**RNAi by *E. coli* Feeding Protocol**

**RNA interference in C. elegans by *E. coli* feeding**

**PROTOCOL:**  This protocol allows the user to knock down the expression of a given gene target in *C. elegans*. *E. coli* containing a specific expression vector can be induced to transcribe a genetic fragment from both the 5’ and 3’ ends. When the resulting double-stranded RNA molecule is ingested by *C. elegans*, an RNA interference reaction targets endogenous transcripts of the same sequence for destruction. In this protocol, the method of testing embryonic lethality (% hatching) is described but observations can also be made in the parent as well. The effects of the RNAi are then observed with smFISH.

**STEPS:**

**\*\*\* streak out RNAi fresh for every experiment \*\*\***

Day 1: Grow one 25cm plate of worms per strain of interest on op50. Prepare RNAi induction plates by making NGM, waiting until solution is cool enough to touch, and adding a final concentration of 1mM IPTG and 50mg/L carbenicillin. Store 4°C until use to prevent degradation of IPTG and carb.

Days 2-4: Seed RNAi plates for strains of interest. 3 RNAi plates/strain of interest. **Only make one small plate for pop-1 (+) control. You will not be doing an embryo prep of this strain, it is only to confirm that the RNAi worked.**

* Culture 10X the volume of *E. coli* you intend to plate using sterile Erlenmeyer flasks for the overnight culture. After growing for ~16hrs spin down at 5000 rpm for 5 min to concentrate. Resuspend in one tenth the volume of M9 and plate directly onto NGM+IPTG+Carb plates. Let dry and induce 1-2 days at room temp.

Day 5: Embryo prep and synchronize worms in prep for RNAi by E. coli feeding. 24 hours in M9 rocking at room temperature. **(DMP plates embryos directly on RNAi rather than synchronizing).**

Day 6: plate L1 worms and let mature for ~24-28 hrs until L4 stage **(you can plate synchronized L1s directly on to RNAi plates if you aren’t expecting a sterility phenotype).**

Day 7: move L4 worms to RNAi acclimation plates for 24-48hrs

Day 9: check for RNAi phenotypes/perform assays

* If *E. coli* is running low at any point, wash worms off plates in M9 and replate on the same strain. **DO NOT LET THE WORMS GET HUNGRY.** RNAi seems to lose efficacy fairly quickly when it’s no longer present.

**KEY CONTROLS:**

* Positive control:  *pop-1* RNAi is a standard positive control that should produce 100% embryonic lethality.
* Negative control: Empty vector (L4440) is a common negative control that should result in near 0 % embryonic lethality.
* Question specific inquiries: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**MEDIA AND SOLUTIONS FOR RNAi BY E. coli FEEDING:**

**NGM Plates (500 ml) –makes about 12 100mm plates**

* Mix the following reagents:

**1.5 g NaCl**

**1.25 g Bacto Peptone**

**8.5 g Bacto Agar**

**Add water to 487.5 ml**

* Autoclave media on 40 minute liquid cycle to sterilize
* Cool media to 50 – 65 C.
* Using sterile technique, add:

**0.5 ml 5 mg/ml Cholesterol**

**0.5 ml 1 M CaCl2**

**0.5 ml 1M MgSO4**

**12.5 ml 1 M Potassium Phosphate Solution (pH 6)**

**0.5 ml 1M nyastatin antifungal solution**

* To make NGM+IPTG+CARB plates, add:

0.5 ml 1M IPTG

0.5 ml 50mg/ml Carbenicillin

* Once all ingredients have been added, mix media thoroughly and then pour into 6 cm plates.
* **Store IPTG/Carb RNAi plates in fridge after they have solidified**

**Carbenicillin Stock Solution (50 mg /ml)**

**0,5 g Carbenicillin**

**10 ml H2O**

* Mix thoroughly in a 15 ml conical vial.
* Filter sterile the solution through a 0.22 um filter.
* Freeze in 1 ml aliquots.

**1 M IPTG**

**2.38 g Isopropyl-β-D-thiogalactopyranoside (IPTG)**

**10 ml H2O**

* Mix thoroughly in a 15 ml conical vial.
* Filter sterilize through a 0.22 um pore filter.
* Freeze 1 ml aliqiuots.

**DAY 1:**

Worm Growth & Embryo Harvest

* Grow **\_\_\_\_\_\_\_\_\_\_\_\_** worms on OP50 plates at a relatively low density until they are gravid.
* Autoclave sufficient 200mL Erlenmeyer flasks for the number of RNAi strains you will be looking at. **Remember that you can just use a culture tube for the pop-1 control.**

**DAYS 2-4:**

Prepare plates for RNAi

* Inoculate \_\_\_\_ ml LB (50 mg/L Carb) liquid cultures with each *E. coli* strain including controls**. For the pop-1 control just grow up a 5ml overnight culture to seed small/medium plate with the 500ul resuspended culture.**
* Grow overnight at 37 C, shaking.
* If 10 cm NGM RNAi plates have not been prepared in advance, pour those plates now.
* Organize 10 cm RNAi plates by labeling them. ~2-3 plates per condition. **Only one small/medium plate for pop-1 (+) control. You will not be doing an embryo prep on this strain. It is only to confirm that RNAi worked.**
* Spin down overnight cultures at 5000 rpm for 5min. Pour out supernatant and resuspend in M9 so that the culture is 10X concentrated.
* Plate ~1 ml of each culture onto a 10 cm RNAi plate.
* Let plates dry overnight (or until dry) at room temp.
* use immediately in step 3 or store in 4°C cold room.

**DAY 5:**

* Harvest worms by washing them off of the plates with M9. Collect in a 15 ml conical tube.
* Spin down at 2000 x g 1 min to pellet.
* Remove supernatant using a pipette or an aspirator.
* Refill conical with M9.
* Repeat wash until supernatant is clear. (*usually 1-2 washes in M9*)
* Spin down worms at 2000 x g, 2 min for the final wash.
* Fill conical with bleaching solution and nutate or hand shake for 7-8 minutes until embryos are released from the mothers.
* Spin down worms quickly at 2000 x g, 1 min.
* Immediately remove supernatant and quench bleaching with M9. Worms should not be in bleach solution for more than 10 minutes.
* Wash with M9 2 – 3 times, depending on the amount of embryos. *(You can typically just dump the supernatant. The embryos tend to stay in a pellet.)*
* After the last wash, remove supernatant, and refill with M9.
* **Starve out in M9 overnight to synchronize to L1. Nutate at room temp. (DMP just plates the embryos on RNAi here and skips all steps from day 6 and 7. It is important to keep a good supply of *E. coli* on the plates so if they start running low on food, the plates are washed and the worms moved to new plates with the same RNAi condition.)**

**DAY 6:**

* Spin down staged worms 2kxg, 1 min, remove sup up to a mL or so
* Plate L1 worms on op50 plates in the morning and wait about 18-24hrs hours until worms are at L3/L4 stage. **Don’t plate too many worms/plate to avoid starving them. Drop worms onto the plate or else they will form little starve clusters even though there is plenty of E. coli on the plate. You can also Plate L1s directly onto RNAi plates if not expecting a sterility phenotype.**

**DAY 7:**

* Once the worms have reached L3 stage, wash worms 2 times in M9
* Move worms onto \_\_\_\_ RNAi plates/strain seeded with strains of interest for 48 hours.

**DAY 9:**

* Check for embryonic lethality phenotype in pop-1 control (little to no embryos should have hatched).
* Proceed with follow-up experiments.

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