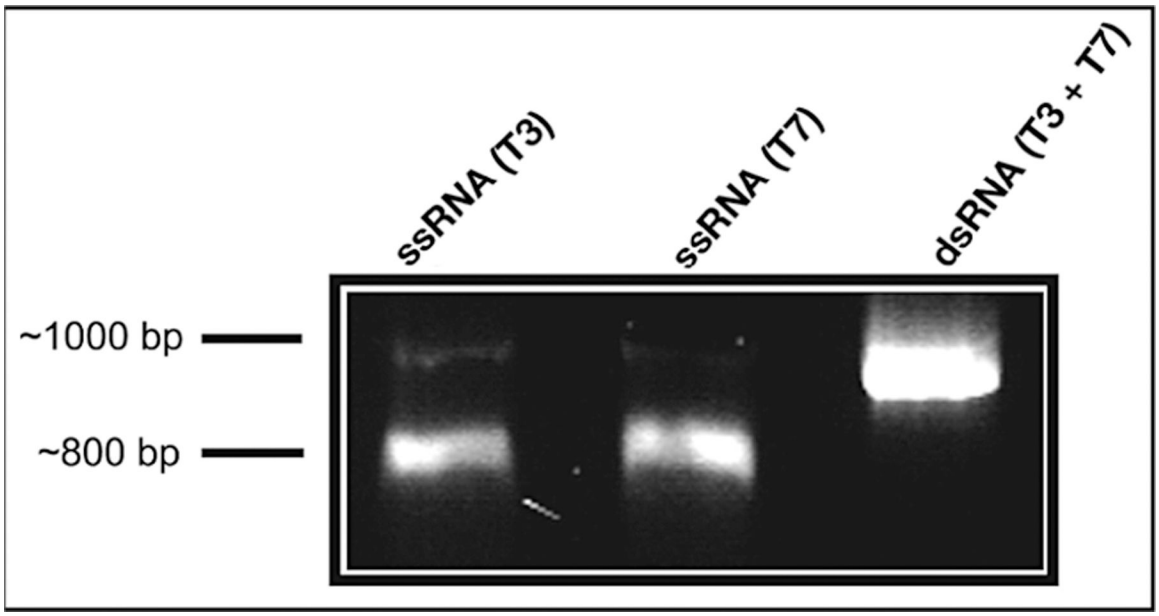

FIGURE 1.

Confirmation of dsRNA formation. Electrophoretic migration patterns of ssRNA and dsRNA in an agarose gel under nondenaturing conditions. Similar amounts of ssRNA (1 μL) were loaded for each transcription reaction (T3 or T7), whereas for dsRNA, only 0.5 μL was loaded. Notice that dsRNA migration is shifted upward compared to ssRNA, confirming hybridization of the T3 and T7 ssRNA molecules. Notice that the approximate size of the bands (*left*) depends on the size of the DNA template used.