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Title: Identification of EGFR and RAS inhibitors using *Caenorhabditis elegans*

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No, all walking distance**

Current Protocol Length

Number of Steps: 26
Number of Shots: 54

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **(Leora) Thuy Nhu L Truong** : This protocol can be used in the initial phase of drug discovery to identify inhibitors that abolish the activities of EGFR and RAS proteins.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **(Leora) Thuy Nhu L Truong**: The main advantage of this assay is that inhibitory effects of anti-EGFR and anti-RAS therapeutics can be easily visualized in *C. elegans*.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Preparation of a Synchronous *C. elegans* Culture

2.1. Begin by spotting 100 microliters of overnight grown *E. coli* OP50 onto the center of each NGM plate **[1]**. Allow the plates to dry for 24 hours in a laminar hood **[2]**, then store them in a polystyrene container **[3]**.

2.1.1. WIDE: Establishing shot of talent adding E. coli to a plate.

2.1.2. Talent leaving the plates in the laminar hood to dry.

2.1.3. Talent storing the plates in a polystyrene container.

2.2. Using a sterile worm pick, gather 10 to 12 gravid adult worms from a previously grown plate **[1]** and transfer them to a fresh NGM plate seeded with *E. coli* OP50 **[2]**. Incubate the plate for 24 hours at 20 degrees Celsius **[3]**.

2.2.1. Talent picking worms. Videographer NOTE: 2.2.1 and 2.2.2 shot together.

2.2.2. Talent adding worms to the fresh plate.

2.2.3. Talent putting the plate in an incubator and closing the door.

2.3. After 24 hours, use a sterile worm-pick to remove adult worms from the plates **[1]**. Incubate the plates at 20 degrees Celsius for approximately 3 days to allow the embryos to develop into gravid adult worms **[2]**.

2.3.1. Talent removing adult worms from the plate.

2.3.2. Talent putting the plate in the incubator.

2.4. Collect gravid adult worms into a 15-milliliter conical tube by washing 2 to 4 plates with M9W **[1]**. Pellet the worms by centrifuging the tube at 450 x *g* for 1 minute **[2]**, then decant the supernatant without disturbing the worm pellet **[3]**.

2.4.1. Talent collecting the worms into a tube.

2.4.2. Talent putting the tube in the centrifuge and closing the lid.

2.4.3. Talent decanting the supernatant.

2.5. Prepare worm lysis solution by combining 400 microliters of 8.25% sodium hypochlorite and 100 microliters of 5 normal sodium hydroxide **[1]**. Add this solution to the worm pellet and flick the tube to mix **[2]**.

2.5.1. Talent preparing the lysis solution, with the sodium hypochlorite and sodium hydroxide containers in the shot.

2.5.2. Talent adding the solution to the tube and flicking it.

2.6. Observe the lysis of the worms under a dissecting microscope to prevent overbleaching of the embryos **[1]**. *Videographer: This step is difficult!*

2.6.1. Talent observing the worms under the microscope.

2.7. When 70% of the adult worms have lysed, add 10 milliliters of M9W to the conical tube to dilute the lysis mix **[1]**. Centrifuge the tube at 450 x *g* for 1 minute **[2]**, then replace the supernatant with 10 milliliters of M9W **[3-TXT]**. *Videographer: This step is difficult!*

2.7.1. Talent adding M9W to the tube.

2.7.2. Talent putting the tube in the centrifuge.

2.7.3. Talent replacing the supernatant with M9W. **TEXT: Repeat wash 2 X**

2.8. After completing the washing steps, add 3 to 5 milliliters of M9W to resuspend the egg pellet **[1]**. Rotate the tube overnight at a speed of 18 rpm on a tube rotator at room temperature **[2]**.

2.8.1. Talent adding M9W to the egg pellet.

2.8.2. Talent putting the tubes on a rotator and starting rotation.

2.9. After overnight incubation, remove the tube from the rotator **[1]** and pellet the L1 larvae by centrifuging the tube at 450 x *g* for 1 minute **[2]**. Aspirate the M9W until 250 microliters is left in the tube **[3]**.

2.9.1. Talent removing the tube from the rotator.

2.9.2. Talent putting the tube in the centrifuge and closing the lid.

2.9.3. Talent aspirating the M9W.

2.10. Shake the tube to resuspend the larvae **[1]** and add three 5-microliter drops of larvae onto a Petri dish lid **[2]**. Using a dissecting microscope, count the number of worms in each drop and determine the number of worms in 1 microliter **[3]**.

2.10.1. Talent shaking the tube.

2.10.2. Talent adding drops of larvae to a Petri dish lid.

2.10.3. Talent at the microscope counting worms.

3. Preparation of Drug Assays

3.1. Grow 30 milliliters of *E. coli* OP50 in a 50-milliliter conical tube at 37 degrees Celsius overnight in an orbital shaker at 150 rpm **[1]**.

3.1.1. E. coli in the shaker.

3.2. On the next day, spin the *E. coli* OP50 culture at 4,000 x *g* for 10 minutes to pellet the cells **[1]**, then remove the supernatant **[2]** and resuspend the pellet in 3 milliliters of M9W to concentrate the culture **[3]**.

3.2.1. Talent putting the bacteria in the centrifuge and closing the lid.

3.2.2. Talent removing supernatant.

3.2.3. Talent resuspending the pellet.

3.3. Prior to preparing the working solutions for the drug assay, add 0.1 milliliter of cholesterol into 100 milliliters of M9W **[1]**.

3.3.1. Talent adding cholesterol to the M9W.

3.4. Prepare working solutions of each experimental drug by diluting the drug and vehicle in 4.8 milliliters of M9W supplemented with cholesterol **[1]**. Dissolve DMSO in 4.8 milliliters of M9W supplemented with cholesterol to prepare the vehicle control **[2]**. *Videographer: This step is important!*

3.4.1. Talent diluting the drug in the M9W.

3.4.2. Talent dissolving DMSO in the M9W, with the DMSO container in the shot.

3.5. Add 200 microliters of concentrated *E. coli* OP50 culture to each tube containing the vehicle control or drugs **[1]** and vortex the tubes to mix **[2]**. *Videographer: This step is important!*

3.5.1. Talent adding E. coli to the tube.

3.5.2. Talent vortexing the tubes.

3.6. Add 2 milliliters of each working drug solution or vehicle control to each well in a 12-well tissue culture plate. Test each drug concentration and vehicle control in duplicate **[1]**. *Videographer: This step is important!*

3.6.1. Talent adding drug solution or vehicle to a few wells in the plate.

3.7. Add approximately 100 L1 larvae per well using a sterile micropipette, limiting the volume to 10 microliters **[1]**. Incubate the plates at 20 degrees Celsius **[2]**. *Videographer: This step is important!*

3.7.1. Talent adding larva to a few wells.

3.7.2. Talent putting the plate in the incubator and closing the door.

3.8. Supplement wells with 50 microliters of 10x concentrated *E. coli* OP50 on day 3 of the assay if needed **[1]**.

3.8.1. Talent supplementing the wells with E. coli.

4. Observation of the Muv Phenotype in the *let-60, let-23 and lin-1* Strains

4.1. Prepare a 2% weight to volume solution of agarose by adding 0.1 gram of agarose to 5 milliliters of deionized water **[1-TXT]**. Heat the agarose in a microwave to dissolve it **[2]**.

4.1.1. Talent adding agarose to water. **TEXT: 5 mL of agarose solution / 20 slides**

4.1.2. Talent placing the agarose in the microwave and closing the door.

4.2. Place strips of lab tape along two glass slides, which will act as spacers limiting the thickness of the agarose pads **[1]**. Then, place a third clean glass slide between the taped slides **[2]**.

4.2.1. Talent placing strips of lab tape on slides.

4.2.2. Talent placing the third slide between the taped slides.

4.3. To make an agarose pad, spot 100 microliters of molten agarose onto the center of the clean slide **[1]**. Place another clean glass slide across the top of the agarose and gently press down to form a pad **[2]**. Remove the top slide when the pad has solidified **[3]**.

4.3.1. Talent spotting the agarose on the clean slide.

4.3.2. Talent placing another slide on top of the agarose. Videographer NOTE: 4.3.2 and 4.3.3 shot together.

4.3.3. Talent removing the top slide.

4.4. When the appropriate stage of the life cycle is reached, remove the plates from the incubator **[1]** and collect the worms in 15-milliliter conical tubes **[2]**. Centrifuge the tubes at 450 x *g* for 1 minute **[3]**, then remove the M9W without disturbing the worm pellet. Wash the worms twice with 5 milliliters of fresh M9W **[4]**.

4.4.1. Talent removing a plate from the incubator.

4.4.2. Talent collecting the worms in a tube.

4.4.3. Talent putting the tube in the centrifuge and closing the lid.

4.4.4. Talent removing the M9W from the tube.

4.5. After removing all remaining M9W, add 500 microliters of 2 millimolar sodium azide or 2 millimolar tetramisole hydrochloride to anesthetize the worms. Allow tubes to incubate at room temperature for 15 minutes **[1]**.

4.5.1. Talent adding anesthetic.

4.6. Add 10 microliters of the anesthetized worm suspension onto the center of an agarose pad **[1]** and place a number 1.5 coverslip gently over the suspension **[2]**. If needed, fix the coverslip with nail polish to prevent drying **[3]**.

4.6.1. Talent adding worms to the agarose pad.

4.6.2. Talent placing the coverslip on the worms.

4.6.3. Talent fixing the coverslip with nail polish. Videographer NOTE: Not needed.

4.7. Use a DIC microscope at 10 and 20x magnifications to observe the *let-60(n1046) (pronounce 'Let sixty n-ten forty-six')*, *let-23(sa62) (pronounce 'let twenty three-s-a-sixty two')* and *lin-1(sy254) (pronounce 'lin-one-s-y-two-fifty four')* strains **[1]**.

4.7.1. Talent positioning a slide on the microscope stage.

4.8. For *let-60(n1046)* and *let-23(sa62),* score the adult worms based on the presence or absence of the Muv *(pronounce 'mauve')* phenotype **[1]**. For the *lin-1* strain, count the number VPCs that adopted 1 or 2-degree cell fates on the ventral side of the L4 larvae **[2]**.

4.8.1. Talent observing the *let-60(n1046)* and *let-23(sa62)* strains.

4.8.2. Talent observing the *lin-1* strain. Videographer NOTE: Not available.

Results

5. Results: R-fendiline Alters *let-60* and *let-23* Function in *C. elegans* in a Dose-dependent Manner

5.1. This protocol was used to demonstrate that R-fendiline is able to suppress the Muv *(pronounce 'mauve')* phenotype in the *let-60(n1046) (pronounce 'Let sixty n-ten forty-six')* mutant strain in a dose-dependent manner **[1]**.

5.1.1. LAB MEDIA: Figure 2 A and B.

5.2. Non-reversal of the Muv phenotype was observed in the *lin-1* null mutant strain in response to increasing concentrations of R-fendiline **[1]**, suggesting that R-fendiline blocks activated *let-60* signaling at the level of RAS in *C. elegans* **[2]**.

5.2.1. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize the lin-1 bars.*

5.2.2. LAB MEDIA: Figure 2 B.

5.3. Similarly, the Muv phenotype was significantly reduced in the *let-23(sa62)* strain in response to 3, 10 and 30 micromolar R-fendiline treatments in comparison to the DMSO treated worms **[1]**.

5.3.1. LAB MEDIA: Figure 2 C and D.

Conclusion

6. Conclusion Interview Statements

6.1. **Ransome van der Hoeven:** During bleach treatment, it is important to observe the lysis of the worms. Prolonged incubation in the bleach mix will lead to death of the embryos resulting in lower yields of L1 larvae.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.2, 2.6.1, 2.7.1*

6.2. **Ransome van der Hoeven:** The localization of LET-23 can be determined in the presence of anti-EGFR inhibitors using transgenic worms expressing GFP fused to LET-23. This allows an investigator to determine if the anti-EGFR inhibitors cause the mislocalization of LET-23 to the plasma membrane.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.