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Larval RNAi in *Nasonia* (Parasitoid Wasp)

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INTRODUCTION

This protocol describes a method to use RNA interference (RNAi) to knock down genes in *Nasonia* larvae. Unlike *Drosophila*, RNAi in *Nasonia* is systemic. Lynch and Desplan (2006) have injected double stranded RNA (dsRNA) in female pupae and successfully knocked down genes in offspring embryos. By injecting dsRNA against the eye color gene *cinnabar* in last-instar *Nasonia* larvae, we have successfully produced adult red-eye-color phenotypes.

MATERIALS

REAGENTS

Taq Polymerase

5' and 3' Gene-specific primers with attached T7 polymerase binding sites at the 5' ends.

5' and 3' *cinnabar* primers with T7 sites:

T7_RNAi_cn_F1:

TAATACGACTCACTATAGGGAGACCACCGGTGAAGGTGAGGTCGTCG

T7_RNAi_cn_F2:

TAATACGACTCACTATAGGGAGACCACCTCGCATCGGGGTAGGGAAG

Megascript T7 RNAi Kit (Ambion cat. no. 1626)

2% Agar in 1xPBS Plates

Capillary Tubes: OD 1.0mm, ID 0.75mm, Length 100mm (World Precision Instruments cat. no. TW100F-4)

Food Coloring, preferably red or blue (McCormick brand)

Microloader Pipet Tips (Eppendorf cat. no. 5242 956.003)

Gelatin Capsules such as Coni-Snap #4 Clear/Clear (Medisca INC. cat. no. NDC 38779-1125-9)

Nasonia to inject (such as wildtype genome strain AsymCX)

Nasonia with known mutant phenotype (such as bl13, or 123)

Fly hosts (*Sarcophaga bullata*, etc.)

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EQUIPMENT

Micro-injection Pump (e.g. PicoSpritzer III from Parker Instrumentation)

Needle Puller (e.g. Flaming Brown Micropipette Puller Model no. P.80/PC, Sutter Instrument co.)

Small paintbrushes

PROCEDURE

A. MAKE dsRNA AND NEEDLES

1. Prepare control and experimental dsRNA according to the protocol in the MEGAscript RNAi Kit. The PCR approach to making dsRNA templates is sufficient with some PCR condition optimization. Run 3 to 5 100µl reactions, pool them and ethanol precipitate the products to get a high enough concentration of the dsRNA template. Do the final elution using the elution buffer supplied with the kit. This buffer alone has no adverse effects on the larva. It is best to elute with the smallest recommended volume as it takes only a very small amount to do many injections and having a higher concentration of dsRNA can be beneficial. dsRNA against *cinnabar* can be used as a positive control.
2. Prepare pulled capillary needles using a needle pulling machine. The best needles will come to a very fine point (only visible under magnification) without having “wispy” ends. The same program in different machines (even of the same model) will produce variable needles, so the protocol will have to be optimized. We used the above listed capillary tubes with a ramp value of 395 (see machine manual).

B. PREPARE LARVAE

1. Host *Nasonia* females to produce larvae for injection. To standardize larval body size, females can be hosted individually on two hosts for 24h-48h and then rehosted. Virgin females will produce all-male sex ratios, while mated females will produce approximately 95% female and 5% male offspring.
2. Collect 6-7 day old larvae raised at 25°C. At this stage larvae should be finished feeding, detached from the host, and their gut material should appear to be gray and condensed. These are referred to as fourth instar pre-defecation or gray-gut larvae. Injections can be done as much as three days earlier or 1-2 days later than this, into the pupal stage. In either case the mortality may go up. When injecting fourth instar pre-defecation larvae the survival rate can be as high as 70%.
3. Carefully open the host and spread larvae onto a 2% agar in 1X PBS plate using a small paint brush.
4. Transfer larvae of the proper stage to fresh plates, dividing into experimental and control groups. On each plate organize the larvae into a line or other pattern.

Injection—

1. Mix 4.5uL dsRNA (experimental, *cn* positive control, elution buffer negative control) with 0.5uL food coloring.

2. Backload injection mix into a needle using a microloader pipette tip, creating as few air bubbles as possible.
3. Mount the filled needle in a micro-injection pump.
4. Hold the needle at a 30°-45° angle with respect to the posterior end of the larva. Gently pin the larva down along its length with the tip of the needle facing the posterior end. The posterior end of *Nasonia* larvae is more pointed than the anterior end (Fig. xx).
5. With the top half of the larvae depressed, slide the needle forward just beneath the cuticle, and inject. Be careful not to puncture the gut. (Fig. xx).
6. If using gray-gut larvae (day 7) or later, these will have stopped feeding and can be left to develop on the PBS plate. Collect pupae at day 11 and transfer to glass vials if desired.

Procedure for injecting earlier larval stages—

Injected larvae of earlier stages must be returned to parasitized hosts to feed. Foster larvae will continue to eat the host and reduce the risk of injected larvae drowning. To avoid ambiguous results, set up foster hosts with females marked by known mutants that differ from the expected results of RNAi knockdown. We used *bl13,or123* which have blue bodies and orange eyes (readily distinguished from the scarlet eyes of the *cn* knockdown). Desiccated or rotted foster hosts are the main causes of larval loss after injection. To prevent this, use sterile techniques when handling foster hosts and be very careful not to puncture the fly carcass.

1. Set up foster hosts with mutant-marked females at the same time that the strain to be injected is set up (*PREPARE LARVAE*, #1, above).
2. Remove the anterior end of the fly puparium using a heavy gauge needle or sharp forceps to crack around the edges, being careful not to puncture the fly within.
3. Before placing the injected larvae into the foster host (5-10 per foster host), remove approximately the same number of the foster larvae.
4. Re-seal the puparium with an empty gelatin capsule, creating a proper seal. Size four capsules are appropriate for large *Sarcophaga*. Create a proper seal over the open foster host.
5. Place the capped pupa in a vial and allow it to develop at 25°C. Mature *Nasonia* (day 14-15) should make their way out of the capsule but when scoring the progeny be sure to open the capsule and check that all of the eclosed *Nasonia* have escaped.

TROUBLESHOOTING

Problem: Injected wasps do not pupate.

Solution: dsRNA treatment or injection stress may have killed the larva or induced diapause. Compare survivorship of treatment and buffer-injected controls. Diapause larvae will continue to wiggle. Diapause can be broken by refrigeration for >8 weeks.

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References

1. Lynch JA, Desplan C. A method for parental RNA interference in the wasp *Nasonia vitripennis*. *Nature Protocols* 1:486–494.

Figure 1. RNAi injection in *Nasonia* larvae. (A) Fourth instar predefecation larvae have a slightly contracted gut with gray contents. The posterior end is more tapered than the anterior. (B) dsRNA marked with food coloring is injected posterior of the gut.

Figure 2.

Phenotype of adult wasps from RNAi of *cinnabar*. Adult wasps on the left were treated as third instar larvae by injection with dsRNA from the eye color gene *cinnabar*; those on the right received control injections. A red-eye phenotype results from *cinnabar* knockdown, whereas controls show the wild-type brown eye phenotype.